Metabolic Changes Associated with Vernalization of Wheat
I. Carbohydrate and Nitrogen Patterns

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Summary. A spring wheat (Triticum aestivum) and an obligate winter wheat (Triticum compactum) variety were each grown for 5 weeks in controlled environments at 20° and 25°. The threshold for flower induction in the winter wheat was 4 to 5 weeks at 20°, whereas the spring wheat had no low temperature requirement for flowering. Changes in the levels of carbohydrate and nitrogen fractions in the wheat leaves were determined during their growth in the cold and warm environments. There was an enhanced accumulation of the 5 carbohydrate fractions in both wheat varieties grown at 20° compared to 25°. Highly significant differences in the levels of sucrose, oligosaccharides, and starch were found between the spring and winter varieties grown at 20°. The winter wheat seedlings grown at 20° accumulated much more of these carbohydrates than the corresponding spring wheat. The carbohydrate patterns in both varieties grown at 25° were nearly identical except for the final 2 weeks of growth.

The level of nitrogenous substances in the tissues grown at 20° was much higher than in the corresponding tissues grown at 25°. The only significant difference between the spring and winter varieties was in the soluble protein fraction. This fraction rose nearly 3-fold in the winter variety grown at 20°, whereas it remained nearly constant in the similarly grown spring wheat. Most of the changing chemical patterns observed in relation to the vernalization treatment appear to be metabolic alterations associated with low temperature rather than alterations directly related with the vernalization response.

Data in the literature suggest that carbohydrate and nitrogen changes occur in cereal plants during exposure to cold. Most reports dealing with these changes have been concerned with metabolic patterns related to frost hardiness, and most of the analyses were performed on field-grown plants. Although we are concerned here with the physiology of vernalization rather than with frost hardiness, the reports on metabolic changes at low temperature are pertinent.

Several papers reported an increase in reducing sugars in winter varieties during cold treatment (1, 4, 5, 12, 14, 16, 18, 20). Some papers indicate few if any differences between the soluble sugar content of spring and winter cereal varieties grown in the cold (2, 9). Others (5, 8) indicate that the winter varieties accumulate more soluble sugars than spring varieties when both are grown in cold environments.

During vernalization of barley, Sparmann (16) found that the soluble protein fraction decreased briefly, then increased considerably while the amino acid content followed the opposite course. Pauli and Mitchell (11) reported increases in the soluble protein nitrogen, soluble nonprotein nitrogen and free amino acid fractions of winter wheat plants during the first 2 weeks of cold treatment at 20°. These 3 fractions gradually decreased during the subsequent 4 weeks of cold treatment, but the nonsoluble nitrogen fraction increased slowly throughout the cold treatment. Comparable plants grown at 20° instead of 25° generally had lower levels of nitrogen in all 3 fractions. Zech and Pauli (20) found no change in the nonsoluble protein nitrogen content of winter wheat leaves during the hardening process in field plantings; but the soluble protein nitrogen, soluble nonprotein nitrogen, and total nitrogen fractions all increased gradually with the onset of winter. Pauli et al. (10) also correlated the increase in soluble protein nitrogen of winter wheat varieties with their cold hardiness. Zech and Pauli (21) demonstrated an increase in total free amino nitrogen in cold-grown winter wheat.

These data suggest major changes in carbohydrate and nitrogen metabolism in cereal plants grown at low temperatures. The qualitative and quantitative evaluation of these changes, however, in spring vs.

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winter varieties and normal vs. cold environments, is still to be investigated for many cereal varieties and several physiological responses. Our experiments were carried out to clarify these questions in relation to vernalization of wheat. Although the carbohydrate and nitrogen fractions analyzed are probably not close to the biologically active key substances associated with the vernalization response, these data should provide background information for explaining changes in specific substances more directly related to the vernalization process.

Materials and Methods

Plant Material. The wheat varieties used in this study were Elgin, a winter wheat (Triticum compactum Host), and Red Bob's, a spring wheat (T. aestivum L.). This winter wheat has an obligate cold requirement for flower induction of about 4 to 5 weeks. Seeds of both varieties, obtained from Dr. R. J. Metzger, United States Department of Agriculture, Corvallis, were planted in washed sand in plastic boxes. Germination and initial seedling growth occurred in the dark at 20°. Six days after seeding, one-half of the boxes containing the young etiolated seedlings were moved into a controlled-environment growth chamber; the other one-half were moved into a controlled-temperature greenhouse. This procedure of germinating and placing the young wheat seedlings in the growth chamber at 2° and greenhouse at 25° was continued once each week until 5 such sets of seedlings were available. Thus, the seedlings ranged in length of time treated from 0 to 5 weeks. In order to correlate the degree of flowering with specific cold treatments, seedlings (15 per variety per week) representing the weeks of cold treatment (0–5 weeks) were planted in sand and grown in the flower-inductive conditions of the greenhouse.

The environmental conditions in the growth chamber were 2°, 1000 ft-c, 12-hour photoperiod, and 80 to 90% relative humidity. The plants grown in the greenhouse had no vernalizing treatment as the growing conditions were 25° day, 17° night, 1000 ft-c, a 16-hour photoperiod, and 50 to 90% relative humidity. The 16-hour photoperiod was required to induce heading in the greenhouse-grown, long-day wheat plants. This experiment was conducted during the winter months under cloudy skies. Under these conditions, with supplementary light, the environmental conditions in the greenhouse were well controlled. The leaves of the plants, grown in the cold or warm environments for 0 to 5 weeks, were harvested 5 hours after the beginning of the photoperiod and used for analyses. The plants transferred from the 2° to the 25° environment were used only to correlate the degree of flowering with specific cold treatments and they were not used for analyses.

Carbohydrate Analyses. To facilitate extraction 4.00 g of leaf tissue were frozen in liquid nitrogen and ground in a mortar to powder form which was transferred to a conical glass-to-glass homogenizer; 40 ml of 85% ethanol at 50° was added and the mixture was homogenized at 1000 rpm for 2 minutes. The supernatant fluid was filtered off and the residue transferred quantitatively to a Soxhlet extraction apparatus where it was extracted with 85% ethanol for 6 to 12 hours. The ethanolic solutions were combined and evaporated to near dryness at room temperature on a rotary evaporator. The sugars were taken up in a small volume of water and transferred quantitatively to small vials. The vials were placed in a lyophilization chamber, the solutions evaporated to dryness, and the sugars taken up in water; thus 8 ml of solution contained the sugars from 4 g fresh weight of leaf tissue.

The sugar components of the solutions were separated by paper chromatography, and identified and determined by the methods of Shallenberger and Moores (15). The alcohol-soluble oligosaccharides were chromatographed as described. They were eluted as a group and determined by the anthrone method (19) with glucose as the standard.

The sugar-free tissue which remained after the Soxhlet extraction was extracted for starch with a 52% perchloric acid solution (3). The resulting solution was filtered and the residue re-extracted with 52% perchloric acid. The filtered solutions were combined and made to known volume, and the starch content was determined by the anthrone method (19).

Nitrogen Analyses. Prior to extraction 5.00 g leaf tissue were frozen in liquid nitrogen (5–30 min after harvest) and ground in a mortar with the aid of small (ca., 100 μm diam) glass beads. The powdered tissue was then transferred to a conical glass-to-glass homogenizer and 30 ml of the extraction solvent (0.01 N NaOH + 0.4 N NaCl) added. The mixture was homogenized at 1000 rpm for 2 minutes. The homogenized mixture was made to 100 ml with the extraction solvent, then stirred magnetically for 10 minutes. Fractions (2.0 ml) of the stirred mixture were centrifuged at 5000 × g for 15 minutes. The supernatant material was discarded and the residue was retained as the nonsoluble nitrogen fraction. Additional fractions (30 ml) of the stirred mixture were also centrifuged at 5000 × g for 15 minutes. Fractions (1.5 ml) of this supernatant fraction were cooled in an ice bath prior to adding an equal volume of cold 20% trichloroacetic acid to each tube. The mixtures were stirred, to initiate precipitation of the proteins, then centrifuged at 5000 × g for 15 minutes. The precipitate was retained as the soluble protein fraction, and the supernatant was retained as the soluble nonprotein fraction. All fractions were dried in a vacuum oven prior to nitrogen determinations. The nitrogen contents of the above samples were determined according to the micro Kjeldahl nitrogen method of Lang (6).

The free amino acids of the tissue were extracted with 80% (v/v) ethanol. The alcoholic extracts were lyophilized, dissolved, in water and neutralized prior to purification via the methods of Thompson.
et al. (17). The total free amino acids in the purified solutions were determined by the ninhydrin method of Rosen (13).

A factorial analysis (7) was computed for each experiment, considering the effects of variety, temperature, time and their interactions. The residual variance (mean square error) was then used to compute the least significant difference (LSD) between any pair of points. One LSD (5%) for each set of 4 curves is presented in each figure.

**Results and Discussion**

The response of the winter and spring wheats used in this study to low temperature is shown in figure 1. The flowering response of the spring wheat variety, Red Bobs, was not affected by the cold treatment as all spring wheat plants flowered irrespective of the cold treatment. In contrast, the winter wheat variety, Elgin, required a cold treatment for flower induction, and the threshold was between 3 and 4 weeks in the 2° controlled-environmental growth chamber.

In following the biochemical patterns during the growth periods it is noteworthy that the spring wheat was induced to flower and was in the boot stage of heading after about 5 weeks of growth in the 25° greenhouse. The 25° greenhouse-grown winter wheat had no cold treatment; hence it remained vegetative. Although the spring wheat plants were induced to flower and were developing flower primordia, this physiological difference was usually not reflected in the carbohydrate content of the leaves until the fifth week of growth at 25°.

**Carbohydrate Patterns.** The changes in the carbohydrate patterns of the spring and winter wheat varieties during growth at 2° or 25° are plotted in figures 2 to 7. In general, the effect of the growing temperature on the carbohydrate levels was greater than the varietal effect of spring vs. winter wheat.

There was no significant difference in the glucose content of winter and spring wheat at either growing temperature throughout the growing periods (fig 2). The glucose content of the cold-grown plants rose significantly during the first week but decreased nearly linearly in the subsequent 4 weeks. The glucose level in the plants grown at 25° decreased sharply during the first week and remained at a low level throughout the growth period.

The amount of fructose present in the wheat seedlings was lower than any other sugar analyzed. Only traces of fructose were present in seedlings grown at 25°. In general, the data indicate a temperature response rather than a varietal response.

The sucrose content of the spring wheat grown at 2° rose 6-fold during the first week and then retained that level during the subsequent 4-week period (fig 4). The cold-grown winter wheat seedlings had 2 sucrose peaks, at the first and fourth weeks, significantly higher than the sucrose levels in the corresponding spring wheats. The sucrose content of the spring and winter wheats grown at 25° was relatively low and showed no change during the 5-week growth period, except for a 2-fold rise in the spring wheat during the final week.

The oligosaccharide content of the winter wheat grown at 2° increased rapidly during the first 3 weeks and retained a high level during the final 2 weeks. In the spring wheat at 2° and 25° and the winter wheat at 25° the oligosaccharide content was low and remained nearly constant throughout the growing periods.

The starch content also differed in a manner that distinguished the winter from the spring wheat (fig 6). In the cold-grown winter wheat the starch content increased sharply in the final 3 weeks, whereas the starch level in the corresponding spring wheat remained constant. At 25° the starch content of the winter wheat varied only slightly, while starch in the spring variety accumulated rapidly during the final 2 weeks of growth.

The data on total carbohydrates (fig 7) clearly distinguish the winter variety from the spring variety at 2° but not at 25°. There was a significant de-
crease in total carbohydrates in the spring variety at 2° compared to a large increase in total carbohydrates in the winter variety at that temperature. The levels of carbohydrates in the cold-grown plants were generally 2- to 6-fold greater than in corresponding plants grown at 25°.

