COMPOSITIONAL CHANGES IN THE DEVELOPING GRAIN OF HIGH- AND LOW-PROTEIN WHEATS. II. STARCH AND PROTEIN SYNTHETIC CAPACITY[1]

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ABSTRACT

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Metabolic properties of the developing kernels of high- and low-protein wheat cultivars (cv. Timgalen and cv. Heron, respectively) were examined in an attempt to account for their genotypically determined differences in protein content. No significant differences in DNA levels were observed in the two cultivars at any stage of development. Both ribosomal and total RNA were significantly higher in Timgalen than in Heron at most stages of development. Only slight differences in ribonuclease activity were observed in the two cultivars. Cut heads of Timgalen wheat, when immersed in solutions of ^{35}S-cystine, incorporated isotope more rapidly into storage protein than did heads of Heron at the same stage of development. The results are consistent with a greater protein synthetic capacity and hence higher rate of conversion of amino acids into protein in Timgalen relative to Heron. Levels of the enzymes sucrose synthase, ADPG pyrophosphorylase, and starch synthase during development suggested that variations in carbohydrate metabolism were not responsible for differences in protein content between the two cultivars, but could be a significant factor in protein variability observed in a cultivar due to environmental influences. Higher growth temperatures, which induced more rapid accumulation of storage carbohydrate, produced higher enzyme levels but lower final storage carbohydrate.

The two Australian wheat cultivars, Heron and Timgalen, differ in their protein content/g dry weight. In an earlier paper (1), it was established that the higher protein cultivar, Timgalen, accumulated more nitrogen and protein per grain. Starch content at maturity, although higher in Heron on a per grain basis, was constant in the two varieties when calculated as a percentage of the total dry weight.

To determine what factors may have contributed to the observed differences, the nucleic acid content and protein synthetic capacity were analyzed during seed development. The results suggest that differences in protein synthetic capacity may be responsible for the variation in protein content of the two cultivars. In addition, three enzymes associated with carbohydrate utilization and accumulation in the seed were assayed during kernel development. The relation of these results to storage carbohydrate accumulation during kernel development is discussed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

High- and low-protein Australian wheats, *Triticum aestivum* cv. Timgalen and cv. Heron, respectively, were grown as described in the preceding paper (1). The methods of dating heads, harvesting, and grain selection are also as described in the preceding paper (1).

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Grain Storage

Grain was stored under liquid nitrogen and was thawed prior to use.

RNA and DNA Estimation

RNA and DNA were extracted from freeze-dried grain using the method of Smillie and Krotkov (2). RNA was estimated by the orcinol method (2), with ribose as a standard. DNA was estimated by the method of Richards (3), with deoxyribose as a standard.

Isolation and Estimation of Total Grain Ribosomes

The method of Lever and Key (4) was modified for the isolation of ribosomes from whole wheat grains. Twenty grains were homogenized in 10 ml of ice-cold buffer (100 m*M* tris-HCl pH 7.5, 400 m*M* sucrose, 50 m*M* KCl, 5 m*M* $MgCl_2$) by an Ultra-Turrax homogenizer (Janke and Kunkel AG) at maximum speed for 10 sec. The homogenizer shaft was washed with 2 ml of ice-cold buffer which was combined with the 10 ml homogenate. The homogenate was further homogenized for 1 min in a glass-Teflon Potter-Elvejham homogenizer using three up-and-down strokes at a pestle speed of 350 rpm, and was then filtered through 9xx flour silk. Subsequent steps were as described by Lever and Key (4). The method of Smillie and Krotkov (2) was used to extract RNA from the ribosomes, except that the initial extraction with methanol $-0.05M$ formic acid was omitted.

Ribonuclease Estimation

Ten grains of wheat were homogenized in 10 ml of ice-cold 0.6*M* sucrose at maximum speed of the Ultra-Turrax for 15 sec. The homogenate was filtered through 9xx flour silk and then assayed for ribonuclease activity according to the method described by Wilson (5). One unit of ribonuclease activity is defined as the amount of enzyme which causes a change in absorbancy of 0.1 under the given conditions.

Culture of Cut Heads of Wheat

Wheat heads were cut 4 cm below the first spikelet and immersed in an aqueous solution of ^{35}S-cystine (0.5 μ Ci/ml, 583 m Ci/mmol), illuminated with fluorescent lighting, and maintained at 22° C for up to 12 hr. When required, 10 grains from each head were removed from the primary and secondary spikelets in the central region of the head.

Extraction of Radioactive Wheat Proteins

Fresh grains were rapidly frozen with liquid nitrogen and freeze-dried. The freeze-dried grain was milled and extracted with sodium pyrophosphate buffer and sodium hydroxide as described in the preceding paper (1).

The cooled sodium pyrophosphate extracts were precipitated by bringing the extracts to 5% trichloroacetic acid (TCA) and the precipitate was collected by centrifugation. The supernatant was stored and the precipitated protein was washed twice with 10 ml of 5% TCA. The washings were pooled with the initial supernatant and an aliquot was neutralized and suspended in Insta-Gel (Packard Instrument Co.) for radioassay. The precipitated protein was resuspended in 50 m*M* sodium hydroxide and divided into two parts. One part was retained for

protein estimation and the other part was precipitated with 5% TCA. The precipitate was dissolved in NCS tissue solubilizer (Amersham/Searle), transferred to a scintillation vial with 10 ml of toluene scintillant (6 g/l. PPO, 75 mg/l. POPOP), and radioassayed. The radioactivity in the sodium hydroxide fraction was assayed without further treatment.

Radioactivities in the two protein fractions are expressed as percentages of the total recoverable radioactivity in the milled grain.

Scintillation Counting

Scintillation counting was carried out using a Packard Model 3003 Tri-carb scintillation spectrometer. Quenching was determined by the channels ratio method.

Protein Estimation

Protein was determined by the method of Lowry *et al.* (6) using a wheat protein extract as a protein standard.

Enzyme Assays

Wheat kernels (5 g) were ground with the Ultra-Turrax for 20 sec in a solution containing 0.05 *M* tris-acetate (pH 7.5), 10 m*M* ethylenediamine tetraacetic acid (EDTA), 2 m*M* dithiothreitol, and 10% sucrose. The homogenate was filtered through flour silk (9xx) and assayed for enzymatic activity. All operations were performed at 4° C. Duplicate extractions were performed at each harvest date.

Starch synthase activity was determined essentially as described by Hawker *et al.* (7). The assay medium contained 20 μmol Bicine buffer (pH 8.5), 5 μmol potassium acetate, 2 μmol glutathione, 1 μmol EDTA, 1 mg rabbit liver glycogen, 160 nmol ADP-glucose-^{14}C (500 dpm/nmol) in 175 μl. The reaction was initiated by addition of 25 μl extract. After 15 min at 37° C, the reaction was terminated by addition of 2 ml of 1% KCl in 75% methanol. One milligram of carrier glycogen was added and the mixture was centrifuged at 1500 × *g*. The pellet was resuspended in 2 ml of 1% KCl in methanol and centrifuged. The washed pellet was dissolved in water, mixed with Insta-Gel to form a gel, and assayed for radioactivity in a liquid scintillation counter. Duplicate analyses were performed on each extract. Standard error of the mean was within 5% in most instances.

Adenosine diphosphoglucose (ADPG) pyrophosphorylase activity was measured by a modification of a method described by Ghosh and Preiss (8). The reaction mixture contained 16 μmol HEPES buffer (pH 7.7), 50 μg bovine serum albumin, 2 μmol $MgCl_2$, 0.2 μmol ATP, 0.1 μmol ^{14}C-glucose-1-phosphate (2.5×10^5 dpm/μmol), 2.5 μmol 3-phosphoglyceric acid, and 25 μl crude extract to give a final volume of 0.2 ml. After incubation for 15 min at 37° C, the reaction was stopped by boiling for 1 min. Excess glucose-1-phosphate was hydrolyzed by incubating the reaction mixture with 100 μg of *E. coli* alkaline phosphatase for 10 min at 37° C. ADP-glucose-^{14}C was adsorbed on DEAE cellulose, the cellulose was washed with water until free of glucose-^{14}C, and the bound ADP-glucose-^{14}C was suspended in Insta-Gel for assay in a liquid scintillation counter. Standard error was within 10% in most instances.

Sucrose synthase was assayed in the direction of sucrose breakdown using uridine-5′-diphosphate as the nucleoside diphosphate. Twenty grains of wheat

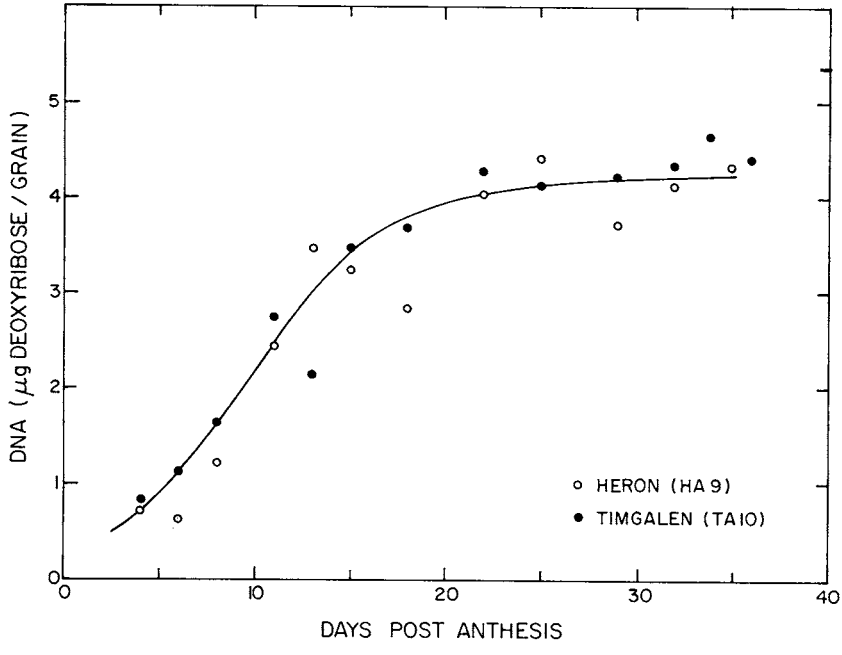

Fig. 1. DNA content of the kernels of Heron and Timgalen during development.

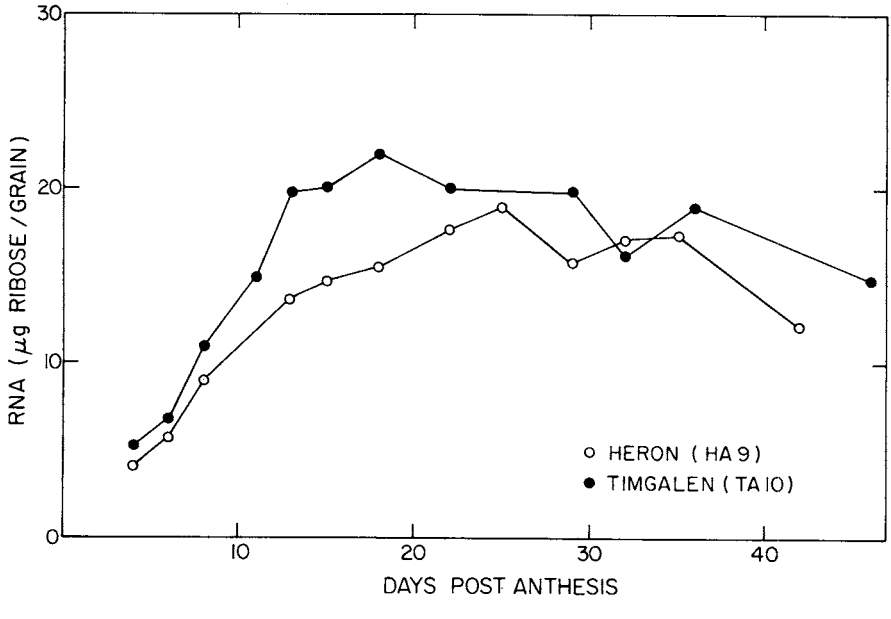

Fig. 2. Total RNA content of the kernels of Heron and Timgalen during development.

were homogenized in 5 ml of buffer (50 m*M* tris-HCl pH 7.5, 100 m*M* EDTA, 2 m*M* dithiothreitol) in an Ultra-Turrax Homogenizer at maximum speed for 20 sec at 0° C, and the homogenate was then filtered through 9xx flour silk. The reaction mixture in a final volume of 250 μl contained magnesium chloride (2 m*M*), sodium pyrophosphate (2 m*M*), ^{14}C-sucrose (42.5 m*M*, 0.05 μ Ci/μmol), uridine-5′-diphosphate (0.2 m*M*), tris-HCl, pH 7.5 (10 m*M*), EDTA (0.2 m*M*), dithiothreitol (0.4 m*M*), and grain homogenate (50 μl). The reaction was commenced with the addition of grain homogenate and the reaction mixture was incubated for 30 min at 37° C. The reaction was terminated by adsorbing the entire reaction mixture onto 4.25-cm diameter discs of Whatman DE 81 DEAE cellulose paper. The discs were washed once on a filter with 50 ml of 0.1 *M* sucrose and then five times with 50-ml aliquots of water. The discs were macerated in 5 ml of water in a scintillation vial and radioassayed in 10 ml of Insta-Gel.

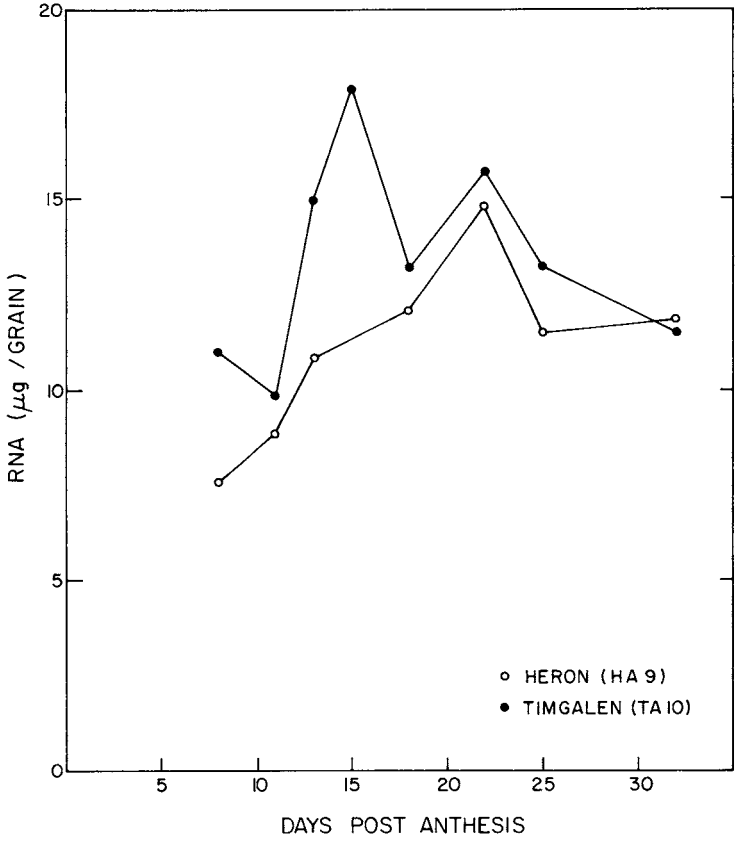

Fig. 3. Ribosomal RNA content of the kernels of Heron and Timgalen during development.

RESULTS

DNA and RNA Levels in Timgalen and Heron

DNA levels per grain in the two cultivars are shown in Fig. 1. At no stage of development were the DNA levels for the two cultivars significantly different. The arbitrary line drawn through the experimental points indicates that the level begins to plateau at approximately 18–20 days.

RNA levels in the two cultivars (Fig. 2) indicated that Timgalen had a higher total RNA content per grain than did Heron.

Isolation of the ribosomes from the whole grain and estimation of their RNA content indicated a higher population of ribosomes in Timgalen than in Heron (Fig. 3).

Ribonuclease Activity

Ribonuclease activity increased rapidly in both cultivars (Fig. 4) and closely

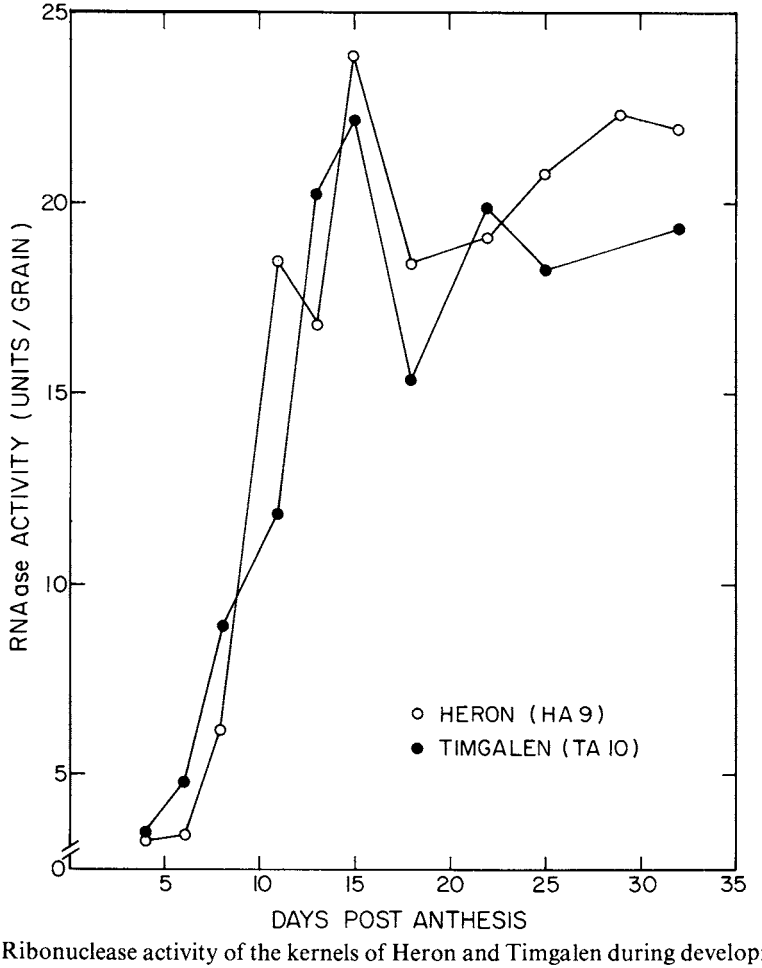

Fig. 4. Ribonuclease activity of the kernels of Heron and Timgalen during development.

paralleled the increase in RNA content (Fig. 2). The two cultivars differed only slightly in ribonuclease activity at all stages of development.

Incorporation of ^{35}S-Cystine in Two Classes of Wheat Protein

The incorporation of ^{35}S-cystine into wheat proteins (with the use of cultured wheat heads) was linear over a 2 to 12-hr period. The distribution of radioactivity in a sodium pyrophosphate fraction and in a sodium hydroxide fraction after 12-hr exposure to the labeled amino acid is shown in Table I. The protein content for these two fractions at various stages of grain development is shown in Fig. 5.

There is a shift in incorporation of radioactive label into the two fractions at 22 days post anthesis for Timgalen and at 29 days post anthesis for Heron (Table I). In both cultivars, a greater percentage of the label appears in the sodium hydroxide fraction relative to the pyrophosphate fraction at these times. This is in agreement with the protein accumulation in the two varieties (Fig. 5). Protein accumulation in the pyrophosphate fraction begins to plateau at approximately 22 days for Timgalen and 29 days for Heron.

There were differences between Timgalen and Heron in label incorporation into the pyrophosphate fraction at 8 and 15 days post anthesis, with the pyrophosphate fraction of Timgalen accumulating a greater proportion of the recoverable label (Table I). The proportion of label appearing in the sodium hydroxide soluble fraction of Timgalen was greater than that of Heron at all times tested. The differences were most pronounced at 15, 22, and 29 days post anthesis.

The specific radioactivity of the sulfur amino acid pool with which the assimilated ^{35}S-cystine was assumed to be in equilibrium was not known. However, the combined pool of disulfides and sulfhydryls in the nonprotein nitrogen fraction (1) as determined by the method of Zahler and Cleland (9) showed no significant differences between Timgalen and Heron.

Sucrose Synthase, ADPG Pyrophosphorylase, and Starch Synthase

Activities of the enzymes sucrose synthase, ADPG pyrophosphorylase, and starch synthase during kernel development of Timgalen and Heron are shown in Fig. 6. Enzymatic activities of two different crops (1) demonstrate the effect of

TABLE I
Incorporation of ^{35}S-Cystine during Kernel Development into Pyrophosphate- and Sodium Hydroxide-Extractable Protein of Heron and Timgalen

	Days after Anthesis				
	8	15	22	29	36
	^{35}S-Cystine Incorporation (Per Cent of Total Recoverable Radioactivity)				
Timgalen					
10 m*M* Pyrophosphate fraction	11.8	28.0	19.2	18.7	11.8
50 m*M* NaOH fraction	10.7	23.3	25.7	26.8	16.9
Heron					
10 m*M* Pyrophosphate fraction	8.9	24.3	19.9	17.0	11.8
50 m*M* NaOH fraction	10.0	16.9	19.2	19.8	15.8

varying growth conditions on enzyme activity.

During periods of most rapid starch deposition, the measured activity of starch synthase, the enzyme transferring glucose from ADPG to a growing starch chain, could have accounted for approximately 50% of the starch deposited in the early crop and 75–80% of the starch deposited in the late crop. Starch synthase activities did not appreciably vary, either between varieties or within crops. There was no apparent relation between the level of starch synthase activity and the initiation or cessation of starch deposition. Throughout development, sucrose synthase, an enzyme capable of converting translocated sucrose to UDP-glucose and fructose, exhibited the highest activity of the three enzymes measured. ADPG pyrophosphorylase, which converts glucose-1-phosphate to ADPG, was generally lower in activity than sucrose synthase but had substantially higher activity than starch synthase. Activities of sucrose synthase and ADPG pyrophosphorylase in the early crop were higher in Heron as compared to Timgalen. In the late crop, the peak sucrose synthase activity was also higher in Heron than Timgalen. In the early crop, there was a slower rate of development of the levels of the two enzymes, as well as lower maximum levels compared to the late crop. Decline in activity of the two enzymes toward maturity occurred before starch deposition ceased in the early crop, while in the late crop the decline in activity appeared to occur nearer the time of cessation of starch deposition (Fig. 5 of ref. 1).

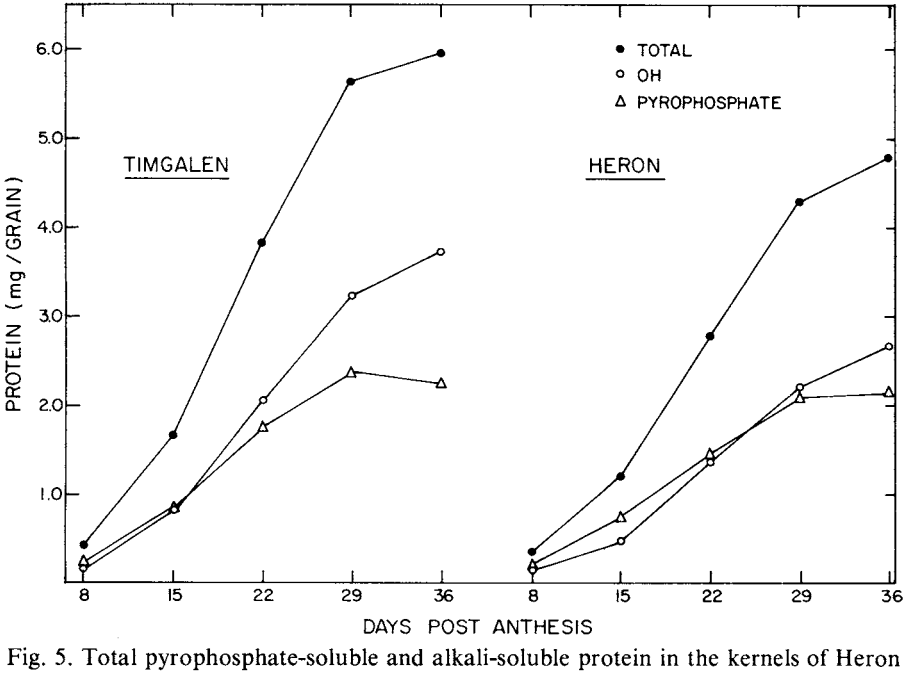

Fig. 5. Total pyrophosphate-soluble and alkali-soluble protein in the kernels of Heron and Timgalen during development.

DISCUSSION

DNA levels in the two varieties were very similar and, assuming the DNA content per nucleus is constant, there is no evidence that the differences in protein content per grain can be accounted for by differences in cell numbers. In contrast, differences existed in RNA levels, especially those associated with the ribosomes. Since ribosome population is often considered an index of the level of protein synthetic capacity, Timgalen appears to be better equipped to make proteins than Heron. Ribonuclease levels in Timgalen and Heron are essentially identical during the stage of development when the rate of protein accumulation is greatest; therefore, ribonuclease activity cannot account for the differences in protein levels or in RNA levels.

Higher RNA levels and higher ribosome populations suggest a greater synthetic capacity and hence a higher rate of conversion of amino acids into protein. The radioactive incorporation experiments reported here are further evidence suggesting faster rates of conversion of protein precursors into protein in Timgalen. Linear rates of incorporation of radioactive amino acids into protein indicate a steady state situation with respect to the specific radioactivity of the amino acid pool, and they also indicate that the grains are behaving

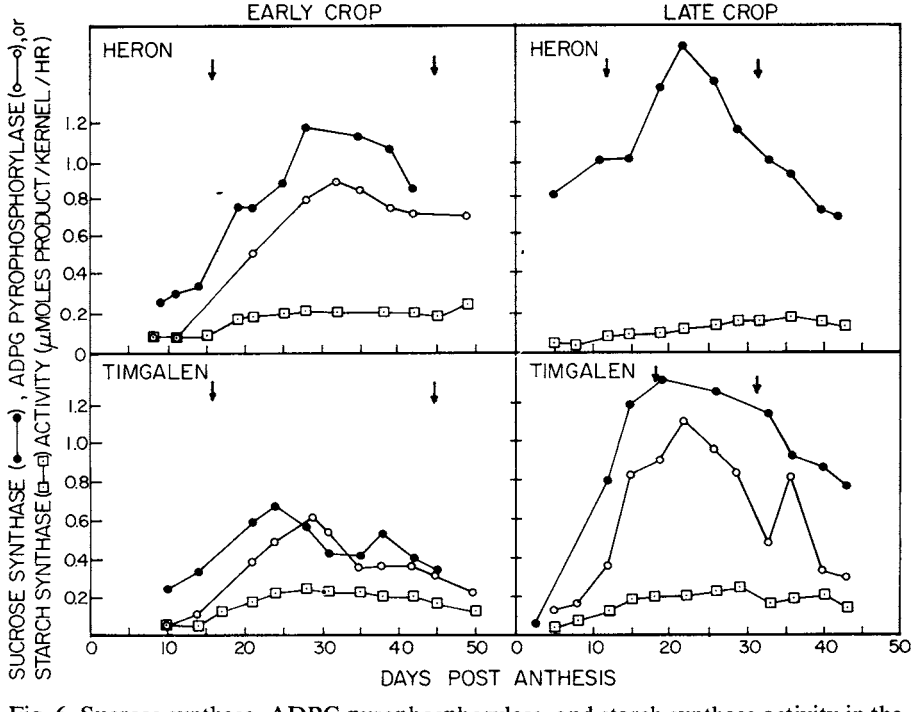

Fig. 6. Sucrose synthase, ADPG pyrophosphorylase, and starch synthase activity in the kernels of two crops of Heron and Timgalen during development. The arrows indicate the approximate commencement and cessation of starch accumulation as determined from Fig. 5 of ref. 1.

physiologically with respect to protein synthesis. The incorporation represents the rate of synthesis, since reincorporation from degraded protein would be expected to be small during this period. This suggests that *de novo* protein synthesis is faster in Timgalen than in Heron.

These and previous results (1) strongly indicate that variations in carbohydrate metabolism are not responsible for differences in protein content between the two cultivars, but can be a significant factor in variability observed in a cultivar due to environmental influences. Our work on field-grown material (1) and Jenner's studies (10) with detached ears of wheat suggest a relation between sucrose concentration and final starch content in the kernel. It could be argued that high sucrose levels might induce increased enzyme activity in the kernel, resulting in higher final starch content, but this would not seem to be the case at least for the three enzymes studied in this work (Fig. 6). Sucrose and starch levels were higher in the early crop (1), whereas the levels of activity of sucrose synthase, ADPG pyrophosphorylase, and starch synthase were lower.

The Km for ADPG of particulate starch synthase is 2–3.3 m*M* (11,12), whereas the Km for soluble starch synthase is about 0.1 m*M* (13). In our studies, we are using 0.8 m*M* ADPG and are therefore predominantly measuring the soluble form of the enzyme. In addition, we have not estimated the contribution of starch phosphorylase to starch synthesis (14). These factors may account for the observation that only 50–80% of the starch synthesized during the period of most rapid starch accumulation is attributable to the soluble starch synthase. Nevertheless, this enzyme is not limiting during the initial or final stages of starch synthesis.

The two cultivars, Heron and Timgalen, have remarkably similar patterns of starch accumulation and respond similarly to variations in environmental conditions. In particular, the termination of growth of the grain of the two cultivars bears some relation to the environment under which the crop is grown. This may be reflected in the activity maxima of the enzymes sucrose synthase and ADPG pyrophosphorylase. In the early crop, maximum enzyme activity occurred 5 to 7 days later than it did in the late crop (Fig. 6). Associated with this was a longer period of starch accumulation of approximately 13–14 days in the early crop (1).

Since the decline in activity of sucrose synthase and ADPG pyrophosphorylase occurred considerably before the termination of starch synthesis, it is unlikely that their levels would be critical determinants in the cessation of starch accumulation. This would not rule out the possibility that these enzymes were under regulatory control by nucleotides, as suggested by Turner (15), and in this manner limited starch accumulation. Our results and those of Jenner and Rathjen (16) indicate that a decline in accumulation of starch is not attributable to reductions in the supply of assimilate.

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