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Enzymes of Carbohydrate Oxidation in Developing Wheat Grains

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Activities of key respiratory enzymes were monitored at different stages in developing grains of low (C-591) and high (WH-157) yielding wheat (*Triticum aestivum* L.) cultivars. Hexokinase activity increased up to day 35 of grain development and then remained almost constant, whereas phosphofructokinase and pyruvate kinase activities peaked at day 28. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase showed biphasic behavior with peaks at days 14 and 28 after anthesis. Activities of soluble as well as mitochondrial malate (NAD^+) and isocitrate ($NADP^+$) dehydrogenases again peaked at day 28. Though the patterns of enzyme activity were similar in the two cultivars, the level in each case, when expressed on a per grain basis, was significantly higher in WH-157 than in C-591 throughout the major period of grain maturation. However, when the results were expressed on a per milligram dry weight basis, the rates were almost identical and there was no substantial difference in the rates for two varieties, indicating that the respiratory processes yielding energy needed for biosynthesis of starch and other reserves in developing wheat grains are not limiting in the small-sized grain cultivar.

Respiration in an actively metabolizing tissue is concerned mainly with oxidation of substrates to provide energy and intermediates required for biosynthetic processes. In this process, the pathways responsible for degradation of respiratory substrates converge ultimately on the Krebs cycle and hence on to respiratory chain phosphorylation. Thus, in the respiration of any class of substrates, four main steps can be recognized: breakdown of polymers and oligomers to their constituents, conversion of these constituents to acetyl-CoA or an intermediate of the Krebs cycle, oxidation of the latter via the cycle, and the reoxidation of cofactors reduced in the above reactions (ap Rees, 1980). Since carbohydrates are generally the principal source of energy, glycolysis attains significance as a major pathway of carbohydrate degradation. However, the oxidative pentose phosphate pathway is an important alternative to glycolysis and is now well-known to supply NADPH for use in biosynthetic reactions in cytoplasm. Additionally, it also serves as a source of pentose phosphates, particularly ribose-5-P for nucleotide and nucleic acid synthesis and erythrose-4-P for synthesis of aromatic amino acids and many other secondary aromatic compounds (Kelly and Latzko, 1980). Glyceraldehyde-3-P released by the transketolase reaction of the above pathway may also be fed into glycolysis and the carbon is eventually released as CO_2 during mitochondrial respiration, thus producing energy. Hence, measurement of levels of key enzymes of glycolysis, the pentose phosphate pathway, and the Krebs cycle will elucidate their role in providing energy, reducing power, and various carbon intermediates needed for varied synthetic activities that occur in developing cereal grains.

Keeping the above in view, the present investigation was aimed at determining whether low- and high-yielding wheat cultivars differ with respect to levels of key enzymes of various respiratory pathways and to associate these parameters further with grain size/starch content. Such information could relate to our previous knowledge of the possible mechanism of starch deposition and physiological and/or biochemical constraints operating in the above process in developing wheat grains (Kumar and Singh, 1980, 1981).

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MATERIALS AND METHODS

Wheat cultivars, namely, WH-157 and C-591, differing in their final grain size (dry weight, 50 and 40 mg $grain^{-1}$, respectively) and starch content (41 and 32 mg $grain^{-1}$, respectively) representing high- and low-yielding cultivars, respectively, were grown in the field as described earlier (Kumar and Singh, 1980). About 500 earheads per replication (three replications in each case) were tagged just at the start of anthesis. Grains were first harvested at day 7 after anthesis and then at weekly intervals until complete maturity of crops. Samples from each replication were analyzed independently. Thus, each value in the figures is the average of three determinations.

Enzyme Extraction. Twenty-five earheads harvested randomly from each replication were brought to the laboratory in a polythene bag buried in an ice bucket. Three spikelets from the upper and three from the lower end of each earhead were discarded. For soluble enzymes, 1 g of grains (whole grains, including both embryo and pericarp) removed randomly from earheads were hand homogenized at 0 °C in a mortar and pestle with 0.02 M Tris–maleate buffer, pH 7.0. The homogenate was centrifuged at 10000*g* at 4 °C for 40 min and the supernatant decanted. The residue was washed once with extracting buffer and centrifuged as before. The combined extract made to a known volume and served as the preparation for soluble enzymes (enzymes of glycolysis, pentose phosphate pathway, and soluble malate and isocitrate dehydrogenases).