The decrease in monosaccharides and increase in di- and polysaccharides at 2° were not merely interconversions of sugars for the quantitative increase in di- and polysaccharides was much greater than the corresponding decrease in glucose and fructose. The enhanced accumulation of carbohydrates in the winter variety (fig 7) suggests a greater capacity to produce carbohydrates or a lower capacity to utilize carbohydrates. The nitrogen data indicate few significant intervarietal differences in seedlings grown at 2°, thus suggesting that the high carbohydrate content of the winter variety is due to a greater synthesis of carbohydrates.

**Nitrogen Patterns.** Data illustrating analyses of nitrogen fractions (fig 8-11) show few significant differences between spring and winter varieties grown at the same temperature. The nitrogen levels are, however, affected by temperature with nitrogen generally lower at 25° than at 2°.

In both varieties grown at 2° the nonsoluble nitrogen (fig 8) increased in a nearly linear manner throughout the entire growth period. At 25° both varieties maintained a nearly constant level of nonsoluble nitrogen.

The soluble protein nitrogen (fig 9) of the spring variety grown at 2° increased slightly during the vernalization period as compared to a more rapid and greater protein increase in the winter wheat grown at that temperature. The soluble protein nitrogen pattern in the spring wheat grown at 25° was nearly identical to that of the similarly grown winter wheat.

Few significant changes were evident in the soluble nonprotein nitrogen. In the cold both varieties maintained a nearly constant level of 1.5 mg soluble nonprotein nitrogen per g of tissue during the vernalization period. At 25° a level 30 to 60% lower continued in both varieties throughout the growth period.

The free amino acid levels (fig 11) under vernalizing conditions were nearly identical in both varieties. The initial increase was followed by a rapid decline during the third week, but this was reversed by the rapid accumulations of the final 2 weeks.

The sudden drop in total free amino acids in the third week suggests an enhanced protein synthesis. The soluble protein data indicate an increase in that fraction during the third week, but this of course represents only a small portion of the total protein content of the tissues. The subsequent increase in free amino acids during the final 2 weeks was not correlated with soluble protein changes.

The changing patterns in total nitrogen levels (fig 12) reveal significant temperature and time differences but no varietal difference at either temperature. A uniform increase in the total nitrogen content of both cold-grown varieties was found compared to nearly constant total nitrogen level in both varieties when grown at 25°.

The moisture content of the wheat seedlings all decreased in a nearly linear manner during the growth periods studied. The analytical data illustrated are based on fresh weight calculations. These data were also calculated and plotted on a dry weight basis. Although the shapes of the curves are slightly different the conclusions regarding carbohydrate levels for the varietal and temperature treatments are the same as those illustrated for the fresh weight data. However, the nitrogen data based on dry weight were inconclusive, compared to the fresh weight data.

Various trends are evident in the changing chemical patterns in winter wheat in relation to progressive cold treatment. If winter wheat alone were analyzed, it might appear that these trends were related to the vernalization response. However, when a spring wheat variety, which did not require cold temperature induction for flowering, was included as a control, few chemical differences were found between the spring and winter varieties. Thus, most of the changing chemical patterns observed in relation to the vernalization treatment appear to be metabolic
alterations associated with low temperature rather than alterations directly associated with the vernalization response.

The ideal wheat varieties to use in a study of this type would be an isogenic pair which differed from each other only by the gene or genes governing spring habit. Such ideal wheat varieties were not available. The spring wheat and winter wheat varieties used in this study, although genetically pure lines, were not closely related genotypes. Nevertheless, in view of the considerable data in the literature on carbohydrate and nitrogen patterns in cereal plants (1, 4, 5, 8, 10, 11, 12, 14, 16, 18, 20, 21), presumably other spring and winter wheat varieties have metabolic patterns similar to those presented in this report.

Since temperature has such a strong influence on carbohydrate and nitrogen patterns in wheat plants it is difficult to compare the results obtained in this study of wheat grown at controlled temperatures with the results of field grown plants. However, general agreement was found with previous reports (1, 4, 5, 8, 12, 14, 16, 18) on increases in reducing sugars in wheat plants during cold treatment, as well as on changes in the major nitrogen fractions (11).

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Literature Cited