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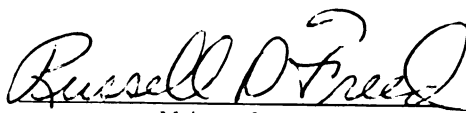
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A Relationship between Carbohydrates and  
Freezing Survival in Winter Barley

presented by

David Palmer Livingston III

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A RELATIONSHIP BETWEEN CARBOHYDRATES  
AND FREEZING SURVIVAL  
IN WINTER BARLEY

By

David Palmer Livingston III

A DISSERTATION

Submitted to  
Michigan State University  
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## ABSTRACT

### A RELATIONSHIP BETWEEN CARBOHYDRATES AND FREEZING SURVIVAL IN WINTER BARLEY

By

David Palmer Livingston III

Past studies have shown that sugar distribution within cells immediately before the onset of stress is critical to a realistic understanding of cryoprotection related to carbohydrates. Because spatial distribution of carbohydrates in cells are difficult to determine, compositional distributions were calculated from total carbohydrate amounts and related to freezing stress. Three barley (*Hordeum vulgare*) cultivars, differing in carbohydrate composition and in response to a controlled freezing test, were hardened under six light levels. At each level plants were freeze-tested and total crown sugars measured by water/ethanol extraction and High Pressure Liquid Chromatography. The log of a sugar to fructan ratio was linearly related to total carbohydrates. Experimental variability was eliminated by calculating simple sugar and fructan amounts at varying carbohydrate levels from the equations for the lines. The relationship between kill temperature and total carbohydrate was different for the three cultivars, suggesting a difference in sugar availability for cryoprotection. At approximately 100 mg carbohydrate/g dry wt. all three cultivars were killed at

approximately  $-10^{\circ}\text{C}$ , the temperature where adhesive stress was reported to begin. When hardened under similar low light conditions, however, the less hardy cultivar Durani accumulated about one half as much carbohydrate as the other two cultivars.  $F_1$ ,  $F_2$  families and backcrosses (BC) of the cultivars were hardened under low and high light and freeze tested. Under low light a dominant effect, causing death in the freeze test, was seen in the cross of Durani with the hardy parent Dictoo. A 13:3 (dead:alive) ratio in the  $F_2$ , 1:1 in  $BC_1$  and 1:0 (dead:alive) in  $BC_2$  implied that the effect was the result of a simply inherited trait controlled by two genes with epistatic action. Carbohydrate data for the parents and  $F_1$  progeny at low light suggest that the trait may be related to supply or demand of sugar or both.

TO

**Maria, Jesse and Rachel**

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS AND DEFINITIONS USED

AOV: Analysis of variance

DF: Degrees of freedom

LSD: Least significant difference

Mean squares: equivalent to variance ( $s^2$ )(42)

$$s^2 = \frac{\sum_i Y_i^2 - (\sum_i Y)^2 / n}{n-1}$$

ns: Differences are not statistically significant at specified probability level.

Simple sugar: sucrose + glucose + fructose

ss/fn: Simple sugar to fructan ratio.

## Introduction

Improving freezing resistance of winter cereals is an important aspect of crop improvement, but environmental and genetic components associated with winterhardiness all interact to produce a complicated system. External factors affecting plants during winter are soil heaving, smothering, snow and ice cover, physiological drought, freeze-thaw cycles, diseases and insects (7,17,44). Infinite levels and unpredictable combinations of these effects make selection of hardy lines in the field very difficult. For example, it is common for winter survival ranking of barley and wheat lines to be different from one year to the next (7,23,38), underscoring the difficulty breeders experience when attempting to improve winterhardiness by selecting in the field. Identifying separate freezing stresses and resistance mechanisms, and then combining favorable characteristics into adapted cultivars may be a more manageable approach.

This reductional approach to freezing resistance has led researchers to correlate almost every measurable biochemical characteristic with the hardening and freezing process in plants, with very little agreement between

authors (17,44). In many instances correlation "between hardiness and a cellular component in either a series of different cultivars or different species or different times of the year has served as justification for the inclusion or exclusion of a particular component in the cold acclimation process.....only if the particular compound were the product of the rate-limiting step in the entire cold acclimation process would such a correlation exist" (44).

The purpose of this study was to examine relationships of carbohydrates to freezing survival and to investigate the genetics of survival in barley.

## REVIEW OF LITERATURE-CARBOHYDRATES

Numerous studies have shown the importance of carbohydrates in cold acclimation and freezing resistance but exact mechanisms are not clear. In fact, the seemingly obscure relationship between carbohydrates and freezing tolerance has prompted some authors to question whether one even exists (45). Sugar cryoprotection seems to fall into three broad categories: 1.) intracellular (protoplasmic) protection 2.) membranes (organelle and protoplast) protection 3.) extracellular and or periplasmic protection (from ice adhesions).

### 1.) Intracellular protection

Intracellular protection mechanisms involving simple sugars are: freezing point depression of cell sap and

prevention of plasmolysis as water is removed during freezing.

Freezing point depression Colligative properties of ideal solutions, depend only on the number of solute particles in solution. One such property is freezing point depression. The freezing point depression constant for dilute, nonelectrolytic solutions of water is 1.86 which means that the freezing point of a one molar solution of water will be lowered by 1.86°C compared to pure water. It seems reasonable then that intracellular sugar accumulation during a hardening phase could, to a certain extent, lower the freezing point and prevent injury by allowing the cell to supercool. Johanssen et al. (quoted by 53) followed the freezing point of wheat at varying solute concentrations and found that freezing points were lowered on a "purely colligative basis" (53). However, Levitt (17) reports the highest recorded vegetative plant cell-sap concentration would lower the freezing point by only 4°C. Therefore, this mechanism, by itself, is not viewed to be very important in freeze protection. While other authors cite similar relationships between solute concentration and freezing resistance (8,17,18,19,53) few attribute protection specifically to freezing point depression. Most simply cite "colligative cryoprotection" as the mechanism which seems to include freezing point depression as well as osmotic protection.

Prevention of plasmolysis: Williams (53) and Levitt (17, and cited by 53) describe a form of stress in plants related to minimum cell volume. Williams defines the term "minimum volume" as:

"some volume below which an individual cell cannot be plasmolyzed without injury which may develop whether during the plasmolysis or during subsequent deplasmolysis and this limit is essentially independent of the concentration of any solute, intracellular or extracellular."(53)

He states that reduced cell volume beyond a critical value "is the principle if not the sole cause of freezing injury" (53). By determining kill temperatures of shrinking cells in solutions varying in solute concentration he found a minimum volume to which cells were reduced before being killed. A study of the relationship of cell volume to freeze damage in wheat not only confirmed the minimum volume hypothesis but showed that wheat changed its lethal cell volume size by altering membrane properties which in turn lowered its kill temperature (53).

Role of carbohydrates Plant cells can use sugars, sequestered or synthesized during hardening, to resist cell volume reduction by changing osmotic pressure. Osmotic pressure ( $\pi$ ) is "the pressure that must be applied to a solution to increase the chemical potential of the solvent to the value of its pure liquid at 1 atmosphere"(4). An equation describing its relationship to solutes (i.e. sugars) is:

$$\pi = -aRT$$

where  $a$  is the activity (equal to molal concentration in

dilute solutions),  $R$  is the gas constant (liters atm mole<sup>-1</sup> °K<sup>-1</sup>) and  $T$  is temperature in degrees Kelvin.

To maintain equilibrium in a system where water is the solvent, net movement of pure water will be from regions of high to those of low water potential (4). Water potential,  $\Phi$ , is defined by:

$$\Phi = \pi + \Phi_p + \Phi_m$$

where  $\pi$  is osmotic pressure (defined above, a negative quantity),  $\Phi_p$  is pressure potential and  $\Phi_m$  is matric potential. If a semipermeable membrane separates two compartments differing only in solute concentration (temperatures are constant) then only solvent (i.e. water) will move from the less to the more concentrated solution. This will cause pressure to increase in the high solute compartment until water potentials are equal in both compartments. At this point net flow of water will cease. In plant cells, with high solute levels in the cytoplasm, differentially permeable membranes, and relatively rigid cell walls, net water movement would be to the cell interior. This would produce a pressure which could resist plasmolysis.

## 2.) Membrane protection

The difficulty of studying a single component of freezing injury in vivo has prompted many researchers to isolate cell constituents, such as membranes, and study their behavior in vitro (12,20,40,45,46). Photosynthetic

phosphorylation associated with chloroplast membranes is a process particularly susceptible to freeze injury and is conveniently measured in vitro. Santairius (40) suggests that one form of freezing injury is an uncoupling of phosphorylation from the electron transport system in thylakoid membranes, preventing photosynthesis. He and other researchers (9,12,45,46) report a protective effect of sugars (sucrose, glucose and raffinose) on phosphorylation. While exact mechanisms are not known they propose that protection is either by reduction of the total number of potentially toxic compounds which come in contact with membranes (dilution) or by direct protein stabilization or both.

Dilution of toxic compounds One commonly recognized, membrane-toxic compound (at elevated concentrations) is NaCl. NaCl caused permanent damage to thylakoids even at temperatures above freezing (12). But in the presence of 0.4 M sucrose, cyclic photophosphorylation was protected 100% when thylakoids were frozen at  $-25^{\circ}\text{C}$  in 0.3 M NaCl (12), while in the absence of sucrose, phosphorylation was completely inhibited at NaCl concentrations below 0.1 M. Other authors (9,45,46) report similar protective effects with glucose, raffinose and glycerol. Other potentially toxic compounds (at elevated concentrations) were  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ , halogenides, nitrates, sulfates, and certain amino acids (12). Precise mechanisms of injury to phosphorylation are not known but Kamienietsky (quoted by 20) was reported

to have obtained subunits of Coupling Factor-1 (CF1) when thylakoids were treated with NaBr. Steponkus reportedly found CF1 released in thylakoid membranes after freezing with no salt treatment (43). He also reported an inactivation of ATPase activity when thylakoids were exposed to elevated NaCl (44) and higher proton uptake in sucrose protected membranes (44).

There is some debate as to whether the protective effect is simple colligative dilution by sugars, preventing, or at least limiting, contact of toxic compounds with thylakoid membranes or whether a more elaborate protection mechanism such as protein stabilization is important (1,12,20,40,44,45,46). Probably a combination of effects occurs depending on freezing processes and concentration of cytoplasmic contents.

Protein stabilization Membranes reportedly are made up of from one-half to two-thirds protein molecules which are imbedded in a lipid bilayer (39). As cytoplasmic water is removed during freezing (see section entitled "Role of carbohydrates"), water of hydration is eventually removed from protein molecules causing permanent alteration of tertiary structure (denaturation) (1,12,20,40,44,45,46). Some authors feel that removal of water alone is enough to cause denaturation (1). Levitt (17) reports that as water is removed sulfhydryl groups lose their hydrogens and will randomly form disulfide bonds. He describes the reaction as shown:



As water is removed one can see that this reaction is favored. The formation of such disulfide bonds may be irreversible and if so will cause severe protein disruption as freezing (or thawing) continues (17). The reaction was apparently inhibited when sucrose was added to the system (17). He has also shown that cells which were hardened have a lower RSSR content in extracted protein when frozen but admits the absolute levels were initially exaggerated due to the extraction procedure (17). Little mention is made by other authors of this theory of injury.

Probably the clearest description of the accepted theory for how sugars stabilize proteins is given by Alden et al. (1).

"Hydrogen bonds between proteins and water...are broken upon removal of water in the formation of ice. Sugars provide hydrogen bonding between the functional water of the lipoproteins or directly to the sensitive lipoproteins of the membrane systems. Sugars, unlike water, are not frozen out and the stabilizing bonds are not ruptured when the plant is subjected to subfreezing temperatures. When water becomes available again in excess, it replaces the bonds provided by sugar because hydrogen bonds are easily reversed." (1)

In support of this theory, the ability of proteins to bind sugar has been demonstrated (12,46) as well as increased sugar levels in chloroplast proteins during hardening in the tea plant (46) and in ivy (quoted by 12). Data are unavailable for sugar binding in winter cereal chloroplast protein but the molecules are similar so a similar sugar binding would probably occur.

### 3.) Extracellular/periplasmic protection:

Olien describes a form of stress called adhesion (25,26,27,29,30,31,32) which is a result of equilibrium freezing [a slow rate of freezing such that very small displacements from equilibrium occur (28)] processes in plants and occurs at temperatures between  $-10^{\circ}\text{C}$  and  $-30^{\circ}\text{C}$  (28). The advancing ice lattice upon reaching the vicinity of the cell wall competes with it for the intervening liquid which causes adhesion between ice and the wall or wall and plasmalemma. As the protoplast shrinks during freezing, adhesions to it can cause considerable damage (29).

The compliance of freezing processes to clearly defined physical laws has led Olien to describe freezing stress using thermodynamic and kinetic principles (28). An explanation of adhesive stress from a thermodynamic viewpoint is shown below.

Figure A illustrates the translational kinetic energy of water molecules at an ice-liquid interface.

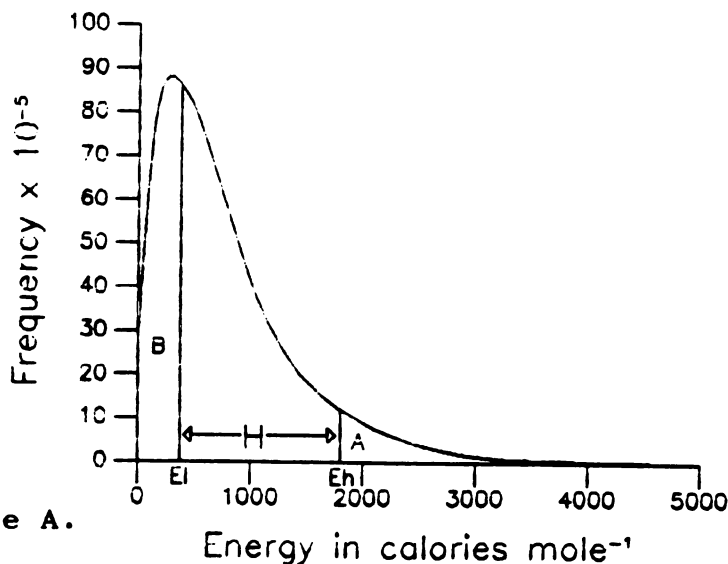


Figure A.

(adapted from 28)

The region "A" represents ice molecules with enough energy to escape the lattice (melt) and "B" represents liquid molecules with energy so low that they will add to the lattice (freeze). As equilibrium freezing progresses the value (energy) of the two integrals represented by regions A and B will be equal. The vertical line at "Eh" is the minimum energy ice molecules must have in order to escape the lattice (activation energy of melting) and "El" is the maximum energy a liquid molecule can have and still add to the lattice (activation energy of freezing). The distance between El and Eh is the energy which must be acquired by a molecule to escape or the amount of energy it gives up as it becomes part of the lattice (latent heat - H).

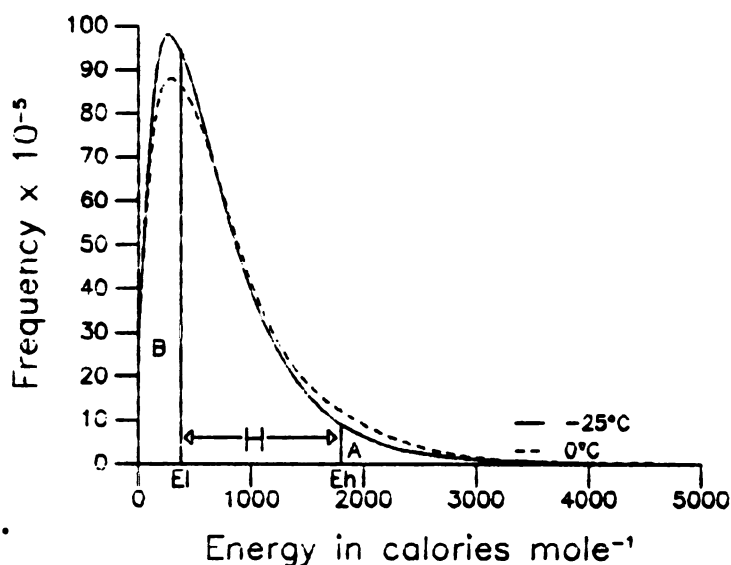


Figure B.

(adapted from 28)

When the temperature is decreased the curve shifts (28), as shown by the solid line in figure B, reducing the

number of molecules with enough energy to leave the lattice (A) and increasing the number that can freeze (B) and freezing continues. Because this is an equilibrium process (A and B are of equal energy) some modification must occur in the system to allow A and B to become equal. One possibility is to decrease B by reducing the number of liquid molecules at the interface through dilution of interfacial water molecules with solutes. A second way for A and B to reequilibrate is to shift the activation energy for melting downward, which increases A. If that occurs one should see a corresponding shift in latent heat -  $H'$  as in figure C. Olien has observed this shift calorimetrically in model cellulose systems (30).

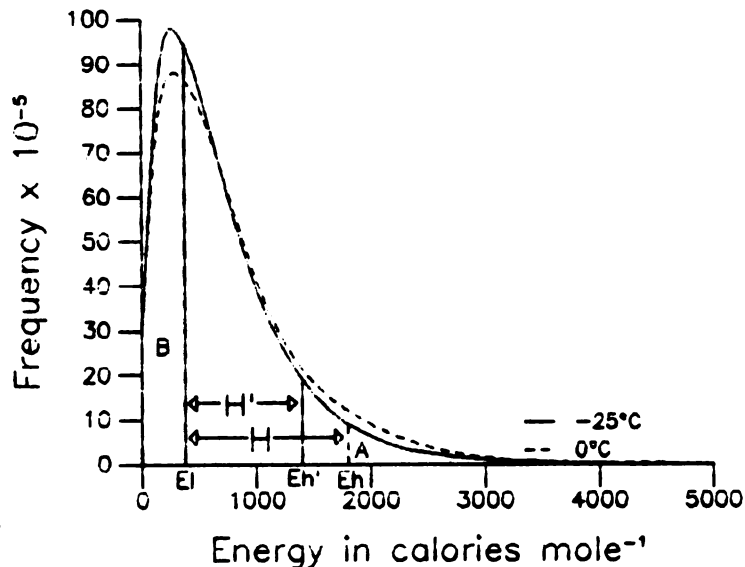


Figure C.

(adapted from 28)

He attributes the latent-heat shift to matrix interaction of ice with hydrophylic polymers as they compete for intervening liquid. This can result in highly

destructive adhesions if competing polymers are part of membranes. Adhesive stress may be relieved by releasing solutes outside the protoplast producing a fluid barrier to adhesions. This causes a change in the number of liquid molecules in the interface without initially affecting ice. Once again A and B are out of equilibrium in figure D.

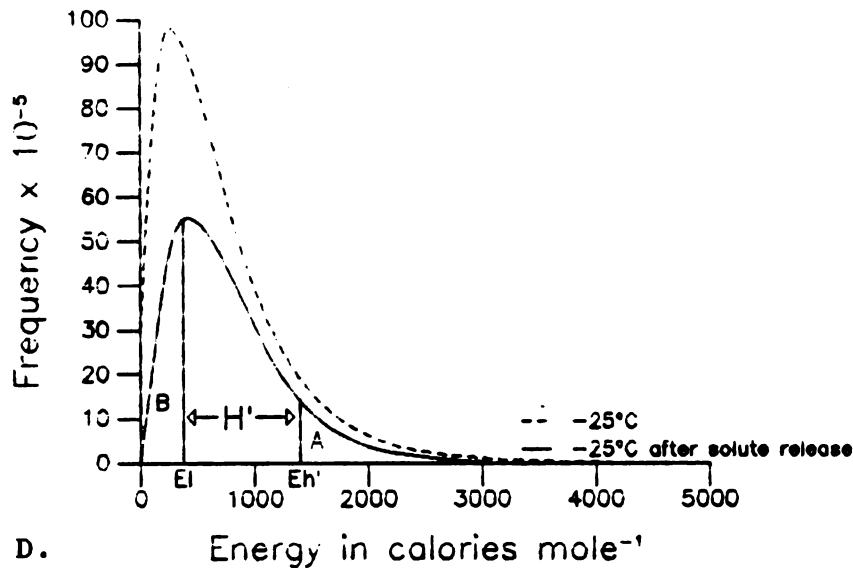


Figure D.

Energy in calories mole<sup>-1</sup>

(adapted from 28)

Molecules with energy to escape are larger in number and melting occurs. For equilibrium to be reestablished the activation energy must again change. A second shift in latent heat [ $H''$ ] (which has been observed experimentally), as shown in figure E, would indicate that this does occur (28)

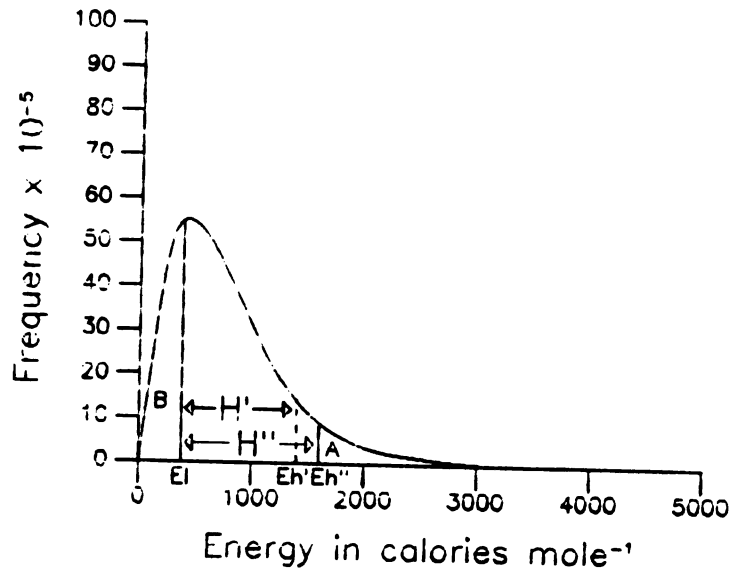


Figure E.

(adapted from 28)

The ice lattice is now some distance from the protoplast with an intervening liquid of higher osmotic pressure than before. This establishes an osmotic gradient between the protoplast and ice crystals. As freezing progresses ice crystals will grow at the expense of protoplasmic liquid causing desiccation.

Once the hypothesis was demonstrated in a model system, Olien showed that this type of protection does occur in winter cereals (26,27). A dilute solute concentration in the extracellular spaces in winter cereals helps prevent the growth of disease-causing microorganisms (29). This allows ice crystals to grow up to hydrophylic cell walls during the freezing process. When frozen, the cell releases sugars outside the protoplast which can maintain a fluid barrier

necessary for prevention of adhesions (26,27). In fact, the hardier cereals release more sugars providing greater protection from adhesion (26,27).

## GENETICS

Most genetic studies involving winter hardiness in cereals have shown freezing resistance to be quantitatively inherited under the control of many genes (2,5,10,13,15,38,51,54). While some authors found freezing resistance to be the result of recessive genes with additive effects (13,15) others reported both additive and non-additive effects controlled by both recessive and dominant genes (5,38). Amirshahi et al. (2) found additive effects with no gene interaction.

In cases where procedures were compared, different hardening and freeze-testing methods revealed different genetic systems for freezing tolerance. Gullord (10,11) and Le (16) reported a difference in ranking of winter wheat cultivars for survival depending on whether a high or a low intensity freezing test was used. In a severe test, Rohde (38) and Worzella (54) reported hardiness was controlled by recessive genes while in a less severe test hardiness was dominant. Roberts (37) found hardiness genes in wheat to be located on chromosomes 2A, 5A and 5B when plants were hardened in the light and on 6A, 3B, 5B, and 5D when hardened in the dark.

## PURPOSE

Two thoughts are important when considering carbohydrate related freeze protection mechanisms: first, sugar distribution in cells, not total sugar [sugar cane contains very high sucrose levels and is not hardy (17)] is fundamental and second, because sugar composition is continually changing (presumably reflecting distributional changes), even below freezing (26,47), it is distribution immediately before the onset of stress that is critical to an understanding of carbohydrate related protection mechanisms.

Distribution: According to Wagner et al. (49,50,52) 80% of the sucrose, and 100% of the fructan, glucose and fructose is contained in the vacuoles of barley leaves. Whether this reflects sugar distribution in crown tissue (the critical region for protection in winter cereals) just before freezing is not known. But Olien has shown that only a portion of total extractable sugars can be perfused from crown tissue without damaging cells (26). If the majority of sugars are sequestered in vacuoles they would presumably not be available for protection. This makes the ability to measure cytoplasmic and/or periplasmic sugar critical if one is to study how sugars are related to winter hardiness.

Sugar changes: Trunova (47) noticed an increase in the hardiness of wheat if plants were exposed to -3°C for three days in the dark. He called this period the "second stage

of hardening"(47). In addition, he observed a decrease in levels of the storage carbohydrate fructan and an increase in fructose and sucrose during this period. He attributed the increase in simple sugars to respiration driven hydrolysis of fructan since photosynthesis was not occurring (47). Olien showed not only the same conversion in rye and barley but an increase in the amount of perfusable sugar after the -3°C treatment (27). An increased perfusable sugar level not only provided critical evidence for an adhesion protection mechanism but also underscored the importance of sugar measurements just before freeze injury.

However, difficulties in using the perfusion technique preclude using it to screen cultivars, so the purpose of this study was to 1) to find an alternate way to measure carbohydrates which would reflect their spatial distribution in crown cells and 2) to determine how that distribution is controlled genetically.

## PROCEDURE

Plant material Three winter barley cultivars were used.

Dictoo: A winter hardy introduction. Parents:  
unknown. Breeder selected.

Hudson: Released in 1951. Parents: Michigan winter  
and Wong. Breeder selected.

Durani: Parents: unknown. Cultivar Introduction # 6316

Eight plants per 12.5 cm pot (the 3 cultivars plus  
Rosen rye which was not extracted; i.e. the four lines were  
planted in duplicate in each pot) were grown in washed sand  
in a growth chamber at 12°C with 18 h/day of light at 175  
 $\mu\text{mol m}^{-2}\text{s}^{-1}$  (80% white fluorescent, 20% incandescent).

Hardening: After five weeks, plants were transferred  
to a hardening chamber at 1 to 2°C and provided with  
continuous light at 3, 7, 25, 45, 80, or 113  $\mu\text{mol m}^{-2}\text{s}^{-1}$  for  
3 weeks. Specific light levels were achieved during  
hardening by covering the appropriate plants with a wire  
frame covered with cotton cheese cloth. A heater coil was  
used to warm pots at lower light levels to bring the  
temperature up to the level of plants under high light.  
Plants were watered daily and fertilized 3 times weekly with  
a Hoagland's solution for the entire growth period.

Prefreeze incubation: After hardening, plants were at  
the three leaf stage with 3 to 4 tillers each. They were  
removed from pots, washed free of sand in ice water, and the

shoots and roots trimmed to four and two cm, respectively. Plantlets (the trimmed plants) were placed in slots cut in slightly damp, circular, cellulose sponges with a pipe flange assembly through the center of each sponge to promote thermostability. One plantlet of each cultivar was placed in a plastic bag for immediate extraction (control) and the other plants from the same pot were placed in sponges for extraction following the prefreeze incubation. This was repeated with 9 more pots per replication [i.e. each sample (replication) contained 10 plants]. A split plot design was used with three replications, two treatments [hardened (H) and hardened then prefrozen (HP)], and three cultivars.

Sponges containing plants to be prefrozen were inoculated with ice, covered with plastic to help prevent desiccation, and placed in a freezer at  $-3^{\circ}\text{C}$  for 60 h in the dark. Thermocouples placed near crowns in the sponges were used to monitor tissue temperature. Because it took 3 to 7 h for the water in the sponges to freeze, actual time at  $-3^{\circ}\text{C}$  was between 53 and 57 h.

After the prefreeze incubation, plantlets were either extracted to determine carbohydrates or freeze-tested to determine survival.

Extraction: Control plants were extracted immediately at  $1^{\circ}\text{C}$  and frozen plants at  $-2^{\circ}\text{C}$  (in a cold room) after 60 h at  $-3^{\circ}\text{C}$  using the same procedure. Plantlet roots were removed and the fibrous tissue at the stem base (zone of

transition between roots and shoots) was grated with a fine-toothed, hand grater. In addition,  $\frac{1}{2}$  cm of the stem, nearest the stem base, was trimmed with a razor blade, combined with the grated tissue and all were placed in a mortar containing 5 ml of aq. 80% (vol./vol.) ethanol. The tissue was mascerated for approximately 1 min with a motor driven mortar and pestle. The ground tissue was transferred to a 100 ml beaker and the mortar and pestle rinsed with 10 ml aq. 80% ethanol which was added to the beaker. The tissue was placed on a 70°C water bath for 10 min to inactivate invertase and shaken for 15 min on a gyratory shaker. The supernatant was transferred to an erlenmeyer flask and the tissue extracted an additional two times with double distilled water (10 ml each) at 21°C. A fourth water extraction contained less than 5% (of total extracted) additional carbohydrate. The combined supernatants were swirled by hand and a 20 ml aliquat was transferred to a 50 ml graduated centrifuge tube; 400 mg of mixed bed resin was added and the mixture was shaken for 10 min (to eliminate ions damaging to the separation column). Samples were centrifuged at 680g for 10 min and 10 ml of the supernate decanted to a preweighed round bottom flask. They were taken to dryness under vacuum at 35°C and placed in a vacuum desiccator over  $P_2O_5$  for approximately 12 h. Samples were weighed, brought to a concentration of 10 mg dry extract per ml double distilled water and passed through a 0.45 micron Millipore filter. Pulp was recovered and dried at 85°C to

constant weight (12 h). Total dry weight consisted of dry pulp plus dry extract.

Chromatography Carbohydrates were separated by high pressure liquid chromatography (HPLC) with a Bio-Rad Aminex HPX-87P column at 85°C. The mobile phase consisted of degassed HPLC grade water (Baker Chemical Co.) and had a flow rate of 0.4 ml/min. A Micromeritics 771 refractometer was used to detect carbohydrates which were quantified by co-chromatography with external standards. Fructan from Hudson barley, collected from the column and freeze-dried, was used as the fructan standard for all cultivars. Because of a consistent linear relationship to absolute amount of standards, peak-height, measured by hand, was used to determine sugar amounts. Retention times in minutes were: fructan 9.2, sucrose 14.8, glucose 18.4, and fructose 25.8. Sugars are reported in mg per gram dry weight of tissue. Simple sugars are sucrose plus glucose plus fructose and total carbohydrate is fructan plus simple sugars.

Freeze test In addition to the three cultivars used in the sugar extractions,  $F_1$ ,  $F_2$ ,  $F_3$  families in the  $F_3$  generation and backcrosses of the cultivars were freeze-tested. Plantlets were treated as above until after the prefreeze treatment at -3°C. The freezer temperature was lowered 1°C per hour to the particular test temperature which resulted in 50% survival (LD-50) of the cultivar being tested. This temperature varied between -22°C and -7°C depending on light levels during hardening (under low light

plants were more tender and were frozen at a higher temperature). A minimum of three freeze tests per cultivar at each light level were used to determine LD-50s. Graphs of temperature verses survival were drawn and the temperature corresponding to 50% survival was taken as the LD-50. Plantlets were held at the test temperature for 1½ hours and then raised 4 to 5°C per hr to 35°C and placed in a cold room.

The following morning plantlets were removed from sponges, trimmed of roots and placed in flats of sand in a growth chamber at 20°C where they were fertilized and watered. After 2 weeks, plants were visually rated on the basis of shoot and root regrowth on a scale of 0 (dead) to 5 (undamaged).

## RESULTS and DISCUSSION - CARBOHYDRATES

Data from the carbohydrate analyses are in three sections: 1) analysis of variances (AOV) for crown sugars at six light levels, 2) the relationship between total carbohydrate and simple sugar to fructan ratio, and 3) the relationship of total carbohydrate to kill temperature.

Four variables were evaluated in the carbohydrate study: 1) fructan, 2) simple sugar 3) total carbohydrate and 4) simple sugar to fructan (ss/fn) ratio.

1) Analysis of variance: In the overall analysis (Table 1), highly significant differences were found for all factors (light, cultivar and treatment) and variables (fructan,

Table 1. Degrees of freedom (DF) and mean squares for carbohydrates in a combined AOV for six light levels.

| Source        | DF | fructan(fn) | sugars(ss) | carbohydrate | ss/fn   |
|---------------|----|-------------|------------|--------------|---------|
| Light (L)     | 5  | 450857**    | 21969**    | 509467**     | 500.8** |
| Replication   | 12 | 4068        | 592        | 2864         | 11.6    |
| Cultivar (C)  | 2  | 17350**     | 38944**    | 7863**       | 39.4**  |
| L X C         | 10 | 2564**      | 1547**     | 4524**       | 9.9**   |
| Error (a)     | 24 | 81          | 59         | 138          | 1.4     |
| Treatment (T) | 1  | 36008**     | 24001**    | 1213         | 204.7** |
| L X T         | 5  | 2883**      | 2978**     | 1067**       | 61.3**  |
| C X T         | 2  | 209         | 455        | 48           | 10.1*   |
| L X C X T     | 10 | 110         | 125        | 273          | 3.1     |
| Error (b)     | 36 | 137         | 158        | 312          | 2.2     |

\*\*, \* Significant at 0.01 and 0.05 levels of probability respectively.

simple sugar, total carbohydrate, and ss/fn) and for some interactions (light x cultivar, light x treatment, cultivar x treatment).

Light: Carbohydrate accumulation occurs during hardening in winter cereals when demand is reduced due to slower growth from lower temperatures (8,17,18,45,47). It is logical then, if other factors are held constant, that higher light levels will increase carbohydrate supply and cause higher overall carbohydrate levels. Studies on shading effects (76,14,24,36,48) as well as Figure 1 confirm this.

light x cultivar: Figure 1 also illustrates the significant interaction between carbohydrate and cultivars (Table 1). At high light Durani has more total carbohydrate than the other two cultivars (not a statistically significant difference, see Table 2) but under low light (7 and 3  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ) Durani has significantly less.

Cultivar: Because an LSD calculated from the overall AOV will bias the mean separation at extreme values, LSD's in Table 2 are calculated from individual experiment AOV's (Table 3). Cultivar differences are discussed in the "ratios" section.

Treatment: At light levels of 113, 80, 45 and 25  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  for every factor (except total carbohydrate)

Figure 1. Total carbohydrate amounts after a 3 week hardening period in three barley cultivars over a range of light levels. Each point represents the mean of 3 replications. Bars above the lines represent LSD values at  $P=.05$ .

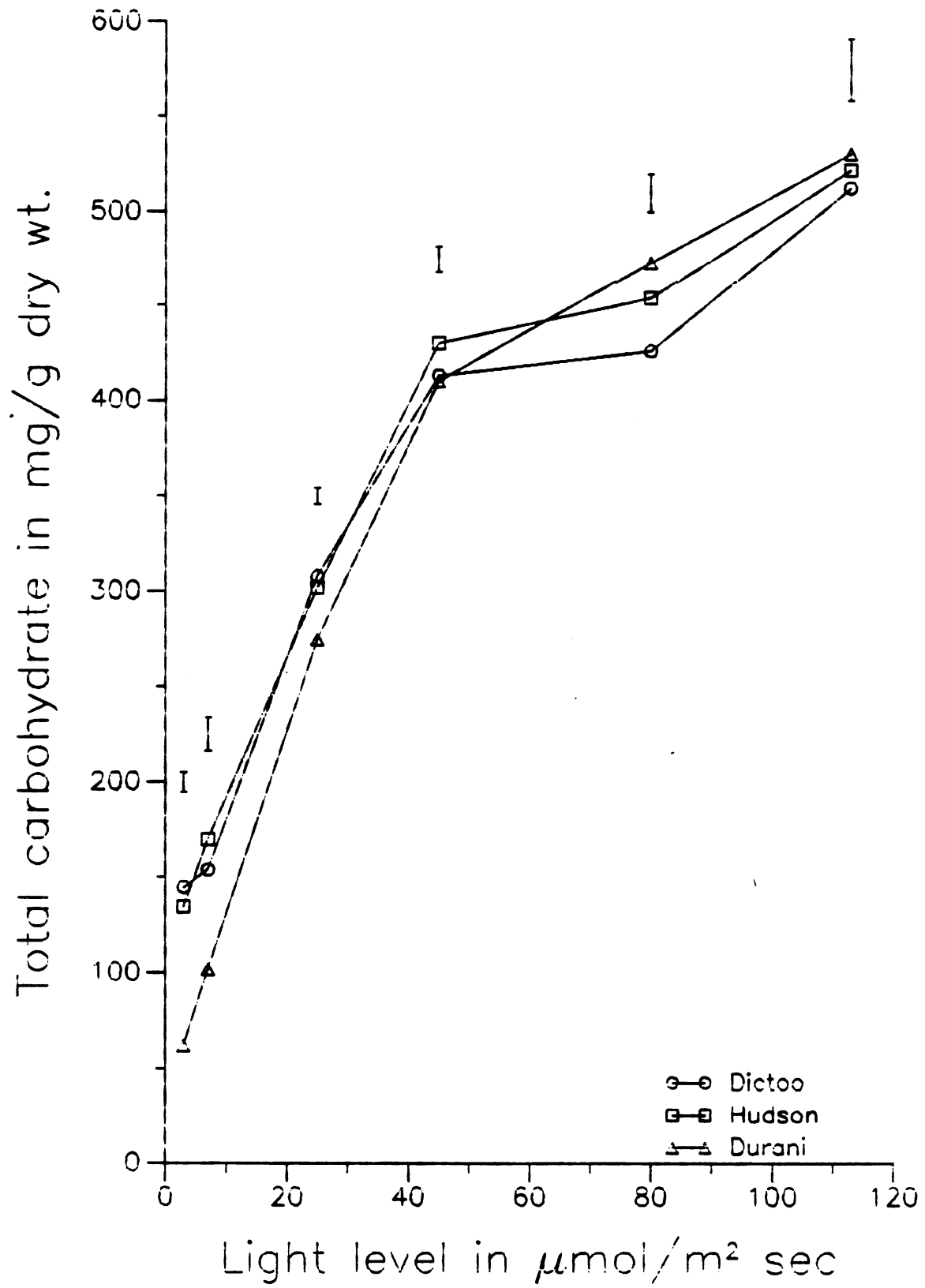


Table 2. Mean (3 replications) carbohydrate amounts and prefreeze changes for three barley cultivars at six light levels.

|                 |          | Mg/g dry weight |             |            |              |             |
|-----------------|----------|-----------------|-------------|------------|--------------|-------------|
| Light           | cultivar | tmt#            | fructan(fn) | simple     | total        | ss/fn ratio |
|                 |          |                 |             | sugars(ss) | carbohydrate |             |
| 113             | Dictoo   | H               | 400bc§      | 113c       | 513a         | 0.28c       |
|                 |          | HP              | 340e        | 165a       | 504a         | 0.49a       |
|                 |          | Change          | -60         | +52        |              | +0.20       |
|                 | Hudson   | H               | 415ab       | 107c       | 522a         | 0.26cd      |
|                 |          | HP              | 350d        | 164a       | 514a         | 0.47a       |
|                 |          | Change          | -65         | +57        |              | +0.21       |
|                 | Durani   | H               | 436a        | 95d        | 531a         | 0.22d       |
|                 |          | HP              | 380cd       | 139b       | 519a         | 0.37b       |
|                 |          | Change          | -56         | +44        |              | +0.15       |
| LSD(.05) Change |          | 26.8            | 7.5         | ns         | 0.04         |             |
| 80              | Dictoo   | H               | 300cd       | 126b       | 426b         | 0.43c       |
|                 |          | HP              | 242e        | 181a       | 423b         | 0.75a       |
|                 |          | Change          | -58         | +55        |              | +0.33       |
|                 | Hudson   | H               | 354b        | 100c       | 454a         | 0.29d       |
|                 |          | HP              | 290d        | 168a       | 457a         | 0.58b       |
|                 |          | Change          | -64         | +68        |              | +0.29       |
|                 | Durani   | H               | 385a        | 88c        | 473a         | 0.23d       |
|                 |          | HP              | 318c        | 140b       | 458a         | 0.44c       |
|                 |          | Change          | -67         | +52        |              | +0.21       |
| LSD(.05) Change |          | 27.4            | 6.3         | ns         | 0.04         |             |
| 45              | Dictoo   | H               | 230c        | 182b       | 413ab        | 0.86b       |
|                 |          | HP              | 158d        | 217a       | 375c         | 1.5a        |
|                 |          | Change          | -72         | +35        |              | +0.64       |
|                 | Hudson   | H               | 277b        | 153c       | 430a         | 0.59bc      |
|                 |          | HP              | 219c        | 178b       | 397b         | 0.85b       |
|                 |          | Change          | -58         | +25        |              | +0.26       |
|                 | Durani   | H               | 304a        | 106d       | 410b         | 0.36c       |
|                 |          | HP              | 272a        | 137c       | 409b         | 0.52bc      |
|                 |          | Change          | -32         | +31        |              | +0.16       |
| LSD(.05) Change |          | 33.2            | 24.2        | 19.1       | 0.15         |             |

\* Light level in  $\mu\text{mol m}^{-2}\text{s}^{-1}$  during 3 weeks of hardening.# Treatment, H=hardened in continuous light, HP=hardened then pre-frozen at  $-3^{\circ}\text{C}$  for 60 hours in dark.

@ sucrose+glucose+fructose

§ Means within a column and light level followed by the same letter are not significantly different from each other at  $P=0.05$ .

Table 2 cont'd

|                 |               | Mg/g dry weight |          |              |             |        |
|-----------------|---------------|-----------------|----------|--------------|-------------|--------|
|                 |               | simple          |          | total        |             |        |
| Light           | cultivar tmt# | fructan(fn)     | sugar@ss | carbohydrate | ss/fn ratio |        |
| 25              | Dictoo        | H               | 92de§    | 216bc        | 308ab       | 2.4b   |
|                 |               | HP              | 72e      | 261a         | 333a        | 3.7a   |
|                 |               | Change          | -20      | +45          |             | +1.3   |
|                 | Hudson        | H               | 118bc    | 184cd        | 302ab       | 1.6bc  |
|                 |               | HP              | 98cd     | 226bc        | 324a        | 2.3b   |
|                 |               | Change          | -20      | +42          |             | +0.7   |
|                 | Durani        | H               | 145a     | 130e         | 275b        | 0.91c  |
|                 |               | HP              | 128ab    | 164de        | 293ab       | 1.3c   |
|                 |               | Change          | -17      | +34          |             | +0.39  |
| LSD(.05) Change |               | 22.9            | 44.7     | ns           | 0.69        |        |
| 7               | Dictoo        | H               | 25a      | 129b         | 154a        | 5.7b   |
|                 |               | HP              | 10b      | 153ab        | 163a        | 15.5a  |
|                 |               | Change          | -15      | +24          |             | +9.8   |
|                 | Hudson        | H               | 32a      | 138ab        | 170a        | 4.4b   |
|                 |               | HP              | 12b      | 157a         | 169a        | 14.9a  |
|                 |               | Change          | -20      | +19          |             | +10.5  |
|                 | Durani        | H               | 23a      | 79c          | 102b        | 3.8b   |
|                 |               | HP              | 10b      | 62c          | 71c         | 8.6b   |
|                 |               | Change          | -13      | -17          |             | +4.8   |
| LSD(.05) Change |               | 10.3            | ns       | ns           | 6.0         |        |
| 3               | Dictoo        | H               | 11b      | 134a         | 145a        | 12.3bc |
|                 |               | HP              | 6b       | 122ab        | 128b        | 19.8a  |
|                 |               | Change          | -5       | -12          |             | +7.5   |
|                 | Hudson        | H               | 20a      | 116b         | 135ab       | 6.7d   |
|                 |               | HP              | 7b       | 114b         | 122b        | 15.4b  |
|                 |               | Change          | -13      | -2           |             | +8.7   |
|                 | Durani        | H               | 7b       | 56c          | 62c         | 9.3cd  |
|                 |               | HP              | 4b       | 43d          | 46c         | 12.8bc |
|                 |               | Change          | -3       | -13          |             | +3.5   |
| LSD(.05) Change |               | 8.9             | 12.8     | 21.8         | 4.0         |        |

\* Light level in  $\mu\text{mol m}^{-2}\text{s}^{-1}$  during 3 weeks of hardening.

# Treatment, H=hardened in continuous light, HP=hardened then pre-frozen at -3°C for 60 hours in dark.

@ sucrose+glucose+fructose

§ Means within a column and light level followed by the same letter are not significantly different from each other at  $P=0.05$ .

Table 3. Degrees of freedom (DF) and mean squares for carbohydrates at six different light levels.

| light | Source        | DF | fructan  | sugar    | carbohydrate | ss/fn ratio |
|-------|---------------|----|----------|----------|--------------|-------------|
| 113   | Replication   | 2  | 762      | 82.0     | 629          | 0.002**     |
|       | Cultivar (C)  | 2  | 2284*    | 833**    | 418          | 0.014**     |
|       | Error (a)     | 4  | 271      | 18.0     | 415          | 0.0004      |
|       | Treatment (T) | 1  | 16,388** | 11,667** | 400          | 0.158**     |
|       | T x C         | 2  | 39.0     | 76*      | 7.0          | 0.002**     |
|       | Error (b)     | 6  | 180      | 14.0     | 275          | 0.0004      |
| 80    | Replication   | 2  | 3493**   | 361      | 2637**       | 0.019*      |
|       | Cultivar (C)  | 2  | 9950**   | 2365**   | 2734**       | 0.100**     |
|       | Error (a)     | 4  | 113      | 91.0     | 153          | 0.003       |
|       | Treatment (T) | 1  | 17,915** | 15,025** | 127          | 0.347**     |
|       | T x C         | 2  | 24       | 101**    | 116          | 0.005**     |
|       | Error (b)     | 6  | 188.1    | 10.0     | 171          | 0.0004      |
| 45    | Replication   | 2  | 19,324** | 819**    | 12,528**     | 0.368*      |
|       | Cultivar (C)  | 2  | 13,321** | 9275**   | 644*         | 0.794**     |
|       | Error (a)     | 4  | 14.4     | 41.6     | 69.6         | 0.047       |
|       | Treatment (T) | 1  | 13,162** | 4154**   | 2527**       | 0.511**     |
|       | T x C         | 2  | 636      | 37       | 620*         | 0.080**     |
|       | Error (b)     | 6  | 276      | 146      | 91.6         | 0.006       |
| 25    | Replication   | 2  | 582*     | 1813**   | 342*         | 1.12        |
|       | Cultivar (C)  | 2  | 4431**   | 12,680** | 2239**       | 5.75**      |
|       | Error (a)     | 4  | 60.7     | 122      | 29.9         | 0.256       |
|       | Treatment (T) | 1  | 1606**   | 7394**   | 2108         | 2.93**      |
|       | T x C         | 2  | 7.60     | 55.0     | 26.3         | 0.350       |
|       | Error (b)     | 6  | 132      | 502      | 910          | 0.120       |
| 7     | Replication   | 2  | 197*     | 385*     | 1075*        | 48.9*       |
|       | Cultivar (C)  | 2  | 62.0     | 10,844** | 12,073**     | 32.3*       |
|       | Error (a)     | 4  | 14.9     | 58.2     | 120          | 3.51        |
|       | Treatment (T) | 1  | 1138**   | 297      | 272          | 312**       |
|       | T x C         | 2  | 21.0     | 747      | 639          | 14.5        |
|       | Error (b)     | 6  | 26.5     | 218      | 207          | 9.05        |
| 3     | Replication   | 2  | 48.0     | 98.0     | 281*         | 19.0        |
|       | Cultivar (C)  | 2  | 109*     | 10,689** | 12,373**     | 50.0*       |
|       | Error (a)     | 4  | 8.91     | 21.3     | 40.3         | 4.31        |
|       | Treatment (T) | 1  | 207*     | 352*     | 1098*        | 195**       |
|       | T x C         | 2  | 35.0     | 61.0     | 4.8          | 11.0        |
|       | Error (b)     | 6  | 19.8     | 57.9     | 119          | 4.01        |

\*\*, \* Significant at 0.01 and 0.05 levels of probability respectively.

differences were found for treatment effects. This confirms past findings (21,26,48,) and could be the result of respiration as suggested by Tumanov (48) or mass action effects as cell contents are concentrated due to water removal during freezing (Olien-personal communication) or both. No significant change in total carbohydrate during the treatment was found except for experiments at 45 and 3  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  (Tables 2 and 3). This suggests a carbohydrate proportional shift without preferential loss of any one component. The two experiments with significant changes are probably a result of procedural error because there is no continuity between the occurrence of the two changes and it is rare for the before and after prefreeze treatments not to have the same carbohydrate levels.

Light x treatment: As light levels increase so do changes in fructan and simple sugars (Table 2). For all three cultivars prefreeze induced changes are at a maximum at 80  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  (Table 2). At very low light levels (7 and 3  $\mu\text{mol m}^{-2}\text{sec}^{-1}$ ) no significant increase in simple sugar was found; the significant change in Table 3 (compared to the mean) at 3  $\mu\text{mol m}^{-2}\text{sec}^{-1}$  is a result of simple sugar decrease in Durani. It is possible that at this light level the demand for simple sugar has exceeded the supply by fructan hydrolysis and sequestered simple sugar is being consumed. However, the change is barely significant at  $P=.05$  so procedural error is also possible.

Cultivar x treatment: While Olien (26) found significant differences in prefreeze-induced fructan hydrolysis between rye and barley, none were found between the three cultivars in this study at any light level. At  $80 \mu\text{mol m}^{-2}\text{sec}^{-1}$ , however, a highly significant interaction between simple sugar increase and cultivars was found (Tables 2 and 3). An LSD infers that Hudson's simple sugar increased to a greater extent than Dictoo or Durani. If the source of increased simple sugars is fructan then there may be genetic differences between cultivars for fructan hydrolysis but experimental variability in this test is too great to detect them.

2) Ratios: All data presented are from a total extraction of crown tissue; therefore no information is available on carbohydrate distribution within and around cells, the critical factor in sugar cryoprotection. One simple way to express carbohydrate data in the form of a distribution is to divide simple sugars by fructan [a distribution between types of carbohydrates (compositional distribution)].

If demand for simple sugar remains relatively constant [respiratory pools are apparently maintained at the expense of storage and exogenous carbohydrate ( 3)] then as carbohydrate supply is increased the storage component (fructan) should increase and the ratio would decrease. At low light levels (see Table 2) the ratio for Hudson after the prefreeze was 15.4 while at high light it was 0.47. In fact, a linear relationship was found when plotting the log

of the ratio against total carbohydrate (Figure 2). For all three cultivars the correlation coefficient was at least 0.97 and was highly significant.

From the equations for the lines, simple sugar and fructan amounts can be calculated (thereby eliminating experimental variability) and plotted over a range of carbohydrate amounts. This allows visual comparison of fructan, simple sugar and their shifts during the prefreeze incubation (Figure 3). The plots show: 1) a simple sugar maximum between 300 and 350 mg carbohydrate which then begins to decrease. Possibly when incoming simple sugar exceeds a certain level it is diverted to the storage pool. This may be analogous to what Pollock (33) found after transferring plants to cold temperatures. Sucrose accumulated to ca. 10 mg/g fresh wt. after 3 days and then began to decrease while fructan levels increased. Labhart et al. (14) suggested a threshold level of sucrose and short chain fructans is necessary before significant fructan synthesis begins. 2) noticeable freeze-induced changes begin at 100 to 150 mg carbohydrate and reach a maximum around 400 mg carbohydrate. It is at this maximum that statistically significant differences between cultivars were seen for simple sugar increase (Tables 2 and 3). 3) Durani accumulates carbohydrate in the form of fructan to a greater extent than either Durani or Hudson (leaving less simple sugar available for cryoprotection). This could be advantageous for a cultivar not specifically bred to cope

Figure 2. The log of simple sugar to fructan ratio at different total carbohydrate amounts in three barley cultivars before and after a prefreeze treatment. Each point represents the mean of 3 replications.

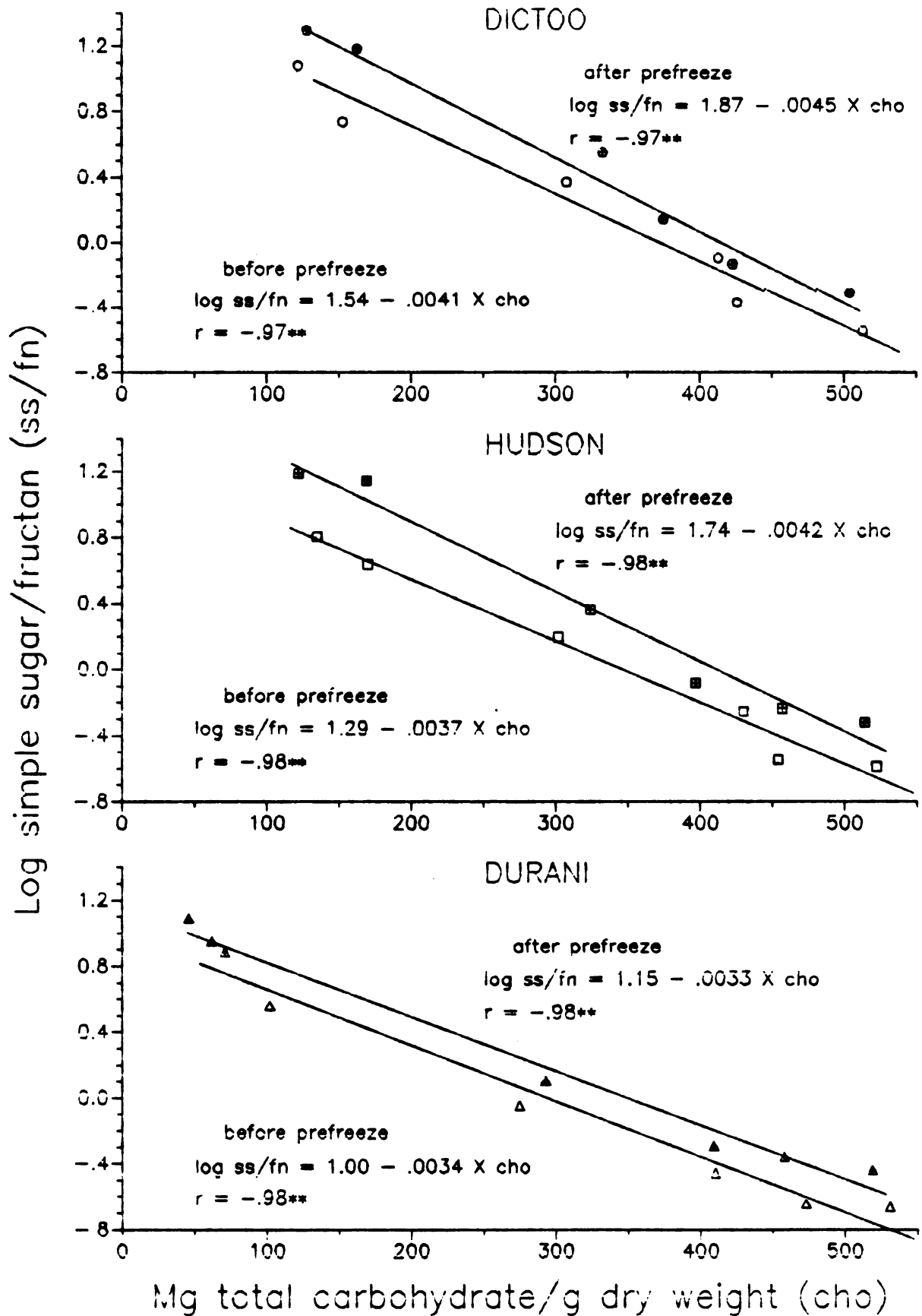
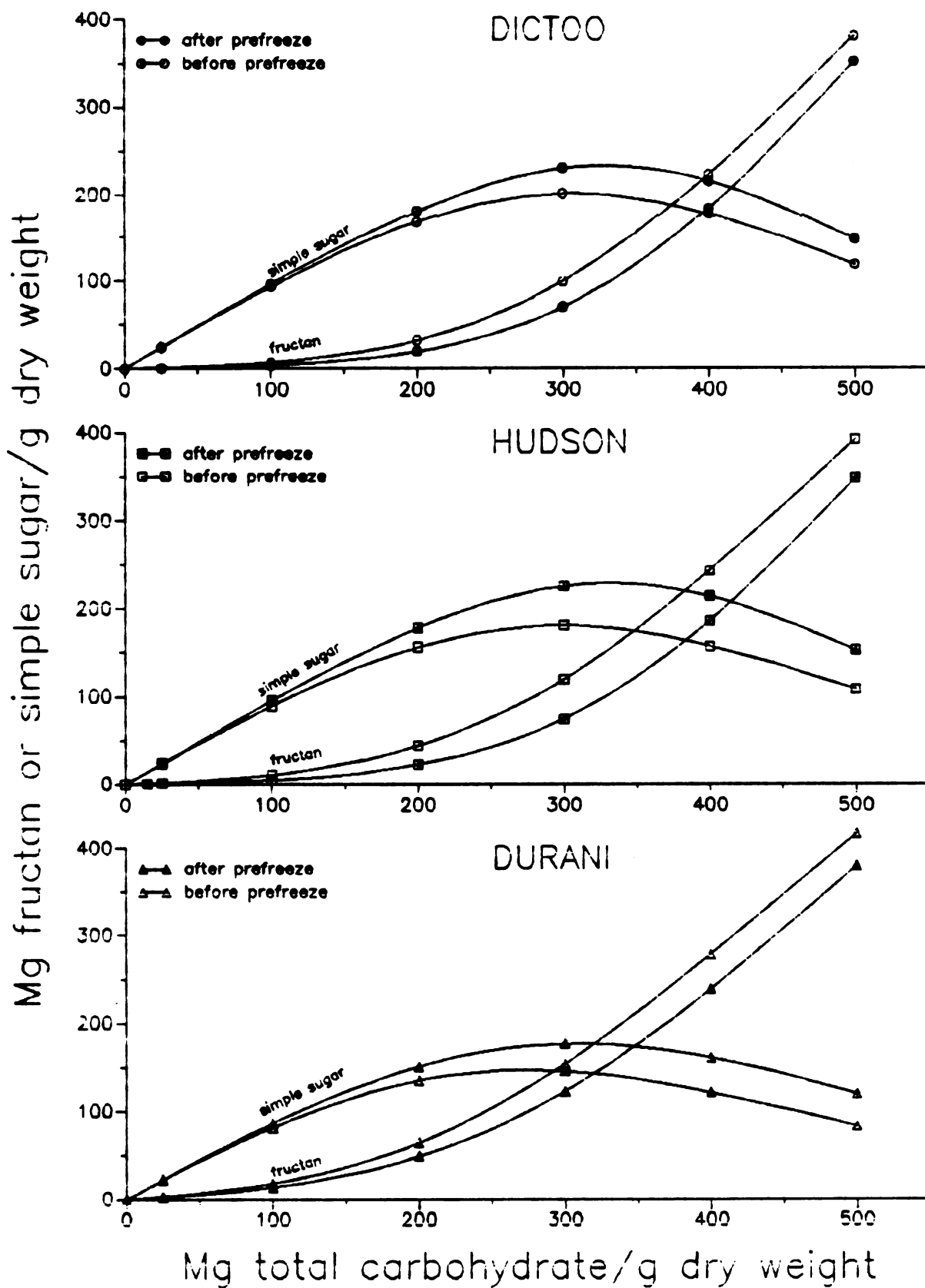


Figure 3. Simple sugar and fructan amounts at different total carbohydrate levels for three barley cultivars before and after prefreeze calculated from the equations for the lines in figure 2.



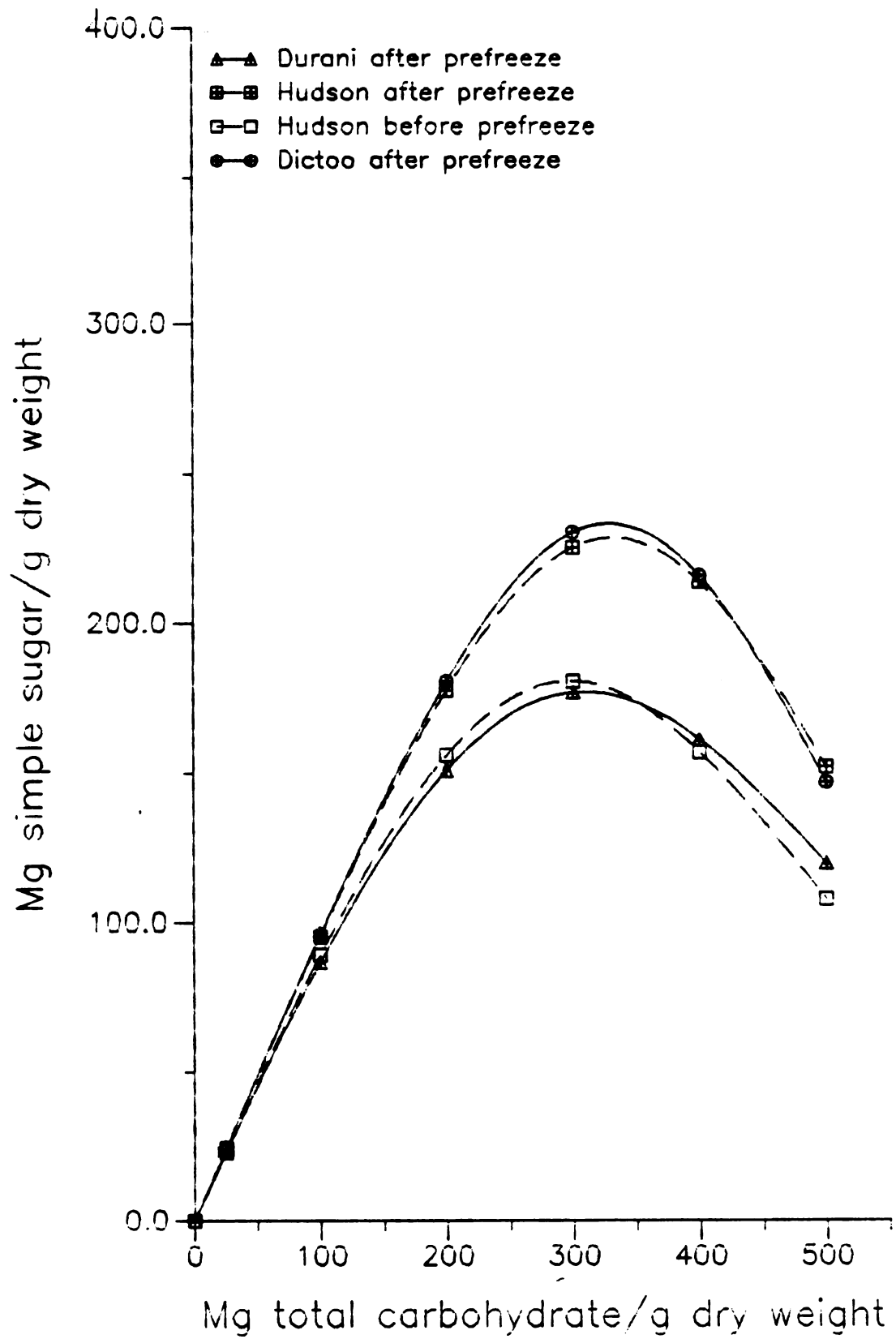
with a harsh winter environment. Where mild winters and favorable conditions for fall carbohydrate storage occur Durani may have an advantage due to high stored fructan levels, allowing more rapid regrowth in spring.

When calculated simple sugars (from Figure 3) for the three cultivars are plotted on the same graph, differences between the three cultivars are apparent (Figure 4). Dictoo has a relatively high simple sugar level both before and after freezing. Hudson has a low simple sugar level before freezing but because its shift is greater than Dictoo it tends to approach Dictoo after the prefreeze. Durani has less simple sugar before and after prefreeze than either cultivar. If simple sugar increase during the prefreeze is important in the cryoprotective process (26,48) then enhancing Dictoo's conversion may improve its freezing tolerance.

**3) Carbohydrate and Freeze tests:** Because these data do not supply information on actual distribution of carbohydrate within plant cells and interstitial spaces expectations of a simple correlation between carbohydrate fractions and kill temperature would be somewhat naive.

However, if total carbohydrate increases, over a range of light levels, and demand (i.e. respiration) for sugar remained the same (3) then the "surplus" sugar would be available for storage or cryoprotection or both. If true, then, while actual distributions are unknown, increasing total carbohydrate should increase overall freezing

Figure 4. A comparison of the three barley cultivars for calculated simple sugar levels from figure 3. Dictoo and Durani are values after a prefreeze; Hudson (dotted line) is before and after a prefreeze.



tolerance. Figure 5 illustrates the relationship of total carbohydrate to kill temperature. If, with increasing carbohydrate levels, a portion of the additional (beyond that needed for respiration and storage) carbohydrate is allocated for cryoprotection then the difference in the relationship of kill temperature to total carbohydrate (slope) between the three cultivars, indicates a difference in the allocation of additional carbohydrate for cryoprotection; this suggests differences in cellular distribution. The three cultivars also appear to converge at about 100 to 150 mg carbohydrate. At this carbohydrate level all three cultivars kill at about -10°C which is the temperature where adhesive stresses (28) begin to be a factor in cell death.

When raised under the same low light conditions, however, Durani has significantly less carbohydrate than the other two cultivars (see Figure 1). Therefore, after being hardened under identical light conditions and freeze tested, Dictoo and Hudson were killed at around the same temperature but Durani was always killed at a much higher temperature.

## RESULTS - GENETICS

At high carbohydrate levels differences between cultivars for total carbohydrate/g dry wt. were not statistically significant (Tables 1 and 2). Most genetic studies of winter hardiness were performed with plants hardened under high light (5,10,15,38,51,54) and genetic

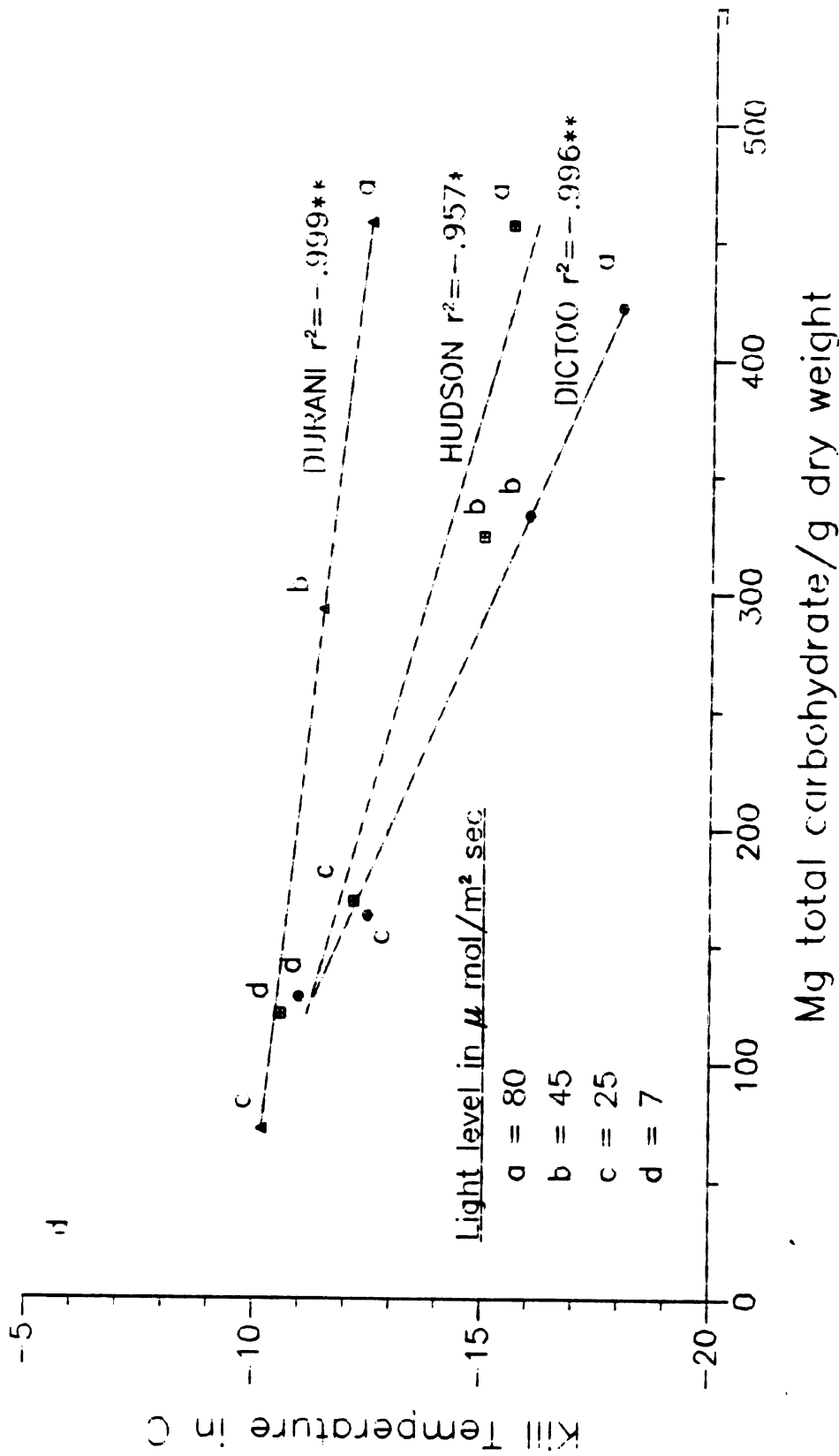


Figure 5. Kill temperature (LD-50) of three barley cultivars over a range of carbohydrate levels after prefreeze. Each point represents a minimum of 3 freeze tests with at least 20 plants (replications) per test. Common letters adjacent to data points indicate freeze tests where plants were hardened under the same light conditions. The letter "d" at ca.  $-6^{\circ}\text{C}$  was a test in which the cultivar Durani was so tender that its kill temperature could not be determined.

effects were found to be complex. Under low light, (see Figure 5) differences in kill temperature between Durani and the other two cultivars were more related to amount of total carbohydrate than at high light. For example, while the kill temperature of the three cultivars converged when carbohydrate was restricted, under the same low light conditions, carbohydrate levels between Durani and the other two cultivars were significantly different (Figure 5 letters c and d).

Freeze test and carbohydrate data for the three parents and their  $F_1$  progeny are shown in Table 4. Differences between reciprocal crosses were not significant so their results were pooled.

Table 4. Survival rating in a freeze-test (see procedure) and carbohydrate amounts for three barley cultivars and their  $F_1$  progeny hardened at a light level of  $7 \mu\text{mol m}^{-2}\text{s}^{-1}$ .

| Cultivar   | survival# | mg carbohydrate/g dry wt |              |              |
|------------|-----------|--------------------------|--------------|--------------|
|            |           | fructan                  | simple sugar | carbohydrate |
| Dictoo(Di) | 2.03a§    | 22a                      | 122a         | 144a         |
| Hudson(HU) | 1.17b     | 17ab                     | 92b          | 109cd        |
| Durani(Du) | 0.00d     | 8c                       | 51e          | 59g          |
| $F_1$      |           |                          |              |              |
| Di/Hu      | 2.42a     | 16b                      | 115a         | 130b         |
| Midparent  | 1.60**    | 19.5ns                   | 107ns        | 135ns        |
| Di/Du      | 0.41cd    | 9c                       | 85bc         | 95de         |
| Midparent  | 1.02**    | 15.0**                   | 87ns         | 101ns        |
| Hu/Du      | 0.28cd    | 6c                       | 64d          | 71fg         |
| Midparent  | 0.58ns    | 12.5**                   | 72ns         | 84*          |

# mean of 60 plants (replications); visually rated 0=dead to 5=undamaged.

§ Means within a column followed by the same letter are not significantly different from each other at  $P=0.05$ .

\*\*, \*  $F_1$  is different from the midparent at 0.01 and 0.05 levels of probability respectively.

Highly significant differences from midparent values in several crosses (Table 4) indicated the presence of dominant effects. While Dictoo had a highly significant dominant effect for hardiness in the Dictoo/Hudson cross, in a Dictoo/Durani cross the effect was apparently masked by a dominant effect for non-hardiness in Durani.

Under conditions of this test, when crossed with Hudson, Durani's dominant effect for non-hardiness was not observed. This could be due to either different genetic effects in that cross or too harsh a test causing Hudson to have a lower mean survival rating and consequently lower midparent value (if frozen at a higher temperature Hudson would have a higher survival rating while Durani could still have a survival of zero; this would raise the midparent value and possibly show a dominant effect in the cross). Durani also showed a highly significant dominant effect for reduced fructan levels.

Because dominance effects were greatest in the Dictoo/Durani cross (under conditions described in Table 4) this cross was considered the most likely to show the effect of a simply inherited system in a freeze test under those conditions; consequently, while some data was taken on all possible crosses (see appendix), only this cross was analyzed in detail. In addition, because the number of samples needed to do an adequate genetic analysis of carbohydrate inheritance was so large only freeze test data was taken.

F<sub>2</sub> Seeds from eighty F<sub>2</sub> families were classified according to rachilla hair length [a simply inherited trait (6)] and were not significantly different from a 3:1 (long:short hair) ratio. They were allowed to self and 12 F<sub>3</sub>-plants per F<sub>2</sub> family (960 total plants) were freeze tested along with 96 backcross<sub>1</sub> (bc)<sub>1</sub> and 40 bc<sub>2</sub> individuals. All plants were hardened under low light and test frozen at -12°C under conditions where survival for Dictoo and Durani was 100 and 0 percent, respectively. Plants were allowed to recover for 2.5 weeks at 22°C and were rated as either dead or alive.

A chi square was performed on observed F<sub>2</sub> ratios. Expected F<sub>2</sub> values were calculated on the basis that F<sub>2</sub> heterozygotes would be segregating in the F<sub>2</sub> generation. So a 13:3 (dead:alive) ratio in the F<sub>2</sub> generation would give a 49:15 (dead:alive) ratio in the F<sub>3</sub>. The results indicated that observed values were significantly different (at p=0.001) from both a 3:1 (dead:alive) F<sub>2</sub> ratio and a 15:1 (dead:alive) F<sub>2</sub> ratio but were not significantly different from a 13:3 F<sub>2</sub> ratio (Table 5).

Table 5. Observed and expected dead:alive F<sub>2</sub> ratios, and Chi square value for segregating plants of the Dictoo/Durani cross after a freeze test.

|            | observed | expected |
|------------|----------|----------|
| Dead       | 746      | 735      |
| Alive      | 214      | 225      |
| Chi square | 0.64     |          |

A 13:3 F<sub>2</sub> ratio implies that two genes control the effect with one epistatic to the other. A model showing possible F<sub>2</sub> genotypes and their proportions is shown below. "A" is a gene whose effect results in death when dominant; it is epistatic to "B", a gene resulting in death when recessive.

| F <sub>2</sub><br>proportion | genotype | gene action                         | phenotypic<br>expression |
|------------------------------|----------|-------------------------------------|--------------------------|
| 9/16                         | A_B_     | B dominant, but overridden by A.... | dead                     |
| 3/16                         | A_bb     | b recessive, A dominant.....        | dead                     |
| 3/16                         | aaB_     | a recessive, B controls expression. | alive                    |
| 1/16                         | aabb     | a recessive but b is also.....      | dead                     |

If Dictoo is the genotype aaBB and Durani AAbb then the F<sub>1</sub> would be AaBb and backcrosses should result in the following combinations:

| Parental<br>gametes |    | F <sub>1</sub> gametes |      |      |      | ratio<br>dead:alive |
|---------------------|----|------------------------|------|------|------|---------------------|
|                     |    | AB                     | Ab   | aB   | ab   |                     |
| Dictoo              | aB | AaBB                   | AaBb | aaBB | aaBb | 1:1                 |
| Durani              | Ab | AABb                   | AAbb | AaBb | Aabb | all dead            |

A backcross with Durani resulted in 39:1 (dead:alive) which was within the confidence limits for a frequency of zero alive (42). A backcross with Dictoo had a ratio of 55:41 (dead:alive) and a chi square of 1.76 which is not significantly different from a 1:1 ratio.

#### DISCUSSION - GENETICS

Martin (22) found lower respiration rates and higher "conserved" carbohydrates in hardier rye and wheat cultivars. While the present study provides no evidence to confirm that low fructan levels are an indication of higher

respiration rates, if fructan accumulates when supply exceeds demand (34,35) then low fructan suggests either higher demand or lower supply of carbohydrates or both. If true then Durani's dominant factor could be a respiration promoter. For example, dominant A would cause high respiration resulting in little or no sugar availability for cryoprotection. Dominant B (in Dictoo) could be a respiration inhibitor which causes low respiration allowing sugar accumulation. If b is recessive respiration would not be inhibited and would result in low sugars and a higher kill temperature (less hardy).

If A is a respiration promoter, which reduces sugars available for cryoprotection, and if adequate sugars are provided, supply would exceed demand and the effect of A could be masked; in this case sugar cryoprotection would be adequate and the plant would die at a lower kill temperature (more hardy) and possibly from different stresses. In fact when hardened under high light Durani's carbohydrate level is not significantly different from Dictoo or Hudson (table 2) and the dominant effect for non-hardiness is not observed (table 6); in contrast, a dominant effect for hardiness is seen in the Dictoo/Durani cross under these conditions (Tables 6-see appendix for complete set of crosses at high light). This could explain Roberts (37) finding genes for hardiness on different chromosomes depending on whether plants were hardened in the dark or in the light. However, more information is needed to confirm this.

Table 6. Mean survival rating for three barley cultivars, their F<sub>1</sub> progeny and midparent values, hardened at 113  $\mu\text{mol m}^{-2}\text{s}^{-1}$ .

| Parents |                  | F <sub>1</sub> |                 |
|---------|------------------|----------------|-----------------|
|         | <u>survival#</u> |                | <u>survival</u> |
| Dictoo  | 3.62a§           | Di/Hu          | 3.45ab          |
|         |                  | Midparent      | 2.56**          |
| Hudson  | 1.50d            | Di/Du          | 2.98b           |
|         |                  | Du/Di          | 2.35c           |
| Durani  | 0.10e            | Midparent      | 1.86**          |
|         |                  | Hu/Du          | 1.08d           |
|         |                  | Midparent      | 0.8             |

# mean of 60 plants (replications); visually rated 0=dead to 5=undamaged.

§ Means within a column followed by the same letter are not significantly different from each other at P=.05.

\*\* F<sub>1</sub> is different from the midparent at 0.01 level of probability.

## CONCLUSION

Difficulties in improving winter hardiness of existing crops have prompted many investigators to try and reduce protective mechanisms to individual simply inherited traits. When considering carbohydrate related protective mechanisms sugar distribution immediately before the onset of stress is critical to an understanding of this important category of cryoprotection (26,47). Because current techniques are not amenable to screening cultivars for sugar partitioning the aim of this study was to derive a measurement from total extractions which would reflect carbohydrate partitioning in plant crowns and would relate to freezing tolerance.

Specific findings were:

1) A simple sugar to fructan ratio, expressed as a log, was found to have a linear relationship to total carbohydrate/g dry wt. When equations for the lines were used to calculate simple sugar and fructan amounts (which eliminated experimental variability) at varying total carbohydrate levels the following differences were apparent:

a) simple sugar/g dry wt. increased to a maximum at about 300 to 350 mg total carbohydrate/g dry wt. and then began to drop as total carbohydrate/g dry wt. increased b) the cultivar Durani allocated more of its total carbohydrate to

the storage pool, fructan, than the other two cultivars c) prefreeze-induced simple sugar/g dry wt. change was at a maximum at the maximum simple sugar level (300 to 350 mg total carbohydrate/g dry wt.) and the change during the prefreeze was greatest in the cultivar Hudson.

2) As the level of total carbohydrate was lowered, kill temperatures increased at different rates in the three cultivars, (suggesting a difference in the amount of sugar allocated for cryoprotection) and converged at a carbohydrate level of approximately 100 mg carbohydrate/g dry wt. At this point all the cultivars were killed at around -10°C, the temperature at which adhesive stress was reported to begin (27). When hardened under the same conditions, however, the less hardy cultivar Durani accumulated significantly less carbohydrate than the other two cultivars and consequently was killed at a higher temperature.

3) When hardened under low light and freeze tested such that survival of Dictoo and Durani is 100 and 0 percent, respectively, chi square tests of  $F_2$  and backcross progeny from the Durani/Dictoo cross indicated that two genes were operative. Durani had a dominant gene expressed as freezing susceptibility and was epistatic to a dominant gene in Dictoo expressed as freezing resistance. Carbohydrate data suggest that the genes may be related to supply or demand of sugars or both, but more study is necessary to confirm this.

## APPENDIX

Table A1. Survival rating and frequency for each rating class of three barley cultivars and their progeny hardened under low and high light

| HIGH LIGHT (113 $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) |           |                          | LOW LIGHT (7 $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) |                |                          |
|-------------------------------------------------------|-----------|--------------------------|----------------------------------------------------|----------------|--------------------------|
|                                                       | survival  | frequency<br>0 1 2 3 4 5 |                                                    | survival       | frequency<br>0 1 2 3 4 5 |
| PARENTS                                               |           |                          |                                                    |                |                          |
| Dictoo                                                | 3.55a     | 0 2 2 5 5 6              |                                                    | 2.90abcd       | 0 6 3 4 1 6              |
| Hudson                                                | 1.55fg    | 8 3 3 3 2 1              |                                                    | 2.35cde        | 7 2 2 1 2 6              |
| Durani                                                | 0.15l     | 18 1 1 0 0 0             |                                                    | 0.00j          | 20 0 0 0 0 0             |
| F <sub>1</sub>                                        |           |                          |                                                    |                |                          |
| Di/Hu                                                 | 3.05abc   | 0 3 3 6 6 2              |                                                    | 3.50ab         | 2 3 0 2 4 9              |
| Hu/Di                                                 | 3.30ab    | 0 1 3 7 7 2              |                                                    | 3.30abc        | 1 3 4 1 3 8              |
| Di/Du                                                 | 2.15def   | 3 3 7 3 3 1              |                                                    | 1.10ghi        | 10 4 3 1 1 1             |
| Du/Di                                                 | 1.65fg    | 6 3 6 2 3 0              |                                                    | 0.80ghij       | 14 2 1 1 1 1             |
| Hu/Du                                                 | 1.15ghijk | 10 5 1 1 2 1             |                                                    | 1.15ghi        | 14 1 0 0 3 2             |
| Du/Hu                                                 | 0.60kl    | 12 6 0 2 0 0             |                                                    | 0.20ij         | 18 1 0 1 0 0             |
| F <sub>2</sub>                                        |           |                          |                                                    |                |                          |
| Di/Hu                                                 | 2.50cde   | 3 3 2 5 7 0              |                                                    | 2.65bcd        | 6 1 0 5 3 5              |
| Hu/Di                                                 | 2.85abcd  | 1 1 5 7 5 1              |                                                    | 3.30abc        | 2 4 0 1 6 7              |
| Di/Du                                                 | 1.35ghij  | 6 5 6 2 1 0              |                                                    | 1.40fg         | 11 1 3 0 4 1             |
| Du/Di                                                 | 1.45fghi  | 9 2 3 4 1 1              |                                                    | 1.10ghi        | 13 1 2 1 1 2             |
| Hu/Du                                                 | 0.80hijkl | 12 3 3 1 1 0             |                                                    | 0.30hij        | 17 2 0 0 1 0             |
| Du/Hu                                                 | 0.50kl    | 16 2 0 0 2 0             |                                                    | 0.10j          | 18 2 0 0 0 0             |
| BACKCROSS                                             |           |                          |                                                    |                |                          |
| Di/Hu//Di                                             | 3.15abc   | 2 2 1 6 4 5              |                                                    | 3.80a          | 0 3 0 4 4 9              |
| Hu/Di//Di                                             | 3.50a     | 0 2 3 3 7 5              |                                                    | 3.60a          | 1 1 2 4 5 7              |
| Hu/Di//Hu                                             | 2.10ef    | 4 4 4 3 4 1              |                                                    | 2.50cde        | 9 0 0 1 3 7              |
| Di/Hu//Hu                                             | 1.50fgh   | 7 4 4 3 1 1              |                                                    | 2.60bcd        | 3 5 2 1 5 4              |
| Di/Du//Di                                             | 2.65bcde  | 2 2 3 8 4 1              |                                                    | 2.20def        | 6 4 1 3 1 5              |
| Du/Di//Di                                             | 2.60bcde  | 1 3 5 6 4 1              |                                                    | 1.65efg        | 5 5 4 4 2 0              |
| Di/Du//Du                                             | 0.65jkl   | 14 2 2 1 1 0             |                                                    | 0.00j          | 20 0 0 0 0 0             |
| Du/Di//Du                                             | 0.65jkl   | 14 2 2 1 1 0             |                                                    | 0.10j          | 19 0 1 0 0 0             |
| Hu/Du//Hu                                             | 0.75ijkl  | 8 9 3 0 0 0              |                                                    | 0.85ghij       | 14 1 1 3 0 1             |
| Du/Hu//Hu                                             | 0.60kl    | 13 4 1 2 0 0             |                                                    | 1.20gh         | 12 2 1 1 3 1             |
| Hu/Du//Du                                             | 0.10l     | 18 2 0 0 0 0             |                                                    | 0.05j          | 19 1 0 0 0 0             |
| Du/Hu//Du                                             | 0.65jkl   | 15 1 1 2 1 0             |                                                    | 0.00j          | 20 0 0 0 0 0             |
| Total                                                 |           | 212 85 75 85 72 31       | 300                                                | 55 30 39 54 82 |                          |

Table A2. Estimates of standard deviation (sd) for carbohydrate factors from Table 2. Values were calculated by taking the square root of variance estimates ( $\theta^2$ ). A nonsignificant f test is assumed to give an expected sd of zero.

| source    | mg/g dry wt. |       |              | fn/ss ratio |
|-----------|--------------|-------|--------------|-------------|
|           | fructan      | sugar | carbohydrate |             |
| Light     | 157          | 34    | 168          | 5.2         |
| Cultivar  | 22           | 33    | 14           | 0.98        |
| L X C     | 19           | 15    | 26           | 0.37        |
| treatment | 26           | 21    | 0            | 1.9         |
| L X T     | 17           | 18    | 9            | 2.6         |
| C X T     | 0            | 0     | 0            | 0.66        |

Model and error mean squares used in calculations for variability estimates in Table A2:

$$Y_{ijkl} = \mu + L_i + R_j + C_k + (LC)_{ik} + \alpha_{jk} + T_l + (LT)_{il} + (CT)_{kl} + (LCT)_{ikl} + \beta_{ijkl}$$

Expected Mean Squares:

| Source        | EMS                                                       |
|---------------|-----------------------------------------------------------|
| Light (L)     | $\sigma^2_b + t\sigma^2_a + ct\sigma^2_r + rct\theta^2_l$ |
| Replication   | $\sigma^2_b + t\sigma^2_a + ct\sigma^2_r$                 |
| Cultivar (C)  | $\sigma^2_b + t\sigma^2_a + rlt\theta^2_c$                |
| L X C         | $\sigma^2_b + t\sigma^2_a + rt\theta^2_{lc}$              |
| Error (a)     | $\sigma^2_b + t\sigma^2_a$                                |
| Treatment (T) | $\sigma^2_b + rcl\theta^2_t$                              |
| L X T         | $\sigma^2_b + rc\theta^2_{lt}$                            |
| C X T         | $\sigma^2_b + rl\theta^2_{ct}$                            |
| L X C X T     | $\sigma^2_b + r\theta^2_{lct}$                            |
| Error (b)     | $\sigma^2_b$                                              |

Legend:

l = 6

r = 3

c = 3

t = 2

$\theta^2$  = estimate of variation

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