For mitochondrial enzymes, the mitochondria were isolated essentially by the method of Bonner (1967) as modified by Krasnook et al. (1979). All steps, unless stated

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otherwise, were carried out at 0–4 °C. The homogenate, obtained after hand homogenizing 10 g of grains in 25 mL of a medium (pH 7.8) containing 0.4 M sucrose, 0.1 M Hepes, 0.001 M EDTA, and 0.05% cysteine hydrochloride, was centrifuged at 500*g* for 15 min. The supernatant obtained was recentrifuged at 3000*g* for 20 min. The pellet was discarded and the supernatant further centrifuged at 24000*g* for 30 min. The mitochondrial pellet thus obtained was further washed twice with isolation medium lacking cysteine. Since the mitochondrial recovery in the two cultivars was almost identical, the results were, therefore, not corrected relative to the recovery of mitochondria.

The washed mitochondrial pellet was suspended in 1.5 mL of 0.005 M Tris–glycinate buffer (pH 8.3) containing 0.2% Triton X-100. This preparation was kept at 0–4 °C for 1.5 h for dissolution of the pellet. The suspension thus obtained was used as the mitochondrial enzyme preparation.

Enzyme Assays. Preliminary assays were done on all enzymes to determine optimum conditions where linear reaction rates with respect to time and enzyme concentration were obtained.

Hexokinase was assayed by the method of Tsai et al. (1970). Phosphofructokinase was assayed by following the procedure of Axelrod et al. (1952) as modified by Brown and Wray (1968). Pyruvate kinase activity was followed by adopting the method described by Dennis and Green (1975). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed essentially according to Glock and McLean (1953). Soluble and mitochondrial malate dehydrogenase activity was followed by measuring oxidation of B-NADH at 340 nm (Davies, 1969). The reduction of $NADP^+$ measured by observing the increase in absorbance at 340 nm was used as the basis for assaying soluble and mitochondrial isocitrate dehydrogenase. The assay mixture for these enzymes was prepared according to Kornberg (1955) and Washitani and Sato (1977), respectively. Unit activity in each case is the amount of enzyme catalyzing formation of 1 nmol of the product or transformation of 1 nmol of the substrate (min of incubation)$^{-1}$ at 30 °C.

RESULTS

In developing wheat grain endosperm, cell division ceases around 14 days postanthesis (Jennings and Morton, 1963; Brocklehurst, 1977). Hence, to arrive at more meaningful interpretations, results have been expressed on a per grain basis.

Enzymes of Glycolysis. Hexokinase activity increased from day 7 onward, attaining maximum value at day 35 after anthesis and then remained almost constant till maturity (Figure 1a). Phosphofructokinase activity was very low during initial stages of grain development but increased sharply from day 21 onward to attain peak at day 28 and then decreased until the end (Figure 1b). Pyruvate kinase activity increased progressively up to day 28 after anthesis and then declined until maturity. The activity dropped sharply after 35 days of grain development. Though, the above enzymes exhibited a similar pattern of activity in the two cultivars, the level in each case, when expressed on a per grain basis, was significantly higher in WH-157 than in C-591 throughout the major period of grain maturity.

Enzymes of Pentose Phosphate Pathway. Both the enzyms (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) of the pentose phosphate pathway showed biphasic behavior with peaks of activities at day 14 and day 28 after anthesis (Figure 2). Thus, the activity first increased sharply from day 7 onward to seek a maximum at day 14 and then suddenly declined to a low value at day 21. It again increased to acquire a second peak at day 28. Hereafter, the two enzymes behaved differently. The activity in case of 6-phosphogluconate dehydrogenase decreased, while in case of glucose-6-phosphate dehydrogenase, it remained almost constant. Though the pattern of activity for the two enzymes was similar in both the cultivars, WH-157 again had significantly higher activity on a per grain basis than C-591 at each stage of development.

