SOME EFFECTS OF LOW TEMPERATURES ON SOLUBLE PROTEIN, PROTEIN-SULFHYDRYL CONTENT, AND ELECTROPHORETIC PROTEIN PATTERNS IN YOUNG COTTON PLANTS

By

JOE TOM COTHREN

Bachelor of Science

East Central State College

Ada, Oklahoma

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Thesis Approved:

Glenn W. Todd
Thesis Adviser

Edwin Basler

George N. Odell

Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Low, non-freezing temperatures after planting often cause considerable damage and even death to young cotton seedlings in some areas where cotton is grown. This type of damage to plants, without ice crystal formation, is known as chilling injury as contrasted to freezing injury where ice crystals are formed. Because of decreased yields and replanting costs, a definite need for ways of preventing this low temperature injury exists.

When cotton seedlings are exposed to a temperature of approximately 15 C for a 2-day period, they become hardened and are able to withstand chilling temperatures (5 C) without apparent injury. At chilling temperatures, the nonhardened plants usually first become flaccid, then necrotic, and are often killed.

Hardening in frost-resistant plants apparently involves a number of factors. Physical and/or biochemical changes in plants during hardening to frost injury have been extensively described. However, the metabolic reactions accompanying the hardening process in thermophilic plants have not been established. Although low temperatures affect cotton seedlings by increasing their susceptibility to disease and altering their metabolism, this study was concerned only with the metabolic processes. The purpose of this study was to investigate changes in the buffer-soluble protein and protein-sulfhydryl content

of hardened cotton tissue as compared with nonhardened tissue. An examination of the electrophoretic protein patterns, due to size and charge, was also made from hardened and nonhardened tissue, using polyacrylamide disc-gel electrophoresis.

CHAPTER II

REVIEW OF LITERATURE

An extensive review of cold hardiness (39), i.e., the development of the ability of an organism to survive low temperatures, revealed that early philosophers believed plant life could not exist without the plant maintaining its "natural heat" (Aristotle). Theophrastus also noted that the myrtle tree, which was without heat, froze rapidly while the laurel was resistant as long as it retained its heat. This theory of plant survival by heat retention was known as the "caloric theory", as the term "caloric" was once used to denote heat. According to Levitt (39), Hunter (1775), who froze many kinds of plants, was one of the first to experiment with low temperature hardiness. After thawing frozen plants, Hunter found they were dead and could be more easily refrozen. Touching frozen plants with unfrozen shoots caused local thawing; thus Hunter concluded that plants must die before freezing, and that living plants produce heat to resist this freezing tendency. The prevalent "caloric theory" (39) did not go unchallenged. In 1741, Duhamel completely discredited the caloric theory by reporting that apples frozen for 2 months did not suffer any injury, and Nau duplicated some of Hunter's experimental results with dead objects. Moreover, plants observed with ice inside actually proved to be alive on thawing.

Interest in the subject of plant hardiness to low temperature began to increase, and the question arose as to what were the lowest

temperatures plants could withstand. Lipman and Lewis (49) exposed seeds to liquid air at -190 C for 60 days and found that they were still viable. Mosses (protonema) exposed to -190 C for 50 hours (48) were also able to survive. This indicates that protoplasm can survive long exposures to very low temperatures. However, in higher plants, and some lower plants, this is true only for protoplasm in the dry state. If plants are hydrated, they freeze and are killed at these low temperatures.

Observations of the varying tolerances of protoplasm to frost injury led to more extensive investigations of the phenomenon. Johansson et al. (30) noticed that wheat plants placed at low temperature in light increased their hardiness more rapidly than plants at higher temperatures and attained maximum hardiness after some weeks. The protoplasm in the hardened wheat plants was more thermostable than that in unhardened plants.

Numerous ideas have been presented correlating hardiness to changes in metabolism and the physical nature of plants. Li and coworkers (47) found that inorganic P decreased and acid-soluble P and total organic P increased in red-osier dogwood (Cornus stolonifera) as the plants were cold acclimated under controlled conditions combining shortened photoperiods and freezing temperatures. These metabolic changes occurred under conditions similar to those occurring naturally in winter.

Apparently the lipid metabolism of alfalfa roots is altered during hardening. Gerloff et al. (19) found an approximate 2-fold increase in the fatty acid contents of two alfalfa varieties. A preferential accumulation of polyunsaturated fatty acids, namely linoleic and linolenic,

caused the average number of unsaturated bonds in fatty acids to increase.

A study of English ivy (Hedera helix) by Parker (57) indicated that an increase in hardiness during autumn was fairly sharp, especially during November. At this time a fairly level plateau of hardiness was reached and maintained until spring. This increase in hardiness was closely related in time to a sharp increase in anthocyanins. Li et al. (47) also observed an increase in shikimic acid during hardening. It is known that shikimic acid is a precursor for a pigment that accumulates in tree bark during hardening.

Changes in free amino acids (4, 14, 29, 34, 59, 89) and amides (59, 89) have frequently been associated with hardening. Wilding et al. (86) found there was a greater change of some amino acids than others in alfalfa as cold weather approached. Analysis on an amino acid analyzer revealed that arginine increased 246% in a nonhardy variety during a four-month period as compared to a 363% increase in a hardy variety. Alanine showed the same kind of increase. During the active growing season both varieties contained approximately the same amounts of amino acids. The amount of free amino acids, especially proline, glutamine, glutamic acid, and asparagine, increased during the second phase of hardening in all vegetative organs of winter wheat plants (4). Phaseolus vulgaris, grown at a suboptimal temperature of 18 C, exhibited increases in all amino acids except gamma amino butyric acid, glycine, phenylalanine, and tyrosine (34). However, Parker (58) did an extensive study of amino acids and amides in Pinus strobus bark that indicated no consistent seasonal changes relating to cold hardiness changes.

Freezing causes a reduction in water content of cells, as a consequence of ice formation, resulting in the concentration of the cell sap (66). This concentration of cell sap is often regarded as the cause of a toxic effect that salts out or directly flocculates protoplasmic colloids. The principal coagulating substances are electrolytes. Scarth and Levitt (66) felt that susceptibility to frost injury might be avoided by reducing the accumulation of toxic products or by addition of protective substances. They showed a decrease in electrolytes as a result of hardening.

Changes in acidity of plant sap have also been considered. Scarth and Levitt (66) demonstrated a very slight reduction in acidity in hardened cabbage seedlings, but not a large enough change to afford any protection. The expressed juice of hardened cabbage was no better buffered than that of the unhardened, and therefore, offered no more opposition to increase of acidity than did the juice of nonhardened plants. Data of Jung et al. (32) indicated that tissue pH was positively related to cold hardiness in alfalfa. They hypothesized that the increase in pH values observed during the development of cold hardiness may be important in causing changes in enzymatic activity. An increase in pH may also render the cytoplasm more resistant to denaturation since negative charges increase colloidal stability. An increase in tissue pH, as cold hardiness increases, may cause the net anionic charge on the protein to become greater (68). This may be associated with an increase in the amount of bound water which has been reported in hardened plant tissue.

It is widely accepted that sugars increase with hardening (5, 26, 54, 57, 64, 73), but the relationship does not always hold (62).

Lidforss, according to Scarth and Levitt (66), put forth the theory that sugars afforded protection against freezing injury. This protection was not merely by increasing osmotic protection, but through a specific protection against coagulation of protoplasmic colloids. However, Lidforss himself pointed out that certain plants like beets and sugar cane, which contain large amounts of sugars, are killed by light frost while some mosses and bacteria with very little sugar are highly resistant.

Glucose and sucrose were tested for their ability to protect cabbage leaf cells from freezing injury by slow cooling and rewarming, and were found to have highly protective action at temperatures below -50 C (64). Addition of sucrose to spinach chloroplasts prior to freezing protected against uncoupling of phosphorylation (24, 26). Therefore, sugars seem to be highly protective in nature. In contrast to this evidence, Siminovitch and Briggs (70) found that sugar content was not closely correlated with frost hardiness. From comparisons made with eleven sweetclover varieties (29), winterkilling was not a result of insufficient carbohydrate reserves. One variety exhibited almost as much survival as two other varieties even though it had the lowest total available carbohydrate.

Foliar applications of certain purines and pyrimidines enhanced the development or maintenance of cold hardiness (31). The content of nucleic acids was higher in treated plants than in controls. When treated plants were less cold hardy than the controls, the reverse was generally true. However, the purines and pyrimidines, in some instances, increased the content of nitrogenous constituents in a nonhardy variety to a level equal to that of hardy plants which had not been treated.

An increase in RNA content began one week prior to the rapid increase of cold hardening in apple twigs (46). In black locust bark cells, RNA content increased without a change in the DNA content, and preceded the period of maximal increase in rate of glycine incorporation into protein (69). During hardening of alfalfa, the content of RNA and DNA increased 85% and 134% respectively in a hardy variety. Increases of 44% and 93% were observed in a nonhardy variety (31).

A study of the resistance of hardened woody plants to dehydration at low temperature indicated that the branches of cold-hardened plants could lose as much as 76-80% of the initial water content without harm (37). In warty birch and goat willow, which are highly cold resistant woody species, only 8.5 to 9% moisture content was essential for the cells to remain alive at below freezing temperatures. The cold resistance of these plants is based on the discharging of water into the intercellular spaces rather than on the water-retaining capacity of the cells (37).

The response of plants to injuring and hardening temperatures was investigated by extracting cell sap from leaves (75). A damaging temperature of 3 to 5 C and a hardening temperature of 10 C were used for studying plants or detached leaves. Quantitatively, the expressed sap decreased by 13 to 50%, and more, after exposure to the hardening temperature in comparison with the unchilled plants. Less sap was extracted from hardened wheat leaves than from control plants--75% less at 10 C and 61.7% less at 3C. Hardened cabbage also yielded less sap than the unhardened--by 65% after 10 C and 35% after 3 C. Thermophilic plants also showed a decrease in sap expressed from them after they were hardened. The sap yield dropped 13% for Klinkskii cucumbers and

26% for hardened cotton.

Photoperiod and temperature have consistently been recognized as two critical factors in hardening (1, 29, 30, 60, 65, 73, 80, 81). Bula and co-authors (6) felt that short photoperiods and low temperature were major climatic factors affecting the development of cold resistance in overwintering leguminous plants. Steponkus and Lanphear (76) reported that light greatly enhanced the degree of hardiness attained in Hedera helix at relatively low light intensities and short photoperiods. Kohn and Levitt (35) determined that frost hardiness in cabbage seedlings (var. Badger Market) was directly related to photoperiod. Sulfhydryl content rose during the first 1 to 2 weeks of hardening in plants subjected to short days. After this both plants subjected to short days and long days showed a decrease in sulfhydryl content. Only the short day plants showed an increase in soluble protein plus nonprotein N. Investigations were also conducted to evaluate the effects of temperature and photoperiod on metabolic changes of two Medicago sativa varieties during the development and maintenance of cold hardiness (68). The two varieties varied widely in inherent cold hardiness. Temperature and photoperiod both played important roles in the metabolic processes. However, cold temperatures were primarily important for the development and maintenance of cold hardiness.

Proteins and Hardening

Most of the past work on low temperatures and plant response has been done on frost injury or resistance to extremely low temperatures (25, 26, 31, 59, 66, 69, 71) at which freezing injury occurs.

According to Parker (58), Zacharowa (1925) noticed the high resistance of various crop plants to cold and the high protein content of their meristematic tissue. This review (58) also indicated that Levitt (1954) noted a marked rise in soluble protein of potatoes at 3 C, although this plant does not become appreciably cold hardy.

The question remains as to how protein increases, in themselves, can account for hardiness increases. Electrophoretic examination of cold-hardened alfalfa material revealed a zone of highly charged and/or low molecular weight protein that was more prevalent than in non-hardened material (9). When bermudagrass was exposed to a hardening environment for more than 15 days, two of the electrophoretic soluble protein bands present in nonhardened material decreased in density and became more widely separated (12). Four well defined and compact bands of protein also appeared near the origin during overwintering. This may indicate the synthesis of a different type of protein to withstand low temperatures.

Gerloff et al. (20) determined that the soluble protein content of alfalfa roots increased during hardening in all varieties examined. Peroxidase and catalase activities also increased during hardening in these varieties. However, electrophoretic patterns did not reveal any major shifts in protein bands. A direct and consistent relationship of water-soluble protein content to frost hardiness was observed in the bark of the black locust tree (70)

Two protective protein factors were found in unwashed chloroplasts membranes from hardy spinach (25). They have not been found in non-hardy tissue. Both factors were heat stable and stable against acidification to a pH as low as 4.6. When compared on a unit weight basis,

the two protein factors were more than 20 times as effective as sucrose in preventing inactivation of photophosphorylation due to freezing of chloroplast membranes.

A study of two woody species demonstrated that protein electrophoretic patterns change in apple bark and arborvitae foliage at times of changes in hardiness (10). Little change was noted in total protein content during hardening and dehardening.

Sulfhydryls and Hardening

The process of hardening against freezing injury requires light and low temperature (39). Levitt (41) maintains that frost resistance is a resistance towards sulfhydryl oxidation. Therefore, a resistance to sulfhydryl $\rightleftharpoons$ disulfide interchange and formation of intermolecular disulfide bonds is the basis of his hypothesis. His mechanism for hardening against freezing injury might possibly be the same for hardening against chilling injury since both require low temperature and light.

Kuraishi et al. (38) found that unhardened pea plants (Pisum sativum) maintained high NADP and low NADPH levels during illumination at 25 C, but the hardened plants had low NADP and high NADPH levels in the light. Upon transferring the unhardened plants to a dark room at 25 C, their NADPH levels decreased immediately, whereas the NADPH content of the hardened plants remained at a high level for a few hours after being placed in the dark. They postulate that at low temperatures the photosynthetically produced NADPH would be available for metabolic functions other than the fixation of CO_2 as there is a decrease in CO_2 assimilation at lower temperatures. This NADPH would

therefore be available for disulfide reduction during the hardening process. If hardening is caused by a resistance to sulfhydryl oxidation, then it is feasible that hardened plants should have a higher ratio of sulfhydryls to disulfides than nonhardened plants.

Morton (56), on the other hand, recently demonstrated significant decreases in the sulfhydryl content of the total water-soluble protein during hardening and frost death of Brassica oleracea var. capitata. When electrophoretic gels were stained with amidoschwarz, there was no evidence of any new protein bands as a result of hardening; however, the relative amounts of proteins in individual bands changed. The decrease in sulfhydryl content reportedly paralleled a significant increase in water-soluble protein.

Levitt (42), citing Quiocho and Richards, says that cross linking of protein chains can lead to denaturation of carboxypeptidase A. It became insoluble and lost half its activity when cross linked by glutaraldehyde. Levitt feels that the disulfide bond should be just as effective as glutaraldehyde in intact protein because it is heat stable, has little tendency to break, and is stable to extremes of pH and action of strong solutions of urea.

Gaff (18) examined the sulfhydryl levels in extracts of cabbage leaves which had been desiccated to various degrees. A progressive decrease in "reactive" sulfhydryl content in the soluble protein fraction to 50% of the control, resulted from desiccation.

CHAPTER III

MATERIALS AND METHODS

Source of Tissue

Gossypium hirsutum L. seeds, cv. Westburn, were germinated in buckets of moist vermiculite. The seedlings obtained by this method were grown hydroponically in a modified Hoagland's solution (27) in the same manner as described by Stewart (79). The controlled environmental chambers used (Sherer--Model CEL 37-14) were equipped with twelve 50-watt incandescent bulbs and 16 fluorescent, cool white lights, which supplied approximately 3000 ft-c of light at the upper level of the plants. The specifications for the temperature of the chambers were $\pm$ 1 C of the desired temperature setting. However, this specification did not always hold, especially with location on the plant bed.

Treatment of Plants

After the cotton seedlings were transplanted to buckets of modified Hoagland's solution, they were grown for 1 or 2 weeks in the greenhouse and then subjected to various treatments. There were variations in the age of the plants subjected to experimentation, and these exceptions are mentioned where applicable. At either 2 or 3 weeks of age the tissues (either leaves or cotyledons or both) were harvested. The treatments consisted of the following: (1) UN--unhardened, not chilled (control plants); (2) HN--hardened, not chilled; (3) UC--

unhardened, chilled; and (4) HC--hardened, chilled. Plants were hardened at 15 C day and 10 C night for 2 days and/or chilled at 5 C day and night for 2 days with 14 hr of light per day. For older plants a 1-day pretreatment of 20 C day and 15 C night was used before lowering the temperature to 15 C day and 10 C night, because the older plants were more sensitive than younger plants to the lower temperatures. All the harvests were made at approximately the same time each day. Harvested tissue was placed on ice until it could be stored in a refrigerator. Extraction of plant material was completed as quickly as possible after harvest.

Problem Encountered in Obtaining Control Plants

A problem was encountered during the late autumn and winter months that hindered my investigation of low temperature and its relation to hardening. Plants grown under greenhouse conditions during these months hardened to some degree and little or no chilling injury was obtained from these plants. Thus, a couple of factors were investigated that might possibly be correlated with hardening so that I could grow control plants. First an investigation was conducted to see if humidity was a factor. In the winter months, as well as late fall, the humidity in the greenhouse was very low (from 10 to 20% and lower), and it was suspected that high humidity in association with warm temperatures was necessary for growing control or unhardened plants. To test this idea a canopy was constructed over a table in the greenhouse where the plants were ordinarily grown. A plastic covering was used that allowed the sunlight to penetrate to the plants inside the canopy. Light meter readings revealed no significant differences in light

intensity in the greenhouse and under the canopy. An evaporative cooler inside the canopy increased the himidity. Air was pulled into the canopy from the greenhouse through an opening behind the cooler. The plants were germinated and transferred to buckets of nutrient solution in the usual manner. After one week of growth under the canopy at temperatures around 90 F day and 72 F night and 50 to 60% humidity, the plants were transferred to growth chambers at 5 C day and night to check for chilling injury. The second investigation made was of temperature as a factor in growing control plants. The results of these tests are discussed in Chapter IV.

An Attempt to Measure NADP:NADPH Ratios

In order to test the idea that increases in NADPH are correlated with cold hardiness in cotton, the method of Yamamoto (88) was used to attempt a measurement of oxidized (NADP) and reduced (NADPH) nicotinamide adenine dinucleotide phosphate in cotton tissue. Whenever fresh tissue was used, it was washed in tap water, rinsed in distilled water, and the excess water was removed by blotting the tissue with paper towels. Initial attempts to measure NADP:NADPH were made with fresh cotton leaves. The enzymes for the assay were obtained from Sigma Chemical Company, with the exception of NADPH diaphorase which was obtained from spinach and cotton chloroplasts by the method of Avron and Jagendorf (3), through step 6 of their procedure.

A 0.5-g portion of fresh cotton leaves was hand homogenized for 2 min at 90 C in either 5 ml of 0.1 N NaOH for NADPH extraction or 0.1 N HCl for NADP extraction. A longer extracting time inactivates the NADP (52). The homogenates were quickly transferred to an ice bath,

and the pH was adjusted to 7.6. Three-tenths ml of 0.2 M Tris[tris(hydroxymethyl)amino methane]-HCl buffer of pH 7.6 was added to each extract, and the homogenates were centrifuged at 12,100xg for 20 min at 0 to 2 C. The total volume of each supernatant fraction was measured after centrifugation.

The assay mixture for NADP:NADPH consisted of the following:

Extract	0.7 ml
0.2 M Tris-HCl, pH 7.6	0.8 ml
1.2 mM dichlorophenolindophenol (DCPIP)	0.2 ml
0.2 M $MgCl_2$	0.1 ml
Water	0.8 ml
Isocitric dehydrogenase (1 mg of protein)	0.1 ml
NADP diaphorase (0.6 mg of protein)	0.2 ml
Isocitrate	0.1 ml
Total Volume	3.0 ml

All of the components of the assay system, with the exception of the isocitrate, were thoroughly mixed and left for 30 minutes to allow the oxidation status of the DCPIP to become stable before continuing the assay. The DCPIP sometimes was reduced immediately after it was added to the extract. Within 30 min it was usually reoxidized and stable enough to start the reaction. Yamamoto (88) noted this same reaction.

The reduction of the DCPIP was followed on a spectrophotometer at 610 nm. The cuvettes were cooled by circulating water refrigerated at 13 C through thermospacers around the cuvette container.

To start the reduction of the DCPIP, 0.1 ml of isocitrate was injected into the cuvette with a microliter syringe. Electrician's tape was used to cover the hole in the lid of the sample compartment, and the needle was inserted through the tape to prevent light from leaking into the sample compartment. The reaction was followed for 10 min on a spectrophotometer.

The attempts to measure NADP and NADPH ratios in fresh cotton plants by this method proved futile. Therefore, a number of variations from the original procedure were tried. The following were some of the attempts made: An extracting solution consisting of 50 mM Tris-20mM EDTA and 5 mM sodium oxalate was used for an extraction of lyophilized cotton leaf tissue. Because previous tests had failed to give any conclusive results, EDTA (ethylenediaminetetraacetic acid), a chelating agent, was used to complex heavy metals. Sodium oxalate was added to combine with the large amounts of calcium which might also be a competitive inhibitor.

A 250-mg portion of lyophilized tissue was extracted in 5 ml of the Tris-EDTA buffer for 2 min at 90 C in the 50-ml cup of a Servall Omni-mixer, but HCl and NaOH were not used. The mixer was regulated by a power supply set at 140 volts. The assay was carried out at room temperature. After the reaction components were mixed and the isocitrate was added, the reduction of the DCPIP began immediately and lasted approximately 10 min. Sodium lauryl sulfate, which is a detergent, had previously been used in the extracting solution to unfold the protein structure and expose any "masked" NADP or NADPH. However, it proved to be a disadvantage because of its "sudsing" action, and it was omitted.

To determine the limitations of the above experiment, 3 ml of the extracting solution were added simultaneously with either 2 ml of NaOH or HCl to obtain NADPH or NADP respectively. The NaOH was added initially, i.e., previous to heating, while the HCl was not added until after the extraction had been made. Heat was not reapplied after the HCl was added. Adjusting the pH of the homogenates to 7.6 resulted in a final volume of 4 ml for the NaOH extract and 6.5 ml for the HCl

extract. The activity of the HCl treated portion when assayed for NADP was poor, and the NaOH portion for NADPH was not much better.

Another possibility examined was the use of an ion exchange resin to purify the sample and perhaps remove substances that would inhibit the reaction. Standard solutions of NADP and NADPH were prepared and run through a Dowex 50W-X8 column approximately 20 x 100 mm. This was to determine if the positive charge on the $NADP^+$ would cause it to adhere to the column and be separated from NADPH in the purification procedure. Spectrophotometric absorption curves showed that both NADP and NADPH were eluted from Dowex, and no additional NADP or NADPH could be eluted from the column with 2 N NaCl. The use of a Dowex column for removal of substances that might be inhibitory to the assay system for NADP:NADPH was not successful as the plant extract purified on the column did not have any more activity than unpurified plant extract.

A 0.1 M Tris-1% Carbowax 6000 buffer was also tried for extracting NADP and NADPH, as older cotton leaves contain excessive amounts of phenols. The Carbowax was used to complex the phenols and reduce any interference that the phenols might cause during the assay. This did not give good results. Chloroform; 0.04 M H_2SO_4 and 0.1 M Na_2SO_4; 0.1 M $NaCO_3$ and 0.5 mM cysteine; and 0.1 N NaOH-0.5 mM cysteine were also used as extracting agents, but none of them gave satisfactory results.

Purification of Polyclar AT

Because excessive amounts of phenolics are found in cotton leaves and cotyledons as well as many other plant tissues, they must be

complexed during extraction of the plant tissue to prevent protein-phenolic complexes and subsequent precipitation of the protein. Since Carbowax 6000 was not satisfactory as a complexing reagent, insoluble polyvinylpyrrolidone (Polyclar AT obtained from Dyestuff and Chemical Division, Rahway, New Jersey) was used. According to Loomis and Battaile (51), the Polyclar AT should be purified before use. A 100-g portion was suspended in 900 ml of 10% HCl and heated for 10 min in a boiling water bath. Occasional stirring was necessary to keep the Polyclar AT suspended. The suspension was filtered with suction and rinsed several times with distilled water before it was resuspended in distilled water and poured into a large chromatographic column for washing. A continuous flow of distilled water was used to rinse the column until the effluent was free of Cl^- ions, as indicated by a negative test with $AgNO_3$. After the Cl^- ions were removed, the Polyclar AT was dried and ground with a mortar and pestle.

Determination of Sulfhydryl Content

In initial attempts to measure sulfhydryl content, a 20 x 300-mm column of Sephadex G-25 was used for separation of the plant extract into a protein fraction and an amino acid fraction. Because proteins were larger, they passed through the Sephadex column faster than the amino acids. A 30-ml portion of 0.1 M Tris-1% Carbowax 6000 buffer, pH 7.5, was used to homogenize 6 g of fresh cotton cotyledons for 30 sec at 120 volts in the 200-ml cup of a Servall Omni-mixer. The homogenate was filtered through 2 layers of nylon mesh, refiltered with suction through Whatman # 1 filter paper, and the filtrate was centrifuged for 10 min at 23,500xg. A 5-ml portion of the centrifuged

supernatant was carefully pipetted onto the column of Sephadex G-25 so as not to disturb the surface of the bed. The column was rinsed with 2 successive 5-ml portions of water to remove any of the sample that remained on the surface of the gel. An aspirator was used to remove the water. The column was placed on a fraction collector and eluted with water. A 5-ml volumetric siphon was used to collect twenty 5-ml portions in test tubes. A determination of amino acid content (16, 55) on a Klett-Summerson photoelectric colorimeter indicated that the principal peak for protein elution was in tubes 8 and 9, and the peak of elution for amino acids was in tubes 17 and 18 (Fig. 1). Tubes containing most of the protein were pooled adjusted to a pH of 7.0, placed in a 500-ml round bottom flask, shell frozen, and lyophilized. Following this the sample was redissolved in extracting buffer, adjusted to a pH of 8.0, and assayed for sulfhydryl content. This procedure was time consuming and was also inadequate for separating the protein and amino acid fractions. It allowed considerable oxidation to occur, and the quantitative results were not satisfactory because the siphon, for one thing, was not accurate. The use of Centriflo membrane ultrafilters, obtained from Amicon Corporation, Lexington, Massachusetts, was tried. They were placed on a conical membrane support that fits in a polyethylene centrifuge tube with a 26 mm inside diameter. Small volumes (maximum of 7 ml) of protein solution can be purified with the membrane ultrafilters. Proteins above 50,000 molecular weight are retained by the membrane whereas molecules below 20,000 molecular weight are essentially not retained. This attempt at protein and amino acid separation was not satisfactory. Thus, the following procedure was used.

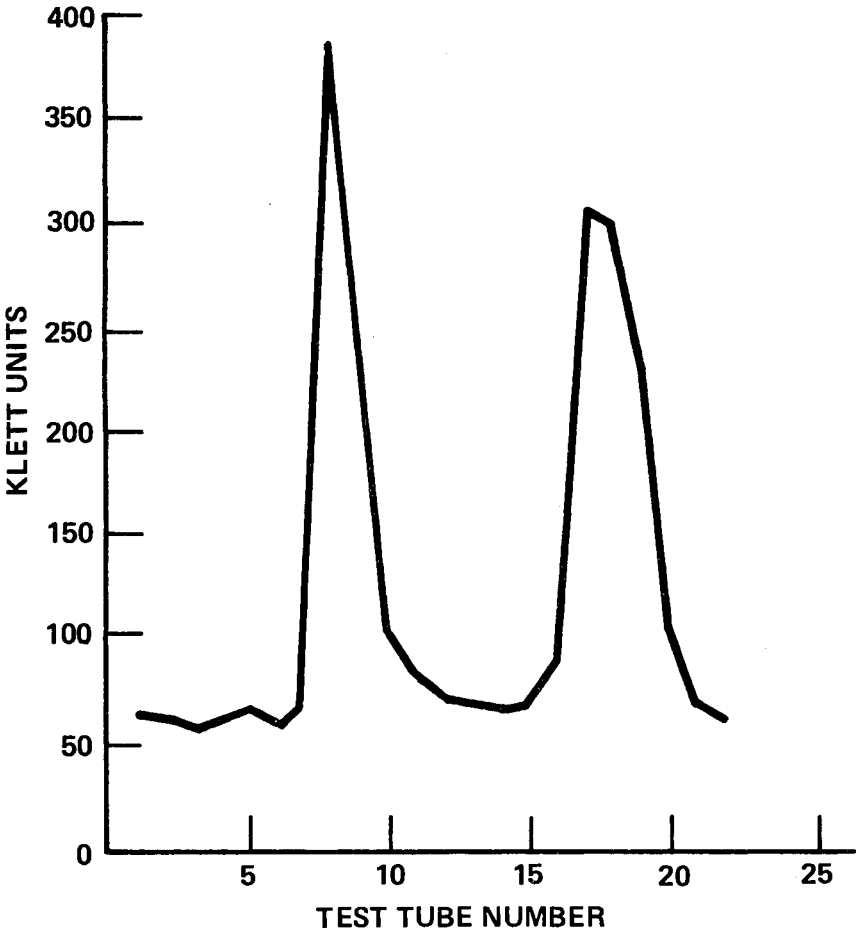

Figure 1. Elution Pattern of Ninhydrin-Positive Substances from a Sephadex G-25 Column

Two buckets of plants containing 6 plants per bucket were combined for each sample of the treatment. Treatments were replicated at least four times. Enough tissue was weighed so that a duplicate 6-g portion of each treatment was obtained. One 6-g portion was extracted in 40 ml of 0.1 M KH_2PO_4-10 mM EDTA buffer, pH 6.5. The other 6-g portion was placed in a tared petri dish and dried at 120 C in a forced draft oven.

Six-g of insoluble polyvinylpyrrolidone were mixed with 25 ml of 0.1 M KH_2PO_4-10 mM EDTA buffer, pH 6.5, at least 24 hours before it was used. The tissue samples were cut into small pieces with scissors and placed in the 200-ml cup of a Servall Omni-mixer. The suspension of insoluble polyvinylpyrrolidone was then added along with an additional 15 ml of KH_2PO_4-EDTA buffer. The cup was purged with nitrogen gas before and during extraction. (Two holes 3 mm and 7 mm were bored near the top of a 200-ml Servall Omni-mixer cup. The 7 mm hole was the inlet for the nitrogen, and the 3 mm hole was the outlet.) The tissue was homogenized in this mixture for 1 min at 120 volts on the mixer. The homogenate was strained through 2 layers of nylon mesh and centrifuged for 20 min at 27,000xg. Calculations were made from dry weights of plants to determine the amount of plant extract (supernatant) needed to contain 150 mg of plant material. These amounts were pipetted into 100-ml beakers, and the pH was adjusted to 8.0 with NaOH. All samples were made to a known volume. Not only was this necessary for sulfhydryl determination, but it also proved to be beneficial in electrophoretic examinations. This will be discussed more fully in results and discussion. The supernatant was recentrifuged for 15 min at 27,000xg, and 1 ml was used for the assay. The following amounts were used in the assay system:

3.8 ml of 0.1 M KH_2PO_4-EDTA buffer, pH 8.0
1.0 ml of plant extract
0.2 ml of DTNB
5.0 ml--Total Volume

The 5,5' Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Calbiochem Chemical Company. It was prepared fresh daily by dissolving 39.6 mg of the reagent DTNB in 10 ml of pH 7.0 KH_2PO_4 buffer. The solution was stored in a refrigerator at approximately 9 C until it was used. The buffer solutions were also refrigerated.

A pH of 8.0 is critical for the assay (15). DTNB reacts with aliphatic thiol compounds at pH 8.0 to produce one mole of para-nitrothiophenol anion per mole of thiol. This anion absorbs strongly at 412 nm and can therefore be used to determine the thiol concentration.

A Klett-Summerson photoelectric colorimeter with a blue #42 filter was used for determining the absorbancy readings. (Klett-Summerson units can be converted to optical density by multiplying by 0.002.) Prior to the addition of the 0.2 ml of DTNB, the 3.8 ml of KH_2PO_4-EDTA buffer and 1.0 ml of plant extract were thoroughly mixed on a Vortex mixer. Some coloration of the plant extract, probably due to extracted pigments, made it necessary to read the samples on the colorimeter before adding the DTNB. Subsequent to the initial reading, the DTNB was introduced, they were again thoroughly mixed, and absorbancies were determined 10 min later. Readings 1 hr after addition of the DTNB were comparable to those developed in 10 min. Therefore, I assumed that the reaction was completed within 10 min. Ellman (15) indicated that the color developed within 2 min.

Duplicate blanks and standards were prepared for each set of samples run. The standard was 1mM L-cysteine monohydrochloride (monohydrate) in water. Fig. 2 shows a typical standard curve obtained

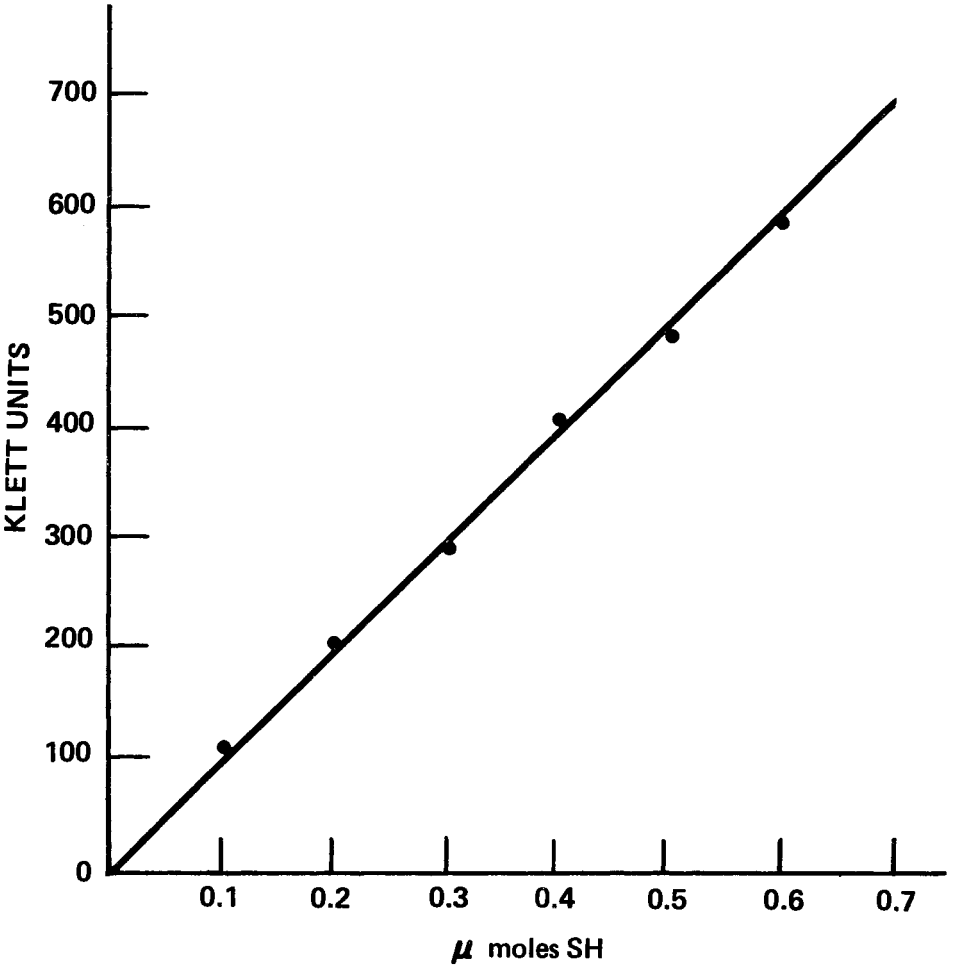

Figure 2. Typical Standard Curve Obtained with L-Cysteine Monohydrochloride

with the cysteine monohydrochloride. No color developed when DTNB was added to a solution of cystine, the oxidized form of cysteine. A value of 198 (±6.6) was obtained with 0.2 micromoles of sulfhydryl in 5 ml of solution.

Because the plant extract contained free sulfhydryls other than those associated with protein, a method had to be devised to measure them. Acetone precipitation was used. A 10-ml portion of acetone that had been stored at -16 C was pipetted into a test tube containing 2 ml of the plant extract that had been adjusted to a pH of 8.0. The mixture was allowed to stand at room temperature for 10 min and was then centrifuged at 27,000xg for 10 min. The precipitated protein was discarded, and the acetone supernatant fraction was placed in a 250-ml round bottom flask and dried in vacuo on a rotary evaporator. Four ml of 0.1 M KH_2PO_4-EDTA buffer, pH 8.0, was used to redissolve the residue in the evaporative flasks, and 1 ml of this was used for sulfhydryl determination.

Determination of Protein

Buffer-soluble protein was extracted with buffers at varying pH levels. Buffers of pH 8.3, 7.5, 6.5, 5.5, and 4.6 were tried. Of these a 0.1 M KH_2PO_4-10 mM EDTA buffer of pH 6.5 was used for most determinations. The extraction procedure was the same as for sulfhydryl determination. Protein content of the supernatant portion was determined by the Folin-phenol assay (53). A standard deviation of ± 5.1 was obtained with 200 micrograms of protein in 7 ml of solution. The standard curve shown in Fig. 3 was used to calculate protein concentrations.

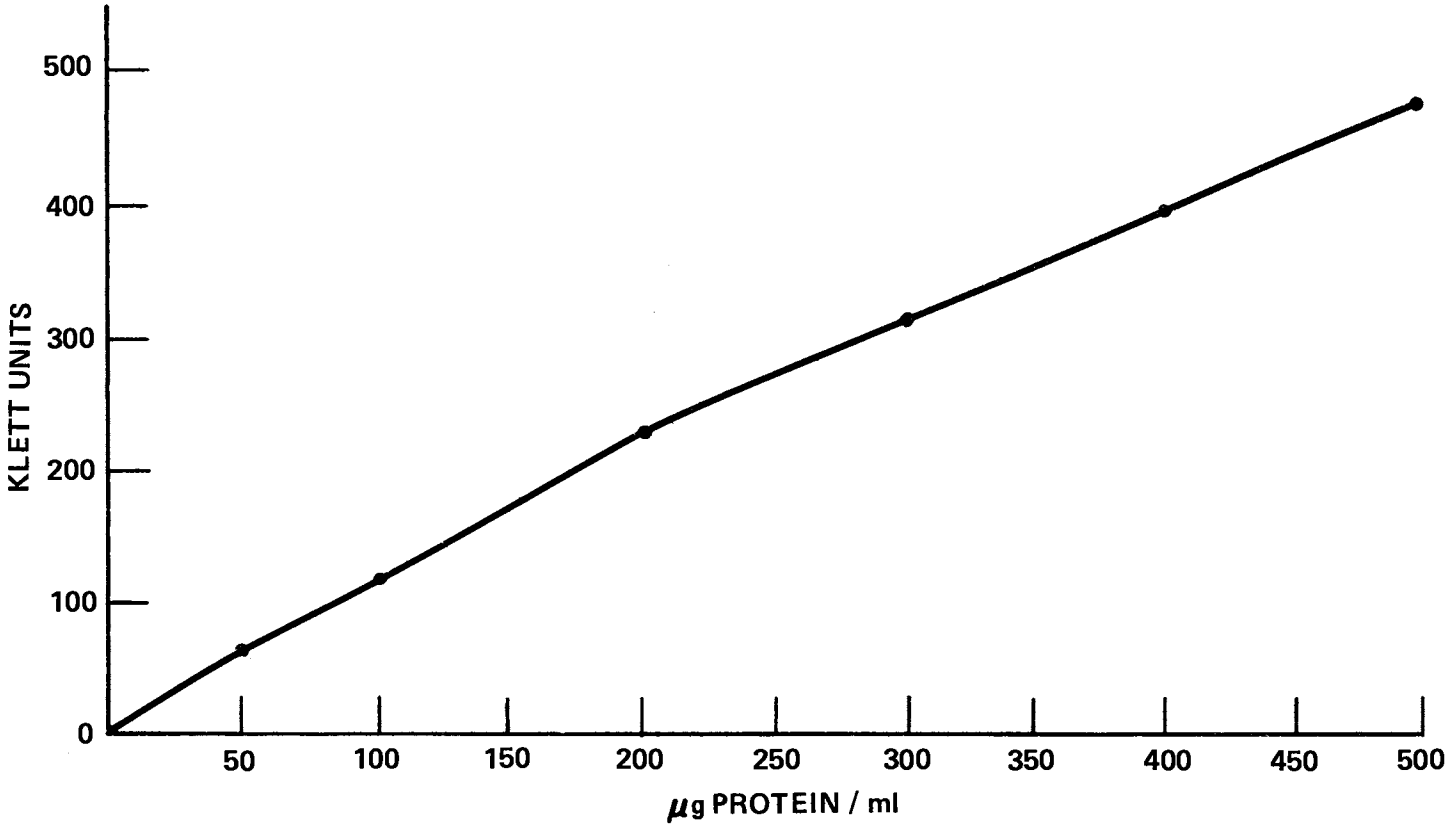

Figure 3. Typical Standard Curve for Protein Determination Obtained with Egg Albumin

Electrophoresis

Polyacrylamide gel electrophoresis (77) was used to note differences in the protein fraction of the various treatments. Loening's (50) methods for purification of acrylamide and bisacrylamide (N,N'-methylenebisacrylamide) were used. For this procedure 70 g of acrylamide were dissolved in 1 liter of chloroform at 50 C, filtered without suction through a hot funnel, recrystallized at -20 C, and the crystals recovered by filtering on a cold funnel. Ten g of bisacrylamide were dissolved in 1 liter of acetone at 40-50 C, filtered and recovered by the same method as the acrylamide.

A buffer of pH 9.3 (anionic gel system) was used. Five percent polyacrylamide gels consisting of a lower and an upper gel were prepared from stock solutions as given in the instruction manual for the electrophoresis apparatus of Buchler Instruments, Inc., Fort Lee, New Jersey: (A) 30 g of acrylamide, 0.8 g of bisacrylamide, and water to 100 ml; (B) 18.15 g of Tris, 24 ml of 1 N HCl, 0.24 ml of N, N, N', N''-tetramethylethylenediamine (TEMED), and water to 100 ml; and (C) 0.14 g of ammonium persulfate and water to 100 ml. These components were mixed in volume ratios of 1, 1, and 2, respectively, to prepare the lower gel. The upper gel was prepared by mixing 1, 1, 1, and 1 volume ratios of: (D) 10 g of acrylamide, 0.8 g bisacrylamide, and water to 100 ml; (E) 2.23 g of Tris, 12.8 ml of 1 M H_3PO_4, 0.1 ml TEMED, and water to 100 ml; (F) 2 mg of riboflavin and water to 100 ml; and (G) 80 mg of ammonium persulfate and water to 100 ml. The ammonium persulfate solutions were prepared fresh weekly. The bisacrylamide and TEMED were obtained from Eastman Organic Chemicals, and acrylamide was obtained from Matheson Coleman and Bell.

The lower gel was allowed to stand for 30 min with approximately 0.1 ml of distilled water layered on it to cause a flat surface rather than a meniscus-shaped surface. After 30 min, any unpolymerized gel solution and water were decanted from the tube, and the tube was rinsed with a small quantity of the upper gel. The upper gel was two times greater in volume than the amount of sample to be applied. After the upper gel was layered on the lower gel, it was overlaid with water, and the gel tubes were placed in a fluorescent light assembly within 3 inches of the lights. Photopolymerization was carried out for 15 min. Following photopolymerization, the gels were placed in the gel rack of the electrophoresis apparatus. The lower chamber was filled with a lower buffer of pH 8.74 consisting of 14.5 g of Tris, 60 ml of 1 N HCl, and water to 1 liter. A refrigerated water bath (3 C), equipped with a pump to recirculate the water, was used to cool the lower chamber. The upper buffer of pH 9.55 consisted of 5.16 g of Tris, 3.48 g of glycine, and water to 1 liter. The plant extract in 10% sucrose (v/w) was pipetted with a microliter syringe onto the gel under a layer of the upper buffer. Layering of the sample under buffer was done to minimize mixing of the samples when the rest of the upper buffer was added. The upper chamber of the electrophoresis apparatus was filled with upper buffer to make contact with the electrode. One ml of 1 mM methylene blue was thoroughly mixed with the upper buffer to serve as an indicator. A regulated power supply furnished 50 volts of electricity to the tubes for 30 min to allow the sample to move through the upper or stacking gel. The voltage was then increased to 110 volts for about 1 to 1.5 hours. Immediately upon removing the gels from the tubes (this can be accomplished by gently rimming the tubes with a syringe

fitted with a 25-gauge needle and filled with 7.5% acetic acid), they were stained for at least 1.5 hr with aniline blue black dye (amidoschwarz) obtained from Matheson Coleman and Bell. The amidoschwarz was prepared by dissolving 1 g of reagent material in 100 ml of 7.5% acetic acid. Fixing and staining was done immediately to prevent diffusion of the protein throughout the gel after the current was turned off. Excessive dye was removed from the "fixed" gels by destaining them for 2 hr at 100 to 150 volts in the electrophoresis apparatus with 7.5% acetic acid.

CHAPTER IV

RESULTS AND DISCUSSION

The young cotton plants shown in Fig. 4 illustrate the ability of hardened plants to better withstand chilling temperatures than unhardened plants. These plants, which were approximately 3 weeks of age, were subjected to a hardening period of 2 days at 15 C day and night with a 14-hr day and/or a chilling period of 5 C for 2 days. Previous treatment of the plants had a marked effect upon the plants. The hardened plants were in some instances less turgid and more wilted than before the hardening treatment. But they were rarely injured to the same extent as unhardened plants by the chilling temperature of 5 C that often caused severe damage and even death to the unhardened cotton plants. Chilled plants were also damaged to different degrees because of their location on the plant bed within the controlled environmental chamber. Although the chambers used were specified to hold temperatures within $\pm$ 1 C of the desired setting, thermometer readings indicated as much variation as 2 to 3 C within the chamber. And this variation was apparently large enough to cause chilling injury to vary widely as shown in later tables.

I observed during harvest of the hardened plants that their leaves and cotyledons were very "sticky", as if they had been covered with a sugary coat. Though there are reports of increased sugar concentrations

Figure 4. Young Cotton Plants Approximately 3 Weeks of Age After Chilling for 2 Days at 5 C (Prior to chilling the plants on the left were kept in a greenhouse. The plants on the right had been hardened for 2 days at 15 C day and night previous to chilling.)

in plants subjected to low temperatures (23, 63), I did not anticipate that sugars would be exuded from these shoot surfaces.

Control Plants

Plants grown in the greenhouse showed marked seasonal changes in their susceptibility to chilling injury. Plants grown during fall and winter were quite resistant to chilling injury, whether hardened in a growth chamber or not. Two tests failed to indicate that lower humidity increased hardiness. Lower greenhouse temperatures and, perhaps, shorter days may have increased resistance to chilling injury during fall and winter. This problem was at least partially overcome by culturing the plants in growth chambers at 35 C day and 30 C night temperatures with 14 hr of light per day, as the plants grown at these temperatures did show chilling injury. The fact that these plants were grown under a 14-hr day as compared to an 11- to 12-hr day for greenhouse plants must also be considered. It is highly probable that photoperiod is associated with hardening. Bula et al. (6) found that Medicago falcata developed cold resistance in late September and early October with the occurrence of low temperatures and a daylength of near 12 hr in Palmer, Alaska. They considered both daylength and temperature as major climatic factors affecting development of hardiness. However, Steponkus and Lanphear (76) reported that the role of light in the cold acclimation of Hedera helix var. Thorndale differs from that reported for winter annuals. They found that, although light greatly enhanced the degree of hardiness attained, photoperiods varying from 8 to 24 hours during the acclimation period were equally effective in promoting maximum hardiness. Paulsen (60) also noted that the photoperiod did

not determine whether winter wheat hardened, but that it did modify the level of hardiness under each temperature treatment. He found that hardening was greater under a constant long photoperiod than under a decreasing photoperiod. Unless the temperature was decreased, hardening did not occur. These wheat plants were grown in a growth chamber and gave results contrary to plants grown under field conditions. Decreasing photoperiod treatment decreased plant hardiness, and except for soluble protein, decreased contents of plant constituents associated with hardening. This is in direct contrast to field studies which indicate that short days (28) are more effective in promoting hardiness.

NADP:NADPH

The system for measuring NADP:NADPH in cotton plants was not satisfactorily developed. Faulty extraction or plant components were apparently the source of the problem, because the assay system worked consistently with a standard solution of NADP. A 0.05 ml aliquot of NADP introduced to the assay system, following each attempted assay of leaf tissue, indicated that the assay system was functional. However, I was unable to successfully measure the pyridine nucleotide content in fresh and lyophilized cotton tissue.

The presence of phenols was also considered as a detrimental problem in the extraction of NADPH since phenols take part in hydrogen bonding unless they are sterically hindered. And they are frequently a problem in plants, because they occur in such high concentrations. Phenolic compounds may be oxidized to quinones which are oxidizing agents that can oxidize essential groups of protein.

The possibility exists that an NADP-ase could affect the extraction and that any NADP would be broken down before it could be assayed. But this does not seem likely because of results obtained with added NADP.

Buffer-Soluble Protein

As mentioned previously, the degree to which unhardened plants were damaged was sometimes dependent upon their location on the plant bed in the growth chamber, as well as on the age of the plants. Table I shows that in the investigation of buffer-soluble protein the cotyledons of 2-week-old control plants contained the highest protein concentration, followed respectively by hardened-chilled, unhardened-chilled, and hardened-not chilled tissue. However, note the extremely high value of 304 in the unhardened-chilled tissue column. This bucket of plants was located near the center of the environmental growth chamber and sustained very little chilling injury. This is why I feel that a few degrees variation in chilling temperature was critical in determining the extent of chilling injury. If the 304 value is disregarded, an average of the other 3 replicates gives a mean of 189 as compared to 218 for the 4 replicates. This average is still higher than values obtained for cotyledons of older plants (Table II), but more nearly agrees with the data obtained from 3-week-old cotyledons. An average of 189 compared to a 266 shows a highly significant difference (74) between the unhardened-chilled and hardened-chilled treatments. Even the 218 average, which includes the 304 value, gives a considerable difference between the two treatments. A decrease in buffer-soluble protein was noted in the plants hardened for 2 days.

TABLE I

BUFFER*-SOLUBLE PROTEIN** IN
2-WEEK-OLD COTYLEDONS

	Treatment			
	Not Chilled		Chilled	
Replicate	Unhardened	Hardened	Unhardened	Hardened
1	277	200	168	255
2	277	188	188	286
3	290	210	304	265
4	264	215	210	257
Mean***	277(±11)	203(±12)	218(±60) 189****	266(±14)

*0.1 M KH_2PO_4 - 10 mM EDTA buffer, pH 6.5.

**Protein concentrations are expressed as milligrams per gram dry weight and are averages of duplicate samples. Standard errors are given in parentheses.

***The Least Significant Difference between treatments at the 5% level is 57.

****This average was calculated when the 304 value was omitted.

TABLE II

BUFFER*-SOLUBLE PROTEIN** IN
3-WEEK-OLD COTYLEDONS

	Treatment			
	Not Chilled		Chilled	
Replicate	Unhardened	Hardened	Unhardened	Hardened
1	140	115	83	120
2	155	103	74	131
3	144	111	41	95
4	136	133	44	105
5	131	125	43	115
6	140	133	47	137
Mean***	141(±8)	120(±12)	55(±18)	117(±16)

*0.1 M KH_2PO_4 - 10 mM EDTA buffer, pH 6.5.

**Protein concentrations are expressed as milligrams per gram dry weight and are averages of duplicate samples. Standard errors are given in parentheses.

***The Least Significant Difference between treatments at the 5% level is 17.

This reduction in buffer-soluble protein disagrees with results obtained with other plant species (57, 71) that indicated soluble protein increased with hardening, but Guinn (22) found similar results in total protein in cotton. Also, in an examination of two woody species, Craker et al. (10) noted little change in total protein during hardening and dehardening. Initial investigations using fresh weight bases for calculations of protein content indicated that hardening did give an increase in soluble protein, but I decided that fresh weights varied enough because of differences in water content, that comparisons drawn from their use were not accurate. Therefore, comparisons were based on dry weights of the plant material.

Why were the cotyledons of the 2-week-old plants not severely damaged by chilling temperatures? Apparently age is a significant factor in determining a plant's resistance to low temperatures. Worzella (87) reported in 1935 that wheat seedlings up to the age of 32 days were more susceptible to cold than older plants. Approximately the same survival was shown by wheat plants 32, 39, and 46 days old when they were subjected to a standard artificial freezing test. Angelo (2) reported that older strawberry plants subjected to low temperatures were only injured 20% while younger plants showed 85% injury. In contrast to this, Crescini and Tettamanzi (11) observed that the younger leaves of wheat were more cold-resistant than older leaves, and Klages (33) observed that wheat seedlings became more susceptible to low temperatures with advancing age. Another explanation for injury to older plants is that the vacuoles of older plants are larger than those of younger plants and are often filled with materials that are highly toxic to the plant cells if the vacuolar membranes become "leaky".

Thus, the younger plants would not have these large accumulations of toxic materials that would be released within the plant even if their membrane structure became disorganized at exposure to low temperatures.

Less buffer-soluble protein was found in 3-week-old cotyledons than in 2-week-old cotyledons (Table II). At the 2-week-old stage of growth the primary leaves have not usually reached maturity, and the cotyledons appear to be the source of metabolic products. However, at the 3-week stage of growth the leaves have become more prominent than the cotyledons, and there appears to be a breakdown and translocation of protein from the cotyledons to the leaves (Tables I and II). The control plants again contained more protein than the other treatments and were followed respectively by hardened-not chilled, hardened-chilled, and unhardened-chilled tissue. A significant decrease was noted in the protein content of the hardened-not chilled tissue as compared to the control tissue. Also, the unhardened cotyledons of the 3-week-old plants were severely injured by the chilling treatment. There was little difference in the protein content of the hardened-not chilled and hardened-chilled treatments.

Heber (25) isolated two protective protein factors during investigations with unwashed chloroplast membranes from hardy spinach. These two protein factors were heat stable and stable against acidification to a pH as low as 4.6. I was curious as to whether the buffer-soluble protein of hardened cotton plants was more resistant to heat and acidification than that of unhardened plants. To determine whether any differences existed, equal portions of the hardened and unhardened supernatant were adjusted to a pH of 4.6, heated for 5 min in a boiling water bath, and assayed for protein content. This treatment of the

supernatants did not give a significant difference between the two treatments.

A comparison was made between the first two replicates and the last four replicates of Table II. Four replicates were normally made, but because of a malfunctioning growth chamber, two replicates of the series were lost. The two replicates that were saved were analyzed and the data obtained were then combined with the four replicates grown the following week. The standard errors within treatments were generally less within the two replicates and four replicates analyzed separately than when all six replicates were combined. However, these differences were not extremely large.

There was a decrease in buffer-soluble protein in the hardened tissue of 3-week-old cotton leaves (Table III), but it was not significantly different from the control at the 5% level. A significant difference in buffer-soluble protein content was noted in the unhardened-chilled treatment when it was compared to the other three treatments. There are at least two possibilities why less protein was found in the unhardened-chilled tissue: (1) Either the hardening treatment caused a change in the metabolism of the plant that prevented the protein from being precipitated, or (2) chilling temperatures caused disorganization of plant membranes that allowed hydrolytic enzymes to break down cellular components, including protein. Todd and Basler (82) reported that drought injury or death of wheat plants was probably due to the breakdown of synthetic machinery rather than to "clumping" or "coagulation" of protoplasm. If the disappearance of protein is due to hydrolytic breakdown, then an investigation of the amino acid content should show a considerable increase in amino acids

TABLE III

BUFFER*-SOLUBLE PROTEIN** IN
3-WEEK-OLD LEAVES

	Treatment			
	Not Chilled		Chilled	
Replicate	Unhardened	Hardened	Unhardened	Hardened
1	175	212	93	250
2	210	190	81	238
3	286	245	66	216
4	386	274	94	230
5	276	253	44	212
6	275	238	40	227
Mean***	268 (±73)	235 (±30)	70 (±30)	229 (±14)

*0.1 M KH_2PO_4 - 10 mM EDTA, pH 6.5.

**Protein concentrations are expressed as milligrams per gram dry weight and are averages of duplicate samples. Standard errors are given in parentheses.

***The Least Significant Difference between treatments at the 5% level is 62.

with chilling injury. Investigations of others have shown increases in amino acids even at hardening temperatures; however, there are contradictions to this in the literature. Therefore, the amino acid content would have to be determined at hardening temperatures before exposing the plants to chilling temperatures. There is the possibility that the amino acids resulting from protein degradation are metabolized even further resulting in the release of ammonia which could cause injury in the plant.

During the weighing of samples, I noticed that the hardened-not chilled material usually weighed more than unhardened-not chilled tissue (Table IV). Fewer leaves and cotyledons of hardened-not chilled plants were needed to make 6-g fresh weights than that of the unhardened-not chilled tissue. There was also an observable difference in weighing out lyophilized material. The hardened-not chilled tissue appeared to have a greater density because a much smaller volume was required to give a certain weight than was required with unhardened-not chilled tissue. Table IV shows that there was a significant difference between the dry weights of all the treatments of 3-week-old leaves. Fig. 5 and Table VI in the Appendix show that the water content of both cotyledons and leaves decreased with hardening and chilling temperatures, and that the leaves were more desiccated than the cotyledons with chilling injury. This decrease in moisture content is generally associated with the hardening process (7, 21).

The association of water with the hardening process is frequently misinterpreted because of the terminology used in discussing water found in the plant and the experimental means used for determining its content within the plant. Levitt (40) found that hardened cabbage

TABLE IV

DRY WEIGHTS* OF 3-WEEK-OLD
COTTON LEAVES

	Treatment			
	Not Chilled		Chilled	
Replicate	Unhardened	Hardened	Unhardened	Hardened
1	0.8133	0.9488	2.2729	1.4135
2	0.7931	1.1120	2.2052	1.5778
3	0.9897	1.1578	2.3979	1.2398
4	0.8803	1.0603	2.0007	1.3109
5	0.7380	0.9620	2.6022	1.2056
6	0.7402	0.9715	2.4142	1.1395
Mean**	0.8258 (±0.096)	1.0354 (±0.088)	2.3155 (±0.206)	1.3145 (±0.160)

*Weights are based on grams of dry tissue per 6 grams of fresh weight and are averages of duplicate samples. Standard errors are given in parentheses.

**The Least Significant Difference between treatments at the 5% level is 0.192.

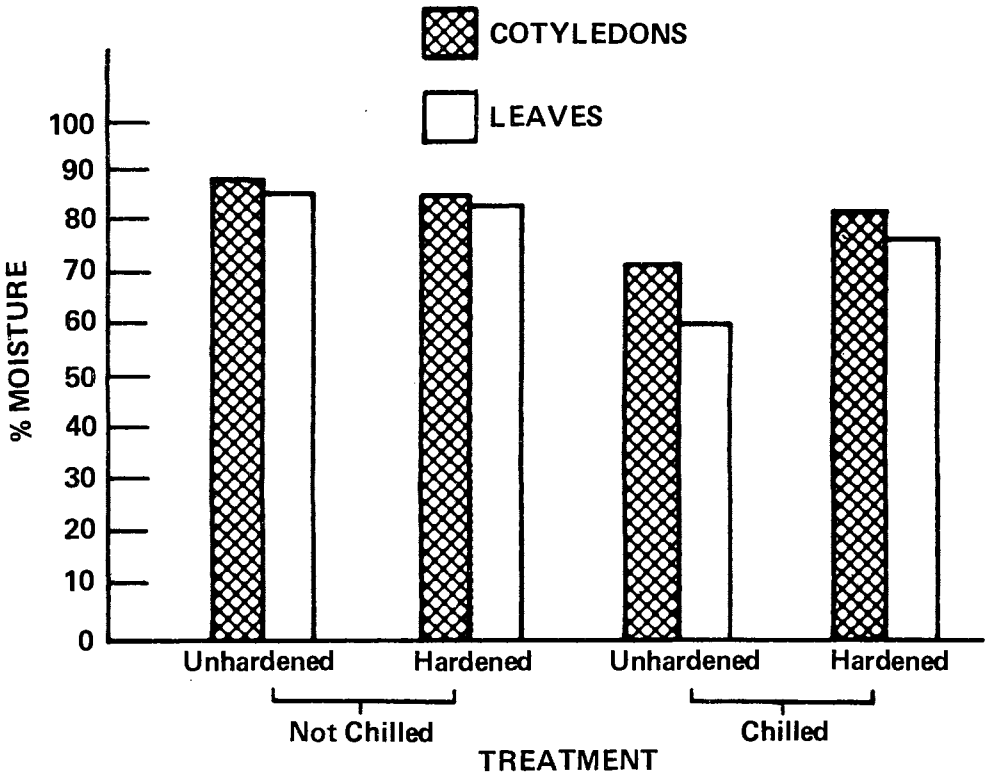

Figure 5. Percent Moisture in 6-Grams Fresh Weight of 3-Week-Old Leaves and Cotyledons Following Various Treatments

leaves dried at 0% relative humidity retained larger amounts of bound water than unhardened cabbage leaves. He defined bound water as that fraction not removed by the force used experimentally to remove water. In his investigations with cabbage, bound water was the fraction not removed when in equilibrium with $Mg(ClO_4)_2$ at 0 to 5 C, but was removed at 105 C.

Hardening and Protein-Sulfhydryls

Sulfhydryls in cotyledons of 2-week-old cotton increased in both the hardened-not chilled and hardened-chilled treatments as compared to the control (Table V). In Saxifraga stracheyi, Levitt et al. (45) found that the sulfhydryl content rose sharply during the first 4 to 11 days of hardening, but only a relatively small rise in frost resistance accompanied the large increase in sulfhydryls. There was actually a decrease in sulfhydryl groups during the second stage of hardening. Other types of hardy plants tested, Brassica oleracea, Pinus sylvestris, and Triticum sativum, always exhibited increases in sulfhydryl content during the first stage of hardening. Levitt et al. (45) also found that sulfhydryl content decreased when hardiness decreased and failed to increase when hardiness did not develop at hardening temperatures. Of the four tender species (incapable of hardening at low temperatures) tested, Begonia was the only one which failed to show a rise in sulfhydryl content on exposure to low temperature, while soybean and sunflower showed increases as large as those in hardy plants. Schmutz et al. (67) found that the sulfhydryl content of the homogenate or the supernatant liquid of wheat paralleled frost hardiness. If the wheat varieties failed to become frost hardy, they also failed to show

TABLE V

SULFHYDRYL CONTENT* OF BUFFER-SOLUBLE** PROTEIN IN 2-WEEK-OLD COTYLEDONS

	Treatment			
	Not Chilled		Chilled	
Replicate	Unhardened	Hardened	Unhardened	Hardened
1	1.93	2.53	0	2.93
2	1.93	1.40	0.33	3.13
3	2.13	2.60	1.27	3.27
4	1.80	2.60	1.77	3.00
Mean***	1.95(±0.14)	2.28(±0.59)	0.84(±0.82)	3.08(±0.15)
Mean Milligrams of Protein per Gram Dry Weight	277	203	218	266
Micromoles of Sulfhydrl per Gram of Protein	7.0	11.2	3.9	11.6

*Sulfhydryl concentrations are expressed as micromoles per gram dry weight and are averages of duplicate samples. Standard errors are given in parentheses.

**0.1 M KH_2PO_4 - 10 mM EDTA, pH 6.5.

***The Least Significant Difference between treatments at the 5% level is 0.79.

increases in the sulfhydryl content. A study of Saxifraga sp. (44) indicated that the sulfhydryl increase during hardening was mainly, if not solely, due to protein-sulfhydryl. The same study revealed that the glutathione-oxidizing activity increased greatly during hardening, but the sulfhydryl content still increased. This may indicate that the glutathione-oxidizing enzymes are unable to oxidize protein-sulfhydryls thereby allowing the plant to maintain a high protein sulfhydryl content though possessing a highly active glutathione-oxidizing system. This information would indicate that if glutathione does increase with hardening that it would be oxidized and could not account for the increase in sulfhydryl content. However, an increase in the non-protein sulfhydryl content may be due to cysteine rather than glutathione, but Levitt and co-workers did not find this (44). I found that the sulfhydryl content of the non-protein fraction was negligible. Waisel et al. (85) proposed that frost hardening occurs in three successive stages: (a) the normal high glutathione-oxidizing activity is decreased during a preparative period; (b) upon exposure to low temperature, the low glutathione-oxidizing activity, which has already begun to increase, allows for an increase in protein sulfhydryl groups; and finally (c) as dehydroascorbic acid accumulates, the glutathione-oxidizing activity increases, and protein sulfhydryl groups decrease. They (85) also found the occurrence of oxidized glutathione and cystine only in the disulfide form. Free cysteine was not detected at any time, and only traces, if any, of free sulfhydryl groups were ever detected during the vernalization of wheat. Kohn et al. (36) concluded that the protein sulfhydryl groups were exposed to significant oxidation during Sephadex separation and that supernate sulfhydryl groups

were actually all or practically all protein sulfhydryl groups. In all three wheat varieties they studied, the increased hardiness was paralleled by an increase in the percent of the total oxidized plus reduced form of protein sulfhydryl content actually found in the sulfhydryl state. My data indicate that the unhardened-chilled tissue had a considerable decrease in sulfhydryl content in all but one replicate (Table V). This agrees with data of Gaff (18) who found only 50% as many "reactive" sulfhydryl groups in the soluble protein fraction of cabbage leaves that had been desiccated to various degrees down to the death of the tissue. This decrease was attributed to configurational changes and a conversion of sulfhydryls to disulfides. Morton (56) reported significant decreases in the sulfhydryl content of the total water soluble protein of cabbage leaves during hardening and as a result of frost death. He did however find an increase in sulfhydryl content per unit high molecular weight protein. The amounts of sulfhydryl (Table V) are even more significant for the hardened-not chilled and hardened-chilled treatments when compared to the mg of protein per g dry weight in all treatments. A determination of the micromoles of sulfhydryl per g of protein gave values of 7.0 for unhardened-not chilled plants, 11.2 for hardened-not chilled plants, 3.9 for unhardened-chilled plants, and 11.6 for hardened-chilled plants. On a proportional basis, with the hardened-chilled tissue as 1.0 because it contained the most micromoles of SH per g of protein, the unhardened-not chilled, hardened-not chilled, and unhardened-chilled treatments gave values of 0.61, 0.97, and 0.33 respectively. This is a relatively substantial difference between all treatments except the hardened-not chilled and hardened-chilled treatments which contained approximately

the same amount of sulfhydryl proportionally. These comparisons would indicate that the hardened-chilled tissue did not increase appreciably in sulfhydryl content per g of protein after 2 additional days at chilling temperatures. Since disulfide content was not measured, I do not know if the decrease in sulfhydryl content in the chilled tissue was paralleled by an increase in disulfide content.

Interpretation of Electrophoretic Gel Separations

Polyacrylamide gel electrophoresis is a sensitive and reproducible method for separating soluble protein extracted from plant material (8, 77), and it has been used extensively for this purpose (13, 17, 61, 78, 83, 84). The gels in Fig. 6 show the reproducible patterns obtained in the electrophoretic separation of buffer-soluble protein of 3-week-old cotton leaves extracted at a pH of 5.5. The intensity of some of the lighter bands in the gels made them difficult to photograph, and they are shown in subsequent illustrations as diagrammatic representations of the bands that were visible with the aid of a laboratory light. An extraction of protein at pH 5.5 was made belatedly, as the gels after electrophoresis show clearer and more distinct bands when the protein was extracted at 5.5 than when extracted at a pH of 6.5. Varying amounts of extract were introduced onto the gels, and they show increased density in bands with increases in the amount of sample used.

A comparison of protein bands obtained from calculations on an equivalent dry weight basis versus an equivalent fresh weight basis (Fig. 7 and 8) shows that there was not a considerable difference between the two methods. The extracts for the electrophoresis were from plants harvested on March 12 and 14 for the equivalent fresh

Figure 6. Electrophoretic Separation of Soluble Protein Extracted at pH 5.5 from 3-Week-Old Leaves (These gels are 50, 100, 100, and 150 microliter aliquots, respectively, of protein extracted with a 0.1 M KH_2PO_4-10 mM EDTA buffer.)

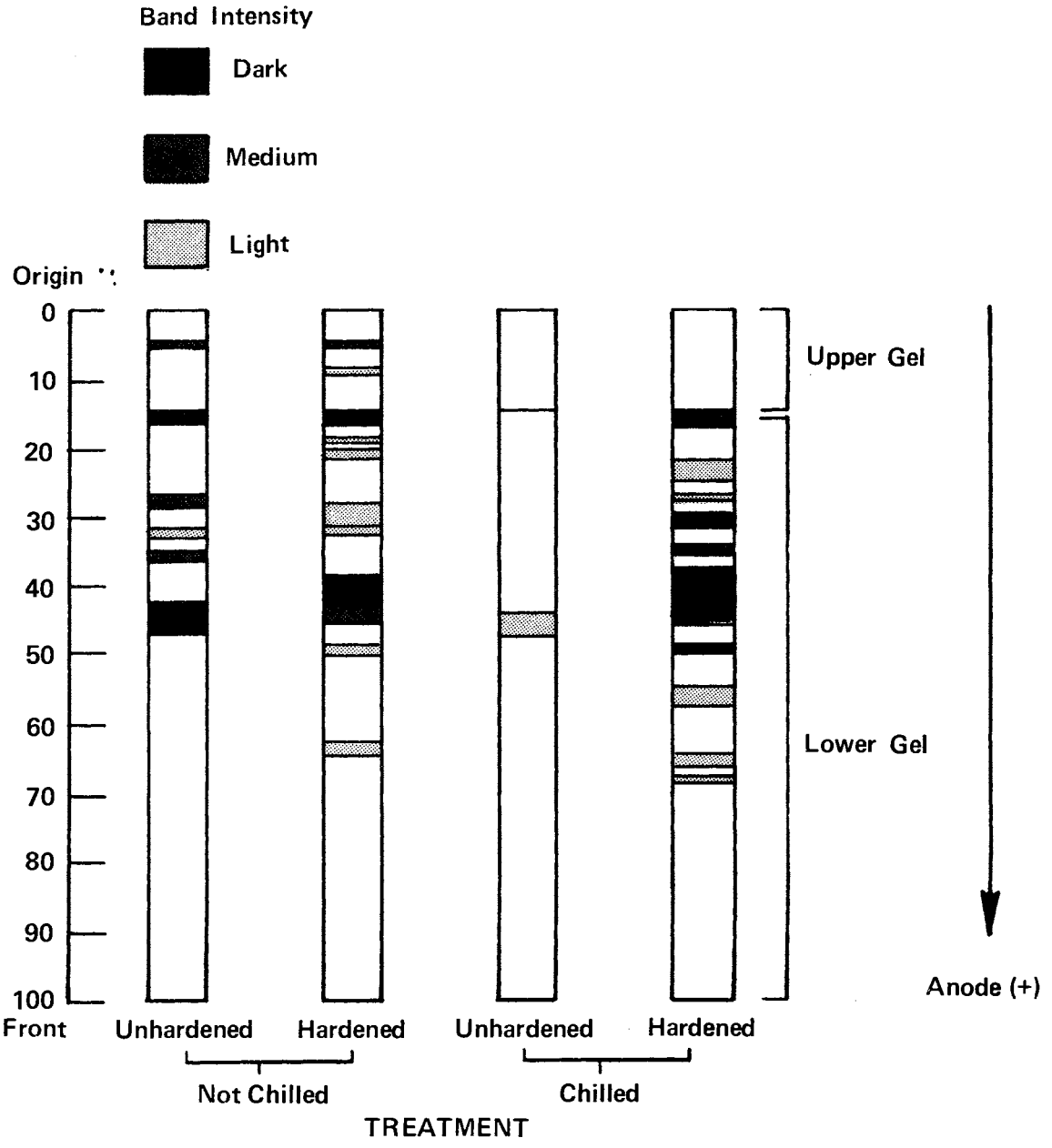

Figure 7. Diagrammatic Representation of the Electrophoretic Separation of Soluble Protein Extracted at pH 6.5 from 2-Week-Old Cotyledon Samples Based on an Equivalent Fresh Weight

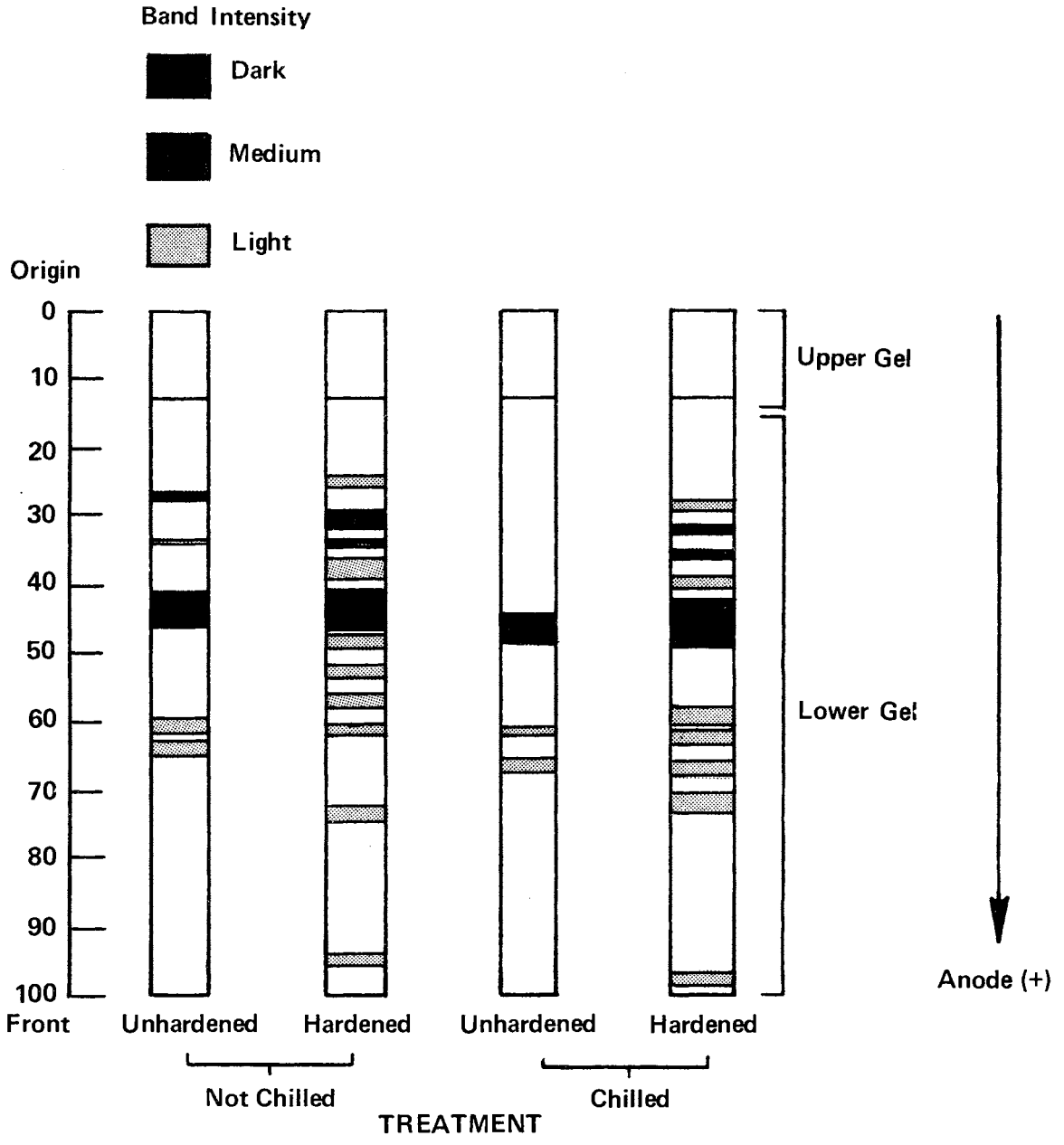

Figure 8. Diagrammatic Representation of the Electrophoretic Separation of Soluble Protein Extracted at pH 6.5 from 2-Week-Old Cotyledon Samples Based on an Equivalent Dry Weight

weight basis and on May 26 and 28 for the equivalent dry weight basis. The differences may be due to inherent characteristics of the particular group of plants or more likely to a slight difference in experimental technique. Some of the apparent differences were due to increases or decreases in intensities of the bands rather than to their appearance or disappearance.

Fig. 8 shows an increase in the number of bands in the hardened-not chilled and hardened-chilled treatments of 2-week-old cotyledons as compared to the control. Most of the protein was found in a dense band in the upper portion of the gel, which indicated that a large portion of the protein of cotton tissue was homogeneous in nature. Four bands were found in the proximal end of the gel in the hardened-not chilled and hardened-chilled treatments as compared to two in the control. Several additional bands also appeared in the distal end of the hardened and hardened-chilled treatments. Hydrolytic enzymes may cause a degradation of the higher molecular weight protein at lower temperatures that would account for the appearance of some of these lower molecular weight proteins. However, this explanation does not seem feasible for the appearance of some of the bands in the upper region of the gel as they are more intense in color than the bands above them. Coleman et al. (9), in their investigation of soluble root proteins of Medicago sativa, found a zone of highly charged and/or low molecular weight protein that was more prevalent in the protein complements of the cold-hardened material than in the non-hardened material. Davis and Gilbert (12) reported that Cynodon sp. exposed to hardening temperatures for more than 15 days showed a decrease in density and a wider separation of 2 bands present in non-hardened material. Four compact

and well defined bands also appeared during overwintering. Morton (56) failed to find any new bands in cabbage leaves as a result of hardening, but he did report a change in the relative amounts of proteins in individual bands. Gerloff, Stahmann, and Smith (20) found two new isoenzymes with peroxidase activities in fully hardened samples of alfalfa but failed to detect any large shifts in the electrophoretic pattern with hardening.

Three-week-old cotyledons (Fig. 9) revealed an additional band when exposed to hardening temperatures and a redistribution of density in bands with chilling temperatures. There was an inversion in the distribution of protein in the control and hardened-not chilled treatments as compared to the chilled treatment. Preceding chilling temperatures, the homogeneous band consisted of a less dense upper portion of protein and a dark lower portion. The inverse relation existed in the chilled tissue. There were fewer bands detectable in the 3-week-old cotyledons than in the 2-week-old cotyledons. This might be expected because protein determinations indicated that more buffer-soluble protein was found in 2-week-old cotyledons than in 3-week-old cotyledons.

In 3-week-old leaves, there was a considerable redistribution of protein within individual bands, but the hardened-not chilled tissue showed only one very faint band (Fig. 10) near the origin that was not found in the control. An additional band was found in the lower portion of the hardened-not chilled gel, and several bands were more diffuse than in the control. At chilling temperatures, two bands that could be seen in the hardened-not chilled tissue were no longer visible in the hardened tissue.

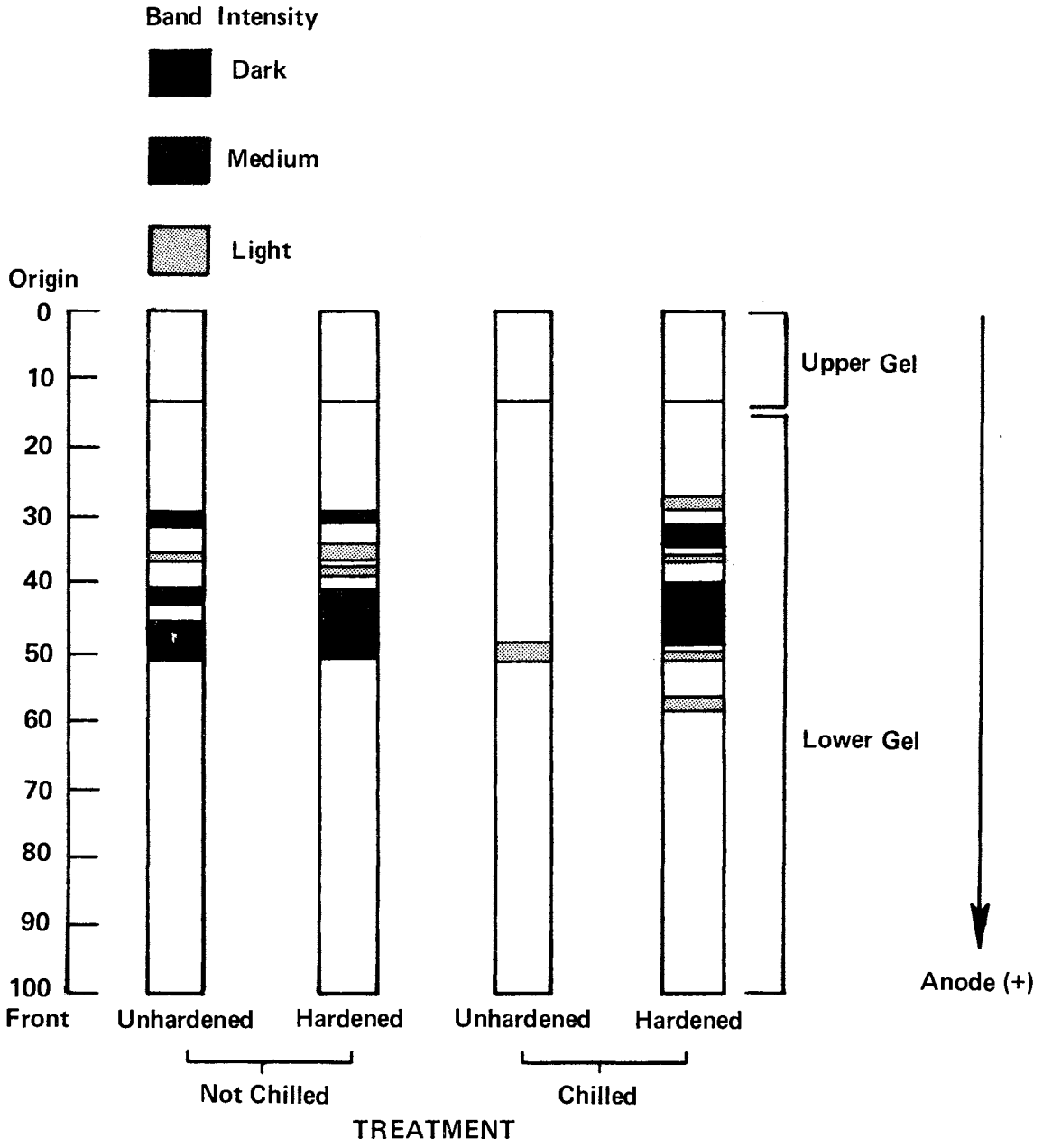

Figure 9. Diagrammatic Representation of the Electrophoretic Separation of Soluble Protein Extracted at pH 6.5 from 3-Week-Old Cotyledons Based on an Equivalent Dry Weight

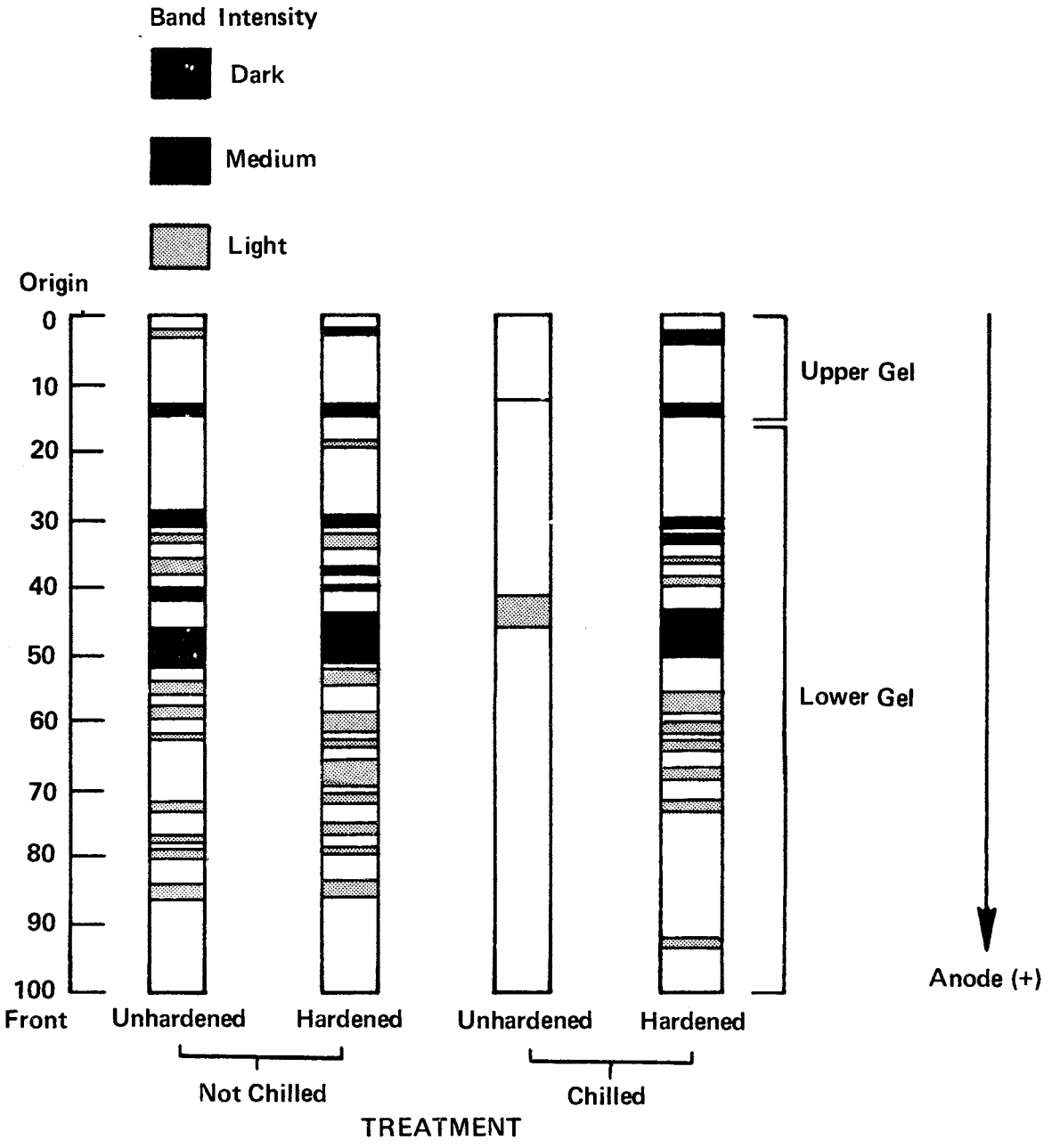

Figure 10. Diagrammatic Representation of the Electrophoretic Separation of Soluble Protein Extracted at pH 6.5 from 3-Week-Old Leaves Based on an Equivalent Dry Weight

There were striking differences in the intensity and number of bands that appeared in the unhardened-chilled treatments as compared to the hardened-chilled treatments (Figs. 7, 8, 9, and 10). In the older plants, only a very faint band appeared in the unhardened-chilled tissue in the region where the dense homogeneous band normally appeared. Very little protein remained in the unhardened-chilled tissues following chilling temperatures (Figs. 8 and 9). Two-week-old plants contained more soluble protein than three-week-old plants following chilling. Visually the two-week-old plants were less chill-injured than three-week-old plants. The increase in sulfhydryl groups during hardening was perhaps a factor that prevented protein disappearance in the soluble fraction during chilling.

The Hardening Mechanism

The physiological process of hardening in thermophilic plants probably involves a number of factors. Levitt's (41) sulfhydryl-disulfide theory of sulfhydryl resistance to oxidation seems to be one likely reason for hardening and increased resistance to chilling injury. Protein sulfhydryls are very important in the metabolic activity of plants as they are often associated with cofactors at the active sites (43), and thus are important in activation of enzymes that could lead to synthesis of proteins or other components that afford protection to the plant against chilling injury. Enzymes, comprised of proteins, are able to change from an active to an inactive form in response to chemical stimuli. Therefore, sulfhydryl enzymes (43) are oxidized to the disulfide form which inactivates them reversibly. The reduction of disulfides would also decrease the chance for formation of disulfide

bridges between protein molecules which often occurs with the dehydration of plant material during both chilling injury and frost injury. Of course the possibility exists that disulfide bridging is the result of rather than the cause for protein denaturation, but this idea is opposed by evidence (41) that the disulfide content increased even when the freezing temperature was too high to cause injury. Protein in vernalized and unvernalized wheat remained soluble long enough after freezing to allow for separation of proteins by gel filtration (41). The proteins showed a marked increase in disulfide content, and were assumed not to be completely denatured as denaturation usually renders them insoluble.

The increases in NADPH and sulfhydryl groups during hardening would make available a larger pool of reducing power that could possibly be involved in the metabolic processes. This again relates to the importance of these two factors in the activation of enzymes and as cofactors in key metabolic reactions.

The hardening process in both thermophilic and cold-hardy plants is probably a combination of several factors such as increases in sugars and sulfhydryl groups, the age of the plant, photoperiod, type of tissue, type of protein, enzyme activation, and previous condition of the plant (drought hardy plants are usually hardy to low temperatures). These factors may be interrelated, and the degree of hardiness maintained by a plant would be dependent upon one or all of the factors, with the maximum hardiness being obtained when all factors are present.

CHAPTER V

SUMMARY AND CONCLUSIONS

The pretreatment given to plants before exposure to chilling temperatures had a marked effect on the degree of chilling injury. Age of the plant also made a difference in the response to low temperatures. Plants that had been exposed to hardening temperatures for two or more days showed very little wilting or injury at chilling temperatures, whereas the unhardened plants wilted and were often killed. This was especially true in the 3-week-old plants. The 2-week-old plants were not usually injured as severely as the 3-week-old plants at chilling temperatures. The growing point or meristematic region of badly chill-injured plants usually survived. Thus, young tissue appears more resistant to chilling injury than older tissue.

There was more buffer-soluble protein in 3-week-old leaves and cotyledons that had been hardened for two days than in unhardened tissues after both treatments were exposed to chilling temperatures for two days. It was assumed that the denatured protein was insoluble. There were significantly large decreases in buffer-soluble protein in the unhardened-chilled tissue of the 3-week-old leaves and cotyledons as compared to the other three treatments. A smaller decrease in protein was also observed during hardening.

An adjustment of the buffer-soluble protein to a pH of 4.6 and a subsequent heating for 5 min in a boiling water bath did not show

significant differences in the amount of residual protein in hardened and unhardened tissue. Thus, this preliminary investigation did not indicate the presence of protective proteins reported in the literature (25).

A decrease in moisture content in hardened, unhardened-chilled, and hardened-chilled leaf and cotyledon tissue was noted. The rate of transpiration may have exceeded the absorption of water by the roots causing this difference. Guinn (22) found that permeability of cotyledons increased during chilling injury. Leaf tissue of 3-week-old plants was desiccated more than cotyledons of 3-week-old plants when exposed to chilling temperatures for two days.

Sulfhydryl content increased in both the hardened-not chilled and hardened-chilled tissue. On a micromole of sulfhydryl per gram of protein basis, the two treatments were approximately equal. Chilling caused a decrease in protein sulfhydryl content of unhardened tissues. The sulfhydryl content in the supernatant portion of an acetone-precipitated sample was negligible, which indicated that most of the increase in sulfhydryl content was due to protein sulfhydryls.

Gel electrophoresis revealed bands in both the distal and proximal ends of the polyacrylamide gels that contained hardened-not chilled and hardened-chilled tissue samples, which were not visible in the control tissue gels. This indicated that the metabolic synthesis of protein was altered with exposure to low temperatures. A large portion of the protein in cotton was homogeneous in nature as one band was significantly larger and darker in color than any others that appeared within any one treatment. Some bands were more compact and more densely colored than others between treatments. Certain enzymes may have

increased or decreased in activity with hardening temperatures. The appearance of new bands during the hardening process does not necessarily indicate the synthesis of new protein, but perhaps the amount synthesized was increased sufficiently so that it was detectable with gel electrophoresis. Hydrolytic cleavage of higher molecular weight protein could have led to the appearance of some of the lower molecular weight proteins, but this does not seem feasible in at least two cases in the appearance of higher molecular weight or more highly charged proteins.

The most striking change was the large decrease in number and intensity of bands when unhardened tissues were chilled. Chilling may have caused coagulation of much of the protein in unhardened leaves and cotyledons. Increases in protein sulfhydryl groups may have been one factor which prevented protein coagulation in hardened tissues during hardening.

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APPENDIX

TABLE VI

TABULAR REPRESENTATION OF FIGURE 5--PERCENT MOISTURE IN 6-GRAMS FRESH WEIGHT OF 3-WEEK-OLD LEAVES AND COTYLEDONS FOLLOWING VARIOUS TREATMENTS

	Treatment							
	Unhardened--Not Chilled		Hardened--Not Chilled		Unhardened--Chilled		Hardened--Chilled	
Cotyledons	Dry Weight	% Moisture	Dry Weight	% Moisture	Dry Weight	% Moisture	Dry Weight	% Moisture
Replicate 1	0.6914	88.48	0.9358	84.40	1.0856*	72.86	1.0766	82.06
2	0.6736	88.78	0.8905	85.16	1.2594	79.01	1.0126	83.12
3	0.6379	89.37	0.7574	87.38	1.2216	38.92	0.9542	84.10
4	0.6560	89.07	0.7728	87.12	1.0852	81.92	0.9889	83.52
5	0.7226	87.96	0.9227	84.62	1.0510	82.49	1.1145	81.42
6	0.7394	87.68	0.8736	85.44	1.0971	81.71	1.0713	82.14
Mean**	0.6868(±0.040)	88.56	0.8588(±0.076)	85.69	1.1333(±0.085)	72.82	1.0364(±0.061)	82.73
Leaves								
Replicate 1	0.8133	86.44	0.9488	84.19	2.2729	62.12	1.4135	76.44
2	0.7931	86.78	1.1120	81.47	2.2052	63.25	1.5778	73.70
3	0.9897	83.50	1.1578	80.71	2.3979	60.03	1.2398	79.34
4	0.8803	85.33	1.0603	82.33	2.0007	66.55	1.3109	78.15
5	0.7380	87.70	0.9620	83.97	2.6022	56.63	1.2056	79.91
6	0.7402	87.67	0.9715	85.39	2.4142	59.77	1.1395	81.01
Mean***	0.8258(±0.096)	86.24	1.0354(±0.088)	83.01	2.3155(±0.206)	61.39	1.3145(±0.160)	78.09

*The first two values of this treatment were 4-gram fresh weight samples, and the third value was a 2-gram sample.
**Least Significant Difference at the 5% level is 0.089.
***Least Significant Difference at the 5% level is 0.192.

VITA

Joe Tom Cothren

Candidate for the Degree of

Doctor of Philosophy

Thesis: SOME EFFECTS OF LOW TEMPERATURES ON SOLUBLE PROTEIN, PROTEIN-SULFHYDRYL CONTENT, AND ELECTROPHORETIC PROTEIN PATTERNS IN YOUNG COTTON PLANTS

Major Field: Botany

Biographical:

Personal Data: Born at Stratford, Oklahoma, May 15, 1944, the son of John and Alma Evelyn Cothren.

Education: Graduated from Maysville High School, Maysville, Oklahoma, in May, 1962; received a Bachelor of Science in Education from East Central State College, Ada, Oklahoma with a major in Chemistry and a minor in Biology, in May, 1966; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in May, 1971.

Professional Experience: Graduate teaching assistant in Biological Sciences at Oklahoma State University, 1966-1967; research technician in the Department of Botany and Plant Pathology at Oklahoma State University, summer, 1967; NDEA Fellow and graduate research assistant in the Department of Botany and Plant Pathology at Oklahoma State University, 1968-1970.

Member: American Society of Plant Physiologists.