A STUDY OF WINTER HARDINESS OF BARLEY PLANTS

by

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TABLE OF CONTENTS

INTRODUCTION

Some plants are capable of withstanding low temperatures better than others. This ability to survive low temperatures has been termed "winter hardiness", "cold hardiness", or "frost hardiness" by those who have investigated the phenomenon. The phenomenon has great economic importance, since soil and air temperatures often determine the ability of a plant to grow in a given region. Even plants which generally are adapted to a region sometimes are damaged or killed by cold weather. Plant breeders have recognized varietal differences in cold hardiness, and have devoted much time to development of more hardy varieties. However, in spite of extensive studies of winter hardiness extending over many years, the fundamental reasons for one variety of plant being more hardy than another is obscure.

The purpose of the study reported here was to determine the changes in some nitrogenous fractions that occur during the winter hardening of barley plants.

REVIEW OF LITERATURE

Cold hardiness has been studied extensively by physiologists and biochemists, and certain qualitative and quantitative changes during hardiness have been reported. Lipids have been investigated by many workers but no relation to winter hardiness has been found (11). Tannins and anthocyanins are not related to hardiness, since both hardy and non-hardy plants of a given variety show the same variations in concentration. Pectins, pentosans, and hemicelluloses show no relation to hardiness except in a very few cases (4, 6, 11). Changes in acidity have been studied also, and many investigators have recorded a reduction in acidity (pH rise of 0.2 to 0.3 units) during the hardening of

plants. However, this phenomenon, although general, seems to be unrelated to hardiness. Some workers attribute pH increases to an increase in soluble nitrogen compounds. Levitt was unable to alter frost hardiness by changing the pH of the plant (11).

The effect of minerals on winter hardiness has been studied. A few workers have reported a direct relationship between salt content and hardiness as measured by electrical conductivity. However, direct measurement of the calcium content has failed to show any relationship (11). Some workers found that boron, manganese, copper, and zinc in the soil increased cold resistance of wheat plants, while others reported the contrary (14, 18). Russian workers found that cold resistance of corn plants increased on lowering the K:Ca ratio in the plant tissue (9).

Respiratory and carbohydrate changes during hardening also have been studied, with contradictory results. In a study of mitochondria isolated from hardened and unhardened winter barley plants, workers at the South Dakota Experiment Station reported a consistently greater oxygen uptake per unit of nitrogen in mitochondrial suspensions from hardened tissue. These workers also obtained some evidence for a greater potential for ATP production in the case of mitochondria from hardened tissue (20). An inverse relation between respiration and hardening has been reported by some investigators while still others doubt that such a relationship exists. Some others found that it varied with the conditions to which the plants were subjected. Thus, when plants were exposed to light for a period prior to the study, respiration was greater in the hardened plants. When the plants were not exposed to light prior to the experiment, the respiration was lower in the hardened plants (11).

Sugars were found to increase during hardening, while starch decreased (16). Apparently both temperature and day length affect the interconversion of starch

and sugars, since exposure to hardening temperatures in the summer brought the sugar content to the winter maximum without producing an appreciable increase in frost hardiness (11). The increase in sugar content in the fall and the increase in starch content in the spring did not coincide closely with the time at which changes in hardiness occured (14).

Workers do not agree on an explanation for the role of carbohydrates in frost hardiness. Some postulate a detrimental effect of a high concentration of starch on the dehydrated cells of hardened tissue (8, 17). Others propose that sugars prevent denaturation of proteins at low temperatures. They believe that the sugars accomplish this by keeping the proteins in solution (10, 19). It is generally accepted that there are increases in sugar content and dry weight during hardening, and it is believed that changes in enzyme activity must be involved.

No definite relation has been found between total nitrogen and frost hardiness. According to Levitt, this may be due to the crudeness of the methods employed (11). Siminovitch and Briggs, working with the bark of the locust tree, found that soluble proteins increased with hardening and decreased with dehardening (15). Similar results have been reported for wheat by other workers (22).

EXPERIMENTAL AND RESULTS

Dicktoo barley, the hardiest variety presently grown in Kansas, was used in the study. The seeds were planted in 6 inch clay pots containing a soil with adequate nitrogen for good growth. The pots were placed in growth chambers in which a light intensity of approximately 1,300 foot candles one foot above the pots was obtained by a bank of fluorescent lights. A ten-hour photoperiod was maintained throughout the experiment. The temperature was maintained at

60° F. ± 1° for three weeks following planting, after which one group of plants was cold hardened at 35° F., so that hardened and unhardened plants of the same calendar age could be compared. After three weeks the plants maintained at 35° F. had assumed a semi-prostrate condition associated with exposure to low temperatures. The hardened plants then were returned to the chamber operating at 60° F. for the dehardening phase of the experiment. Analyses were made at the beginning of the hardening period and at intervals during hardening and subsequent dehardening.

Plants were harvested by clipping the stems at soil level. The number of plants per sample varied from one to four, depending on the age of the plants. The number of plants per sample was such that fresh weights varied from 5 to 10 grams. The water content was determined by drying a sample from each treatment in an oven at 70° C. overnight.

The degree of cold hardiness was determined by Dr. A. W. Pauli, Department of Agronomy, by the electrical conductivity method of Dexter (7), who found a close association between this measurement and cold hardiness. Three grams of tissue were frozen 4 hours at 20° F., and then were thawed for 20 hours at 35° F. in 100 mls. of water. After the liquid surrounding the tissue was brought to room temperature, resistance readings were made and were converted to conductivity ($ohms^{-1}$).

Total nitrogen was determined by the Kjeldahl-Gunning method (2). Water insoluble nitrogen, water soluble protein nitrogen, and water soluble non-protein nitrogen were determined by the method of Briggs and Siminovitch (5), as follows: the sample was desintegrated in cold water in a Waring blendor. The mixture was filtered and the nitrogen content of the filtrate was determined. The difference between total nitrogen and soluble nitrogen represents insoluble nitrogen. Trichloroacetic acid was added to an aliquot of the

filtrate and the precipitate which formed was removed by centrifugation. Nitrogen determinations on the precipitate and supernatant gave values for the soluble non-protein nitrogen contents.

Free amino acids of the water extracts of the tissues were estimated by the procedure of Woiwood (21), as modified by Beauchene and coworkers (3): Five milliliters of the extract were placed in a 15 ml. graduated centrifuge tube. Five milliliters of copper phosphate suspension were added, the volume was adjusted to 15 ml. with water, and the contents of the tube were mixed. The mixture was centrifuged at 2000 rpm. for 5 minutes to sediment the excess copper phosphate. Since the plant extracts were slightly yellow in color and often were slightly turbid, no attempt was made to determine the amino acids by measuring the intensity of the blue color produced at this stage. Instead, the complexed copper was determined by placing 2 ml. of the blue solution in a 40 ml. glass stoppered centrifuge tube and adding 10 ml. of water and 0.1 ml. of 2 per cent sodium diethyldithiocarbamate solution. Twenty milliliters of isoamyl alcohol were added and the tube was shaken vigorously to transfer the yellow copper diethyldithiocarbamate to the alcohol phase. The mixture was centrifuged for 5 minutes to break the emulsion, and the absorbance of the alcoholic phase was measured at 435 mμ with a Beckman DU spectrophotometer. A blank was carried through the procedure to correct for traces of copper in the extract and reagents. The readings obtained were evaluated with a standard curve prepared with alanine solutions.

Changes in electrical conductivity are shown in Figure 1. Conductivity decreased sharply when the plants were grown at 35° F., but did not change appreciably when the growing temperature was 60° F. Based on Dexter's correlations between cold hardiness and decreases in conductivity (7), it was concluded that the cold grown plants had become hardy after three weeks. From previous expe-

riences, they would be expected to survive sudden sub-freezing temperatures better than the plants grown at 60° F. When the hardened plants were returned to the 60° F. chambers, conductivity increased very rapidly, indicating that barley loses hardiness quickly.

Changes in water content are shown in Figure 2. As hardening progressed, the water content decreased and as dehardening occured, the water content increased. However, it did not reach the level of the control plants after seven days of dehardening. The changes in water content were similar to those reported for wheat by Pauli and Mitchell (12).

Total nitrogen content is shown in Table 1. Pauli and Mitchell (13) have discussed the importance of the method of expressing nitrogen values, in view of the concurrent changes which occur in water content. When expressed as milligrams of nitrogen per plant, the hardened plants contained less nitrogen than the unhardened. This indicates a somewhat slower rate of nitrogen absorption per plant from the soil, which would be expected because of the lower metabolic activity. On a fresh weight basis, the hardened plants contained more nitrogen than the unhardened plants. On a dry weight basis the hardened plants in general contained more nitrogen also. Although certain of the values appear to be contradictory, due either to analytical difficulty or to poor sampling, the general trends of the data indicate that the manner of expressing the results may govern the conclusions to be made. Although the hardened plants contained less nitrogen per plant than the unhardened plants, the opposite was true on a weight basis, due to a higher water content and a greater rate of carbohydrate synthesis at the higher temperature.

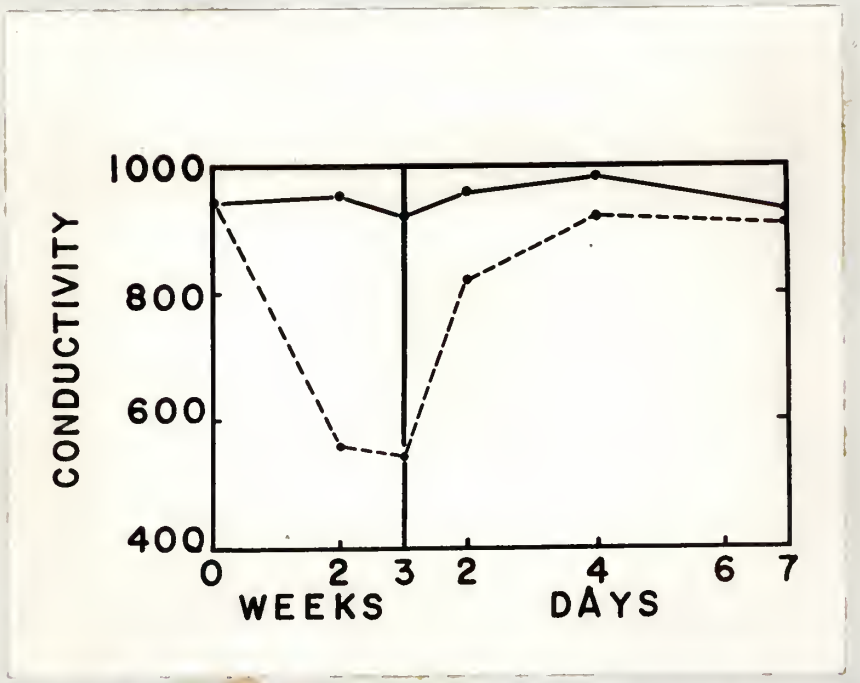

Fig. 1. Effect of hardening and dehardening on the electrical conductivity (ohms $^{-1}$ X 10^{6}) of barley plants.--The broken line refers to hardened plants. The solid line refers to unhardened plants.

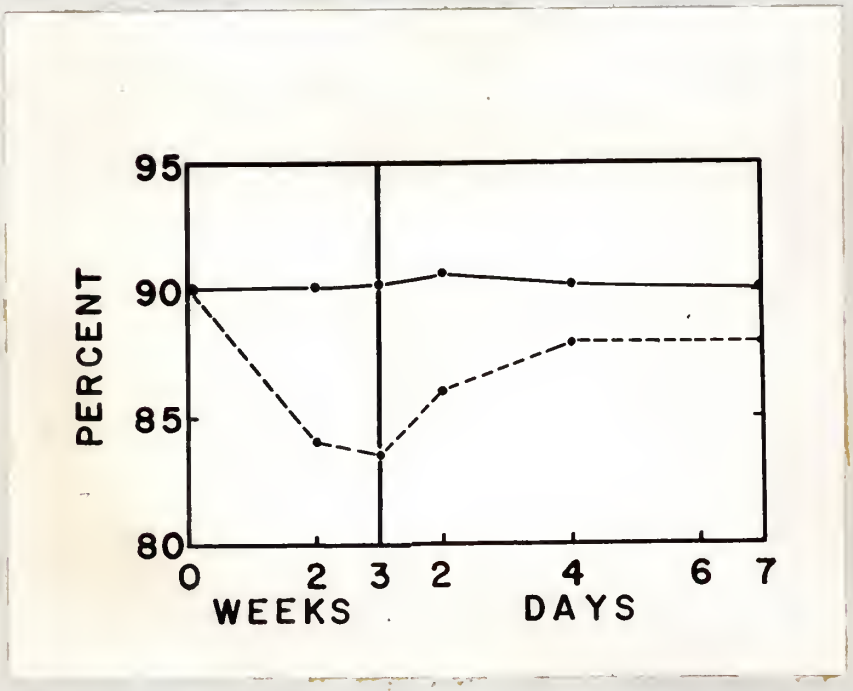

Fig. 2. Effect of hardening and dehardening on the water content of barley plants. —The broken line refers to hardened plants. The solid line refers to unhardened plants.

Table 1. Effect of growth temperature on the total nitrogen content of barley plants.

Method of expression		weeks of hardening			days of dehardening		
		0	2	3	2	4	7
mg./ plant	H	7.6	11.6	13.1	11.2	13.2	13.1
	U	7.6	18.7	18.0	13.3	14.0	15.9
mg./10 g. fresh weight	H	47.2	68.1	61.1	70.4	80.2	51.2
	U	47.2	55.7	28.4	34.1	26.6	46.8
mg./10 g. dry weight	H	467	428	375	509	658	420
	U	467	566	316	369	274	466

H=hardened plants, U=unhardened plants.

The data for soluble protein nitrogen are shown in Table 2. Earlier workers consistently expressed their results on a fresh weight basis, and were in agreement that soluble protein content increased during hardening. The fresh weight data of Table 2 would support this conclusions also. Pauli and Mitchell first proposed expresing the amount of a given nitrogen fraction as per cent of total nitrogen. When this was done, they found no significant change in soluble protein nitrogen during hardening of wheat. Since the data of Table 1 indicated changes in dry matter composition and water content during hardening, the soluble protein nitrogen was calculated as per cent of total nitrogen to eliminate these changes. When expressed in this manner (Table 2), it became clear that growth of barley at hardening temperatures did not alter the proportion of the total nitrogen that was soluble protein nitrogen.

Table 2. Effect of growth temperature on soluble protein of barley plants.

Method of expression		weeks of hardening			days of dehardening		
		0	2	3	2	4	7
mg. nitrogen/ 10 g. fresh weight	H	32	34	35	38	46	28
	U	32	28	16	20	15	27
% of total nitrogen	H	68	51	57	54	57	55
	U	68	51	56	58	57	58

H=hardened plants, U=unhardened plants.

Changes in free amino acids are in Figure 3. When expressed on a dry weight basis, the data indicate much more free amino acids in the hardened plants than in the unhardened plants. On a "per plant" basis, however, there appears to be little difference between the two groups of plants. In view of the moisture changes which occured, it is clear that part of the apparent increase on a fresh weight basis during hardening was due to the greater quantity of dry matter per gram of sample taken for analysis. Since the unhardened plants contained more total nitrogen per plant than the unhardened plants, expressing the free amino acid content as "mg. per 10 plants", or as "mg. per 10 g. dry weight" also may lead to erroneous conclusions because of lack of a constant basis for comparison. However, expressing the data as "per cent of total nitrogen" eliminates the effects of changes in water content, total nitrogen, and proportion of the major components of the dry matter. Such an expression should be a more accurate indication of possible changes in equilibria among the nitrogenous constituents of the plant.

When the data were expressed as per cent of total nitrogen, the hardened

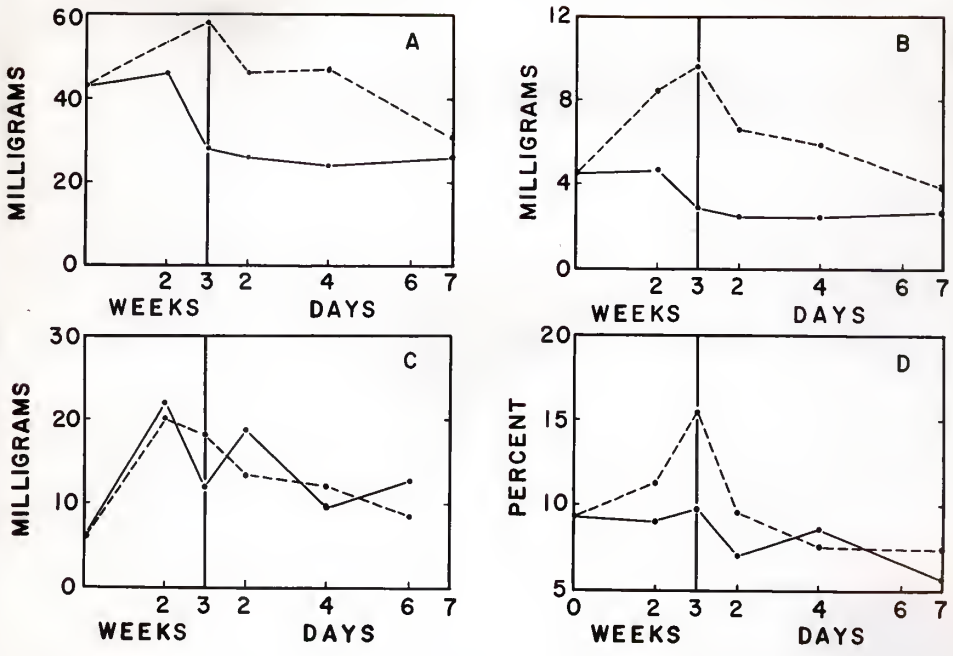

Fig. 3. Changes in free amino acid content with hardening of barley plants.
A. mg. nitrogen per 10 g. dry weight.
B. mg. nitrogen per 10 g. fresh weight.
C. mg. nitrogen per 10 plants.
D. per cent of total nitrogen
The broken line refers to hardened plants. The solid line refers to unhardened plants.

plants contained more amino acids than unhardened plants. As the plants dehardened, free amino acids decreased until there was little difference between the two groups of plants. The free amino acid changes thus were similar to the changes previously reported for wheat (12). An increase in free amino acids thus may be a general characteristic of the hardening process.

The increase in free amino acids during hardening suggests an alteration in enzyme activity. The alteration may consist of a change in enzyme concentration, or it may be that, with lowering of the growth temperature, the activities of certain enzymes decrease more than others. Either possibility could result in an increased proportion of amino acids.

An exploratory study of a method for detecting changes in enzyme activity was made. It seemed desirable to use native substrate and to avoid loss of portions of the enzyme systems by conducting the study without extracting the enzymes. An *in situ* method thus was needed. The usual procedure for plant tissue is to hasten autolysis of the tissue by various killing agents.

Samples of barley tissue were cut into small pieces with scissors and placed in screw-cap wide-mouth jars. Three milliliters of toluene, ether, acetone, or chloroform were added to a jar, after which the jar was capped and placed in an incubator operating at 36° C. After 18 hours the samples were analyzed for free amino acids. A sample of unautolyzed tissue was analyzed to determine free amino acids initially present in the tissue. The results of this study are shown in Table 3.

Toluene was the most effective agent for enhancing enzyme activity in the tissue. Ether and acetone may have been less effective because of their greater volatility and loss around the jar cap. Chloroform also was tested, but the results were extremely variable from one experiment to the other, and hence was

not suitable for use.

Proteolytic enzymes often become inactive by oxidation when extracted, and frequently activators such as cyanide and sulfide are added during an assay to prevent such inactivation (1). Even though the enzymes are not extracted in an *in situ* method, it seemed possible that oxidation could occur following death of the tissue.

Table 3. Production of free amino acids in barley tissue during incubation with various autolyzing agents at 36° C. for 18 hours.

Treatment	Free amino acids	Increase
	mg. nitrogen per 10 g. fresh weight	per cent
Unautolyzed	6.06	-
Autolyzed with toluene	9.82	62
Autolyzed with ether	7.71	27
Autolyzed with acetone	7.77	28

Addition of 2 ml. of 0.1 N sodium sulfide and 3 ml. of toluene prior to incubation did not result in greater amino acid production than toluene alone. Addition of 2 ml. of 0.1 N sodium cyanide appeared to cause a marked increase in free amino acids. However, when this quantity of sodium cyanide was carried through the procedure in the absence of plant tissue, it became obvious that the cyanide solubilized some of the insoluble copper phosphate reagent. Since the amino acid method also depends on solubilization of copper phosphate by complex formation and determination of soluble copper, it was clear that addition of cyanide interfered with the extimation of amino acids. Efforts to increase amino acid production by addition of activators, therefore, were discontinued. The method finally employed for studying enzyme activity was as follows: Samples of barley tissue at the various stages of growth were incubated for 24 hours at 36° C. in the presence of 3 ml. of toluene. The samples then

were analyzed for free amino acids by the method described earlier. The increase in amino acids due to enzymic were obtained by substracting the amino acid values for fresh samples from the values for the incubated samples. These differences are presented in Table 4.

Table 4. Effect of growth temperature on amino acid increase during incubation of barley tissue in the presence of toluene.

Condition of plants	Amino acid increase* weeks of hardening			days of dehardening		
	0	2	3	2	4	7
Hardened	3.7	7.9	7.6	7.6	5.3	12.9
Unhardened	3.7	4.9	10.5	9.8	17.6	6.9

* mg. amino N/ 100 mg. total N

Although no sharp trend in enzyme activity is to be noted from the data, there was a tendency for amino acid formation during incubation to increase as the plants became older. There is some indication also that activity was greater in the unhardened plants. However, the latter trend was not clear cut, since the values for the 2-week and the 7-day unhardened samples appear to be low when compared with the remaining values in this series. It was not possible to duplicate this experiment because the growth chambers were no longer available for use on this project. However, the data are sufficiently consistent to warrant additional study of the method.

This activity method would detect differences due to changes in enzyme concentration. However, it would not detect changes due to differential effects of growth temperatures on reaction rates of the various enzymes involved in nitrogen metabolism. This is true because both hardened and unhardened plants were assayed at the same temperature.

DISCUSSION

The increase in amino acids during hardening of plant tissue is of great interest, inasmuch as it is one of the few chemical changes which has been correlated with the hardening process. However, the fundamental relationship of this change to the hardening process is obscure. Presumably it is only a manifestation of hardening, rather than the cause of it.

In general terms, the metabolism of nitrogen compounds in plants can be summarized as follows:

NO_3^- (from the soil $\longrightarrow$ NH_4^+ $\rightleftharpoons$ amino acids $\rightleftharpoons$ proteins

amides

Since the hardened plants absorbed less nitrogen from the soil than did the unhardened plants, it must be assumed that the increase in amino acids resulted from degradation of protein. The soluble proteins would seem to be the most likely immediate contributors. However, no consistent decrease in soluble protein was detected during hardening. Determination of soluble protein nitrogen is less precise than determination of amino nitrogen. Hence, slight changes in soluble protein content might not be detected. It is conceivable also that soluble protein is maintained at a constant level by solubilization of some of the insoluble protein. Here also the methods available for the detection of any change in this fraction is not sensitive enough.

Undoubtedly the amino acid increases occurred because of unequal changes in activities of the various enzymes involved. The exploratory autolysis study indicated that changes in enzyme concentrations were not responsible. The unequal changes therefore may be due, among other things, to the effects of lowered temperature on rates of the individual enzymes reactions, or perhaps to changes in cell permeability. The latter might alter the effective concentra-

tion of a substrate in the vicinity of its enzyme. The basic cause of the amino acid increase undoubtedly will be difficult to determine.

SUMMARY

Two groups of barley plants were grown, the control group at 60° F. continuosly, and the other group at 35° F. during the hardening period. Hardening was determined by means of electrical conductivity. Changes in dry matter, moisture, and in certain nitrogen fractions during hardening and dehardening were determined.

The water content of barley plants grown at 35° F. was less than that of plants grown at 60° F. The cold grown plants absorbed less nitrogen per plant from the soil.

It was concluded that the best method of expressing the content of a given nitrogen fraction was as per cent of total nitrogen, to eliminate effects of differences in water content and changes in dry matter composition. When expressed in this manner, there was no change in soluble protein nitrogen when the plants were hardened at 35° F. There was an increase in free amino acids as the plants hardened, and a decrease as the hardened plants were dehardened.

An exploratory study of a method was made to determine differences in enzymatic production of amino acids in both groups of plants during autolysis. Under the conditions of autolysis, amino acid production seemed to be more enhanced in the unhardened than in the hardened plants.

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The ability of some plants to survive low temperatures is known as "winter hardiness" or "cold resistance." This phenomenon has great economic importance since soil and air temperatures determine, in part, the ability of a plant to grow in a given region. The fundamental reasons for one variety of plant being more hardy than another still is obscure. The purpose of the study reported here was to determine the changes in some nitrogenous fractions that occur during development of winter hardiness in barley plants.

Dicktoo barley, the hardiest variety presently grown in Kansas, was used in the study. The seeds were planted in soil contained in clay pots, and the pots were placed in a growth chamber operating at 60° F. with a ten-hour photoperiod. After three weeks, half of the plants were moved to a second chamber operating at 35° F., and were grown for an additional three weeks. At the conclusion of this hardening period, they were returned to the chamber operating at 60° F. for dehardening. Samples were removed for analysis at intervals during the hardening and dehardening phases. Samples were taken at the same times from the plants grown continously at 60° F.

The conductivity method of Dexter indicated that the cold-grown plants were in a hardened condition after three weeks, and that dehardening occurred rapidly when the plants were transferred to the 60° F. chambers. Moisture content decreased during the hardening phase and increased during the dehardening phase. On a "per plant" basis, the hardened plants contained less total nitrogen than the unhardened plants, indicating less absorption of nitrogen from the soil.

On a "fresh weight" basis, soluble protein nitrogen increased as the plants hardened, in agreement with results of other workers. However, when calculated as "per cent of total nitrogen", to eliminate changes in moisture content and changes in composition of the dry matter, the relative amount of soluble protein in the hardened plants was essentially the same as in the unhardened plants.

Free amino acids, in a water extract of the plant tissue, were determined by complexing with copper and measuring the amount of complexed copper with sodium diethyldithiocarbamate spectrophotometrically. The free amino acid content was found to increase with hardening and to decrease with dehardening of the hardened plants, independent of the method of expression used.

An exploratory study was made of a method for measuring enzyme activity under in situ conditions, by autolyzing the tissue in the presence of toluene. Under the conditions used, amino acid production seemed to be slightly greater in the unhardened than in the hardened plants.