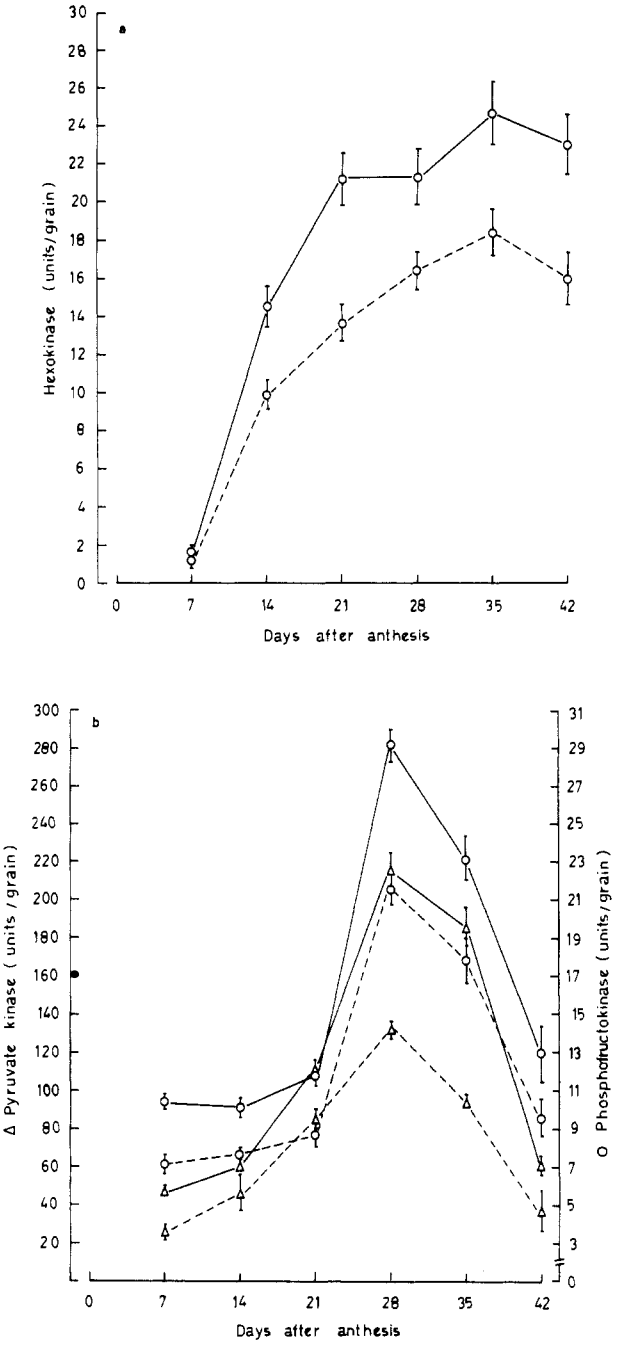

Figure 1. (a) Hexokinase activity in developing grains of WH-157 (—) and C-591 (---) at different days after anthesis. (b) Phosphofructokinase and pyruvate kinase activity in developing grains of WH-157 (—) and C-591 (---) at different days after anthesis.

Soluble and Mitochondrial Malate and Isocitrate Dehydrogenases. The activity of soluble malate (NAD^+) dehydrogenase increased progressively with time after anthesis, attaining a peak at day 28 (Figure 3). The activity of isocitrate ($NADP^+$) dehydrogenase was low during the initial stages but increased sharply after 21 days to attain peak at 28 days after anthesis (Figure 3). Here-

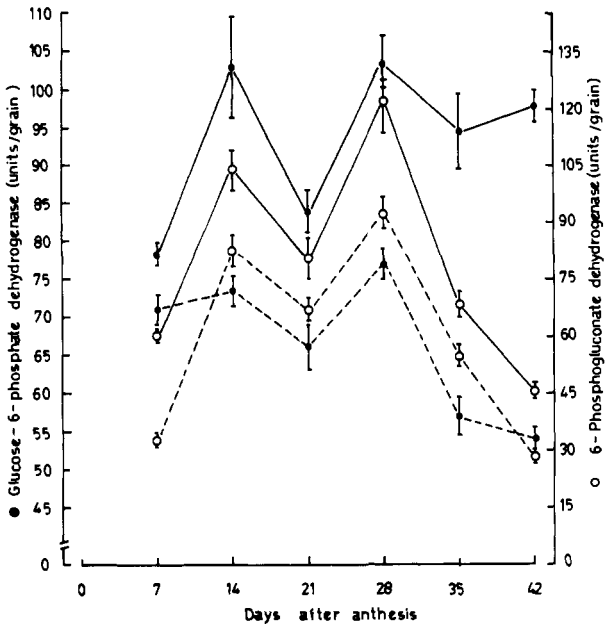

Figure 2. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity in developing grains of WH-157 (—) and C-591 (---) at different days after anthesis.

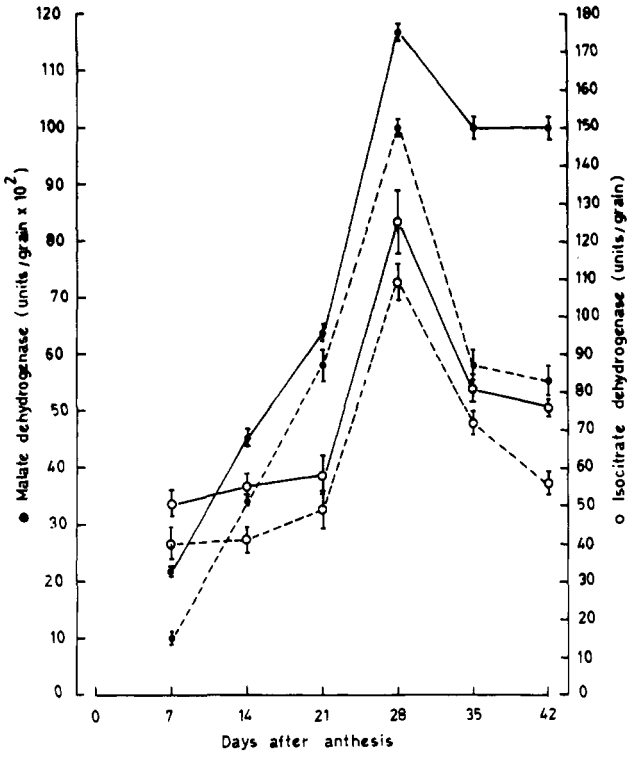

Figure 3. Soluble malate dehydrogenase and isocitrate dehydrogenase activity in developing grains of WH-157 (—) and C-591 (---) at different days after anthesis.

after, the activity declined. However, the decline in the activity of soluble malate dehydrogenase in last interval (35–42 days) was not pronounced. Similar was the case for isocitrate dehydrogenase of WH-157. Expressed on a per grain basis, this cultivar again showed significantly higher activities of these enzymes when compared to C-591 throughout the ripening period. NAD^+-specific isocitrate dehydrogenase could not be detected at any stage of grain development in the soluble fraction.

Activities of mitochondrial malate (NAD^+) and isocitrate ($NADP^+$) dehydrogenases expressed on a per grain basis similarly increased from the initial stage onward, reaching

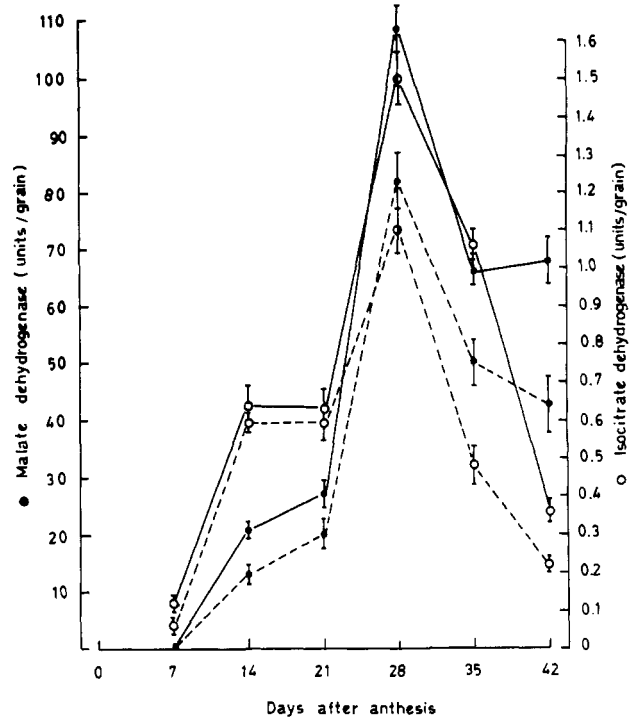

Figure 4. Mitochondrial malate dehydrogenase and isocitrate dehydrogenase activity in developing grains of WH-157 (—) and C-591 (---) at different days after anthesis.

maximum at day 28 after anthesis, and then decreased (Figure 4). The increase in the activity of isocitrate dehydrogenase during the time interval day 14–21 was not, however, significant. Though WH-157 again had significantly higher activities of these enzymes as compared to C-591, the differences were more pronounced during later (28–42 days) stages of grain ripening. In this fraction, also, only $NADP^+$ specific isocitrate dehydrogenase activity could be traced, indicating that NAD^+-specific isocitrate dehydrogenase is perhaps not involved in grain metabolism.

DISCUSSION

A continuous increase during the earlier stages and retention of high activity even at later stages (Figure 1a) indicate an active role for hexokinase in grain development. The hexose phosphate pool generated by this enzyme not only is metabolized by glycolytic reactions but can also be diverted toward starch synthesis (de Fekete, 1969) and the pentose phosphate pathway. However, the observed higher activity of this enzyme in later phases of grain development could not be attributed to respiration or starch synthesis, as at this stage both these activities were insignificant (Kumar and Singh, 1980, 1981). Thus, the higher activity in maturing grains may be ascribed to its relatively low sensitivity to desiccation. The initial lag phase in activity of phosphofructokinase (Figure 1b) indicates its regulatory nature as is known for other systems (Axelrod et al., 1952; Dennis and Coultate, 1966). The peak activity of this enzyme along with that of pyruvate kinase coincided very well with rate of O_2 consumption and the active phase of dry matter accumulation (Sangwan, 1981), indicating active participation of glycolysis in ensuring energy and metabolite supply for synthetic activities of developing endosperm.

As cell division in wheat endosperm continues until 14 days after anthesis (Jennings and Morton, 1963), the high activity of the pentose pathway enzymes during this phase (Figure 2) suggests involvement of the above pathway in providing energy (through triose phosphates) and pre-

cursors for synthesis of nucleic acids, levels of which increase during this period (Kapoor and Heiner, 1976). The peak activity again at day 28 coincided with the active phase of reserve (mainly starch) accumulation. Similarly, a good amount of activity retained by these enzymes during the later phase of grain maturation indicates that the pathway might be supplying energy, reducing power, and precursors for synthesis of storage proteins, endosperm lipids, and embryonal nucleic acids. These synthetic activities are known to be late events in cereal grain maturation (Jennings and Morton, 1963; Ingle et al., 1965).

The importance of soluble malate and isocitrate dehydrogenases lies in their participation in the dicarboxylate shuttle, transferring reducing equivalents from cytosol to mitochondria or vice versa. These reducing equivalents may be utilized as such or oxidized to produce energy. Again the observed coincidence of peaks of mitochondrial enzyme activities (Figure 4) with the rate of respiration and the active phase of starch synthesis (Sangwan, 1981; Kumar and Singh, 1981) strongly supports the view that active respiratory processes yielding energy in the form of ATP from oxidative phosphorylation, etc., accompany grain development in cereals, as inferred earlier from biochemical (Duffus, 1970; Duffus and Rosie, 1977) and ultrastructural (Buttrose, 1960, 1963; Williams and Duffus, 1978) studies. Low levels of mitochondrial enzymes observed at early and later stages of grain maturation may reflect the biogenesis (Williams and Duffus, 1978) and disintegration (Kolloffel, 1970) of mitochondria at these stages, respectively.

From the preceding discussion, the general picture that emerges is as follows: just after anthesis, endosperm cell division persists for up to 14 days. During this phase, the pentose phosphate pathway may be providing energy and precursors for the synthesis of nucleic acids and for cell division. Participation of this pathway does not seem to be of much importance in grain metabolism during the time interval 14–21 days postanthesis. A sharp increase in the activity of all respiratory enzymes studied here, after 14 and/or 21 days with a peak at 28 and/or 35 days after anthesis coincided with the peak of the respiration rate and commencement of the active phase of starch synthesis (Sangwan, 1981; Kumar and Singh, 1981). However, since all of the enzymes examined here occur in vivo as isozymes and the assay methods used reflect only the sum of the activities, it was not possible to ascertain which of the isozyme's activity goes up or declines. Also, during this period of reserve matter accumulation, the ATP pool, adenylate energy charge, and total potential energy pool increased rapidly (Sangwan, 1981), indicating the necessity of active energy metabolism during the peak phase of dry matter accumulation. Retention of sufficiently good amounts of activities of pyruvate kinase and pentose phosphate pathway enzymes together with the high-energy charge, during the later stages of grain ripening, might be supplying energy, reducing power, and precursors to sustain the synthesis of endosperm lipids, storage proteins, and embryonal nucleic acids.

When the results were expressed on a per milligram dry weight basis, the two varieties did not differ significantly in respect of any of the parameters studied here. This indicates that the levels of various respiratory enzymes, vis-à-vis energy-yielding respiratory processes, are not limiting in small-sized grain variety and are thus not directly involved in determining grain size.

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Registry No. Hexokinase, 9001-51-8; phosphofructokinase, 9001-80-3; pyruvate kinase, 9001-59-6; glucose-6-phosphate dehydrogenase, 9001-40-5; 6-phosphogluconate dehydrogenase, 9001-82-5; NAD malate dehydrogenase, 9001-64-3; NADP isocitrate dehydrogenase, 9028-48-2.

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