Periodicity of Growth and Starch Deposition in the Developing Wheat Grain

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ABSTRACT

Three cultivars of bread wheat have been serially harvested from ear emergence to beyond harvest ripeness in three seasons. Deposition of dry matter in the kernel does not occur at a uniform rate but shows periodic fluctuations of rate of deposition. The periodicity is complex, having frequency of maxima about 3 to 4, 6 to 7, and 10 days. Similar periodicity is observed for rate of starch deposition and for concentrations of some enzymes and metabolites. Transmission electron micrographs are presented showing, without Lintnerization, the periodic shell-like formation within starch granules, and showing the groove reticulum that is present within the plastid and related to starch synthesis.

The rate of gain of dry matter in developing cereal grains has not been studied as extensively as one might have expected, considering the simple techniques involved. In a previous publication (1) we observed that the dry matter content of some wheat cultivars did not remain constant after ripeness. The same experiments showed an apparent scatter of experimental points about an assumed smooth curve in the development stage. Differentiation of the curves to give the rate of gain of dry matter showed a periodicity of rate of gain with time and this coincided with a periodicity of activity of starch metabolizing enzymes that we have been studying (2).

Periodicity of dry matter gain can be observed in data of earlier workers (3–6) so long as observations are of sufficient frequency. Saunders (7) indicated a smooth curve through his data and suggested that his observed departures from the smooth line might be caused by variation of daily temperature. He further suggested repeat experiments “with a view to eliminating all irregularities in the observations.” The more recent work of Walpole and Morgan (8) showed considerable standard errors and departure from smooth linearity for their five harvestings, leaving plenty of latitude for the periodicity of growth that we propose.

In several series of developing wheat grains we have examined the dry weight, starch content, and, in a few instances, the blue value of the starch. The development of the starch granule and structural changes in the pericarp accompanying growth of the grain have also been studied using optical and electron transmission microscopy.

MATERIALS AND METHODS

This work is part of a series carried out on wheats grown and harvested as we have described previously (1,2,9). Here we present data for the three cultivars Cappelle Desprez, Aotea, and Hilgendorf 61 grown in harvest seasons 1970 through 1972 and sampled at 2- or 3-day intervals. We also present data for daily sampling of cv. Hilgendorf 61 in harvest season 1972. Each sample is the average

\[1\] Presented at the 58th Annual Meeting, St. Louis, Nov. 1973. Reprint requests to Dr. P. Meredith, P.O. Box 1489, Christchurch, New Zealand.

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for a large number of plants, as discussed later.

Kernel weights were determined by counting and weighing batches of about 100 grains drawn from the bulks of cleaned and dried grains. Successive batches were taken until reasonably concordant results were obtained, as indicated in Fig. 2. Ripeness was judged from the moisture content of entire ears by drying at 110⁰C. for 16 hr. All other analyses were made on finely ground whole grain.

Total carbohydrate was determined by boiling 100 mg. of ground grain in 25 ml. of 0.75M sulfuric acid for 2 hr. and measuring the liberated reducing compounds by a ferricyanide-meric titration method (10). Free reducing compounds were determined in buffered aqueous extracts of the ground grain by a ferricyanide-iodometric titration (11). Starch was assumed to be the total carbohydrate less the free reducing compounds. Blue value of washed-out granular starch was determined according to Gilbert and Spragg (12), the starch content of the dissolved granules being determined as above.

Pericarp amylase, and stable and labile amylase activities, were determined by the methods we have described (2). Total soluble fructose was determined in aqueous buffer extracts of ground grain by a colorimetric anthrone reaction at 50⁰C. according to Johnson et al. (13). Green pigment content was determined spectrophotometrically at 413 nm. in an ethanolic extract of ground grain.

Development of starch granules and changes in pericarp structure were followed in median transverse sections of wheat grains at successive stages of development. Material for optical microscopy was prepared according to the

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**Fig. 1.** Rate of gain of dry matter per kernel with time. Dashed curves = cv. Hilgendorf, and full curves = cv. Cappelle Desprez, both of the 1970 harvest. The experimental points are only shown once for each cultivar. Upper curves = calendar time scale; lower curves = scale of days after ear emergence.
Fig. 2. Experimental points of average kernel weight for each 100 kernels counted to illustrate accuracy of kernel weight determinations (cv. Hilgendorf of 1970 and 1971).

Fig. 3. Total amylase activities analyzed shortly after harvesting (full lines) and after storage in the dry, ground, frozen state for 14 months (dashed lines). Comparison for two cultivars illustrating reproducibility of analyses and stability of stored samples. The upper scales are the sums of daily mean temperatures from anthesis (17).
methods of Feder and O'Brien (14) and sections cut at 5 to 8 μm. Electron microscopy tissue was fixed in either aqueous permanganate or glutaraldehyde-osmium tetroxide following established procedures (15), embedded in Araldite (16), cut on an ultramicrotome with a diamond knife, and examined in an Hitachi HS-7 electron microscope.
RESULTS AND DISCUSSION

Dry Matter Gain

We have already presented (1) curves of kernel weight that are typical of those to be discussed. They are generally sigmoid, but the points show a slight scatter to either side of the line in a rhythmic manner as seen in Fig. 2. The meaning of this scatter is evident when we consider the differentials of the curves, i.e., the rate of gain of dry matter per kernel per day. These have been calculated simply by dividing the difference between successive average kernel weights by the number of days involved.

As an example, the experimental points are shown for the rate of weight-gain curves of two cultivars in 1 year in Fig. 1. The fluctuations of observations are far beyond what we believe to be experimental error. The actual errors have not been determined from replicate wheat plots, but we believe the fluctuations to be real for the following reasons:

1) The general patterns of fluctuation are similar for several cultivars and in several years (e.g., Figs. 1, 5, 6, 9), and are supported by the more detailed daily observations of Fig. 6a.

2) The fluctuations show corresponding periodicity in measurements that have no obvious analytical relationship such as dry matter (starch) gain, two amylase activities, and blue value of the starch.

3) The fluctuations are analytically reproducible, illustrated in Fig. 2 for weight gain and Fig. 3 for amylase activity.

![Graph showing dry matter gain and amylase activities](image)

Fig. 6. a) Rate of gain of dry matter during development of wheat grains, cv. Hilgendorf, 1972 harvest. Five-day sliding mean against days after ear emergence. Upper scale is sum of daily mean temperatures from anthesis. b) Comparison of curve a, dotted, with stable amylase activities (concentration basis), full line. Amylase abscissa shifted 2 days to left to give best coincidence. c) Similar comparison with labile amylase activities, full line. Amylase abscissa shifted 5 days to left to give best coincidence.
4) Fluctuations can be seen in the results of the few workers who have presented sufficient detail (Fig. 4). All these patterns included a final loss of dry matter (the “overshoot” phenomenon) similar to that we have already described (1).

The similarity of pattern of fluctuation between cultivars is seen only when similar physiological dates are considered. Thus in Fig. 1 the two cultivars present coincident patterns when time after ear emergence is used as the abscissal scale, but not when calendar date is used. This also indicates that weather conditions are not primarily responsible for the fluctuations. Agreement between curves for two cultivars can be obtained in less degree by other time shifts, because of the periodicity of the phenomena, as discussed below. Some “phase” shift in time is required when comparing, say, amylases with starch deposition, this shift having a possible relationship to the sequence of reactions involved in starch synthesis and deposition.

Cerning and Guibbot (17) have considered that calendar time scale is not appropriate for the presentation of developmental changes, and have used a climatic expression, the “cumulative daily mean temperature after flowering.” We have calculated such scales for our results and include them in Figs. 3 and 6. An overall seasonal effect has been noted, but this is small compared with the fluctuations we are now considering. The observations of two seasons are compared in Fig. 5 for composite curves of three cultivars.

The precision of the calendar time scale is about 0.5 hr., samples being taken at almost the same time each day, but time is also quantized in 1-day blocks since we have not determined what diurnal variation there may be. Most of our data has been for sampling at 2- or 3-day intervals, each sample being of a large number of plants to give an average of plant to plant, ear to ear, and within-ear variations. Ear emergence has been preferred as the datum, rather than anthesis, for reasons already discussed (9). For the cultivar Hilgardorf in harvest season 1972 (i.e., Oct. 1971 through Feb. 1972) we sampled daily. With this larger amount of data at more frequent intervals the random error was more noticeable and the natural frequencies of fluctuation are most clearly seen only when a sliding mean is plotted rather than the raw data (Fig. 6a). This plot showed an average period between maxima of 3.75 days, about half that shown by three cultivars in 3 years and tabulated in Table I. The uniformity of maxima found in the daily plot is seen in Fig. 7. As a generalization, we have observed a 7-day period through the main development stage of each cultivar, with a 3.5-day period in the early stages and a 10-day period in the post-ripe stages. There has also been a tendency for one

<table>
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<th>Cultivar</th>
<th>1970 days</th>
<th>1971 days</th>
<th>1972 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hilgardorf</td>
<td>6.2</td>
<td>5.0</td>
<td>(3.7)</td>
</tr>
<tr>
<td>Aotea</td>
<td>6.0</td>
<td>6.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Cappelle Desprez</td>
<td>5.6</td>
<td>7.7</td>
<td>5.2</td>
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1Mean of eight = 6.1 days.
maximum to be missed in the immediately pre-ripe period, or at ripeness. These additional trends are illustrated also in the daily plot of Fig. 6a, where in the early stages each maximum is equally accentuated, in the main development stages alternate maxima are accentuated, and in the post-ripe stages every third maximum is accentuated.

**Starch Deposition**

We found surprisingly high concentrations of starch even in the earliest stages of development because, as shown by Bice et al. (18), the pericarp contains starch deposits (Fig. 8). This starch occurs in small spherical amyloplasts, each containing several granules.

From the initial level, around 40%, not illustrated, starch concentration rose steeply to about 63 to 68%, then abruptly leveled off. Figure 9 shows one example of this pattern, which was repeated in all cultivars and seasons. The smooth curves are only an approximation, a fluctuation being observable in starch concentration. When the deposition of starch per kernel per day is calculated it shows a periodicity similar to that found for dry matter, as we would expect since starch is the major component. Composite means for three cultivars and two seasons are illustrated in Fig. 10, the features being similar to those of Fig. 5.

Rate of deposition of starch in cereal grains is only available from the results of two groups of workers that we are aware of. Akazawa et al. (19) recorded starch contents of developing rice from which we can calculate a rate that shows three maxima and a negative period. Merritt and Walker (20) give data for barley from which we can show four maxima and a negative period of a general pattern similar to that of Akazawa et al. Both are consistent with the observations we describe. Curves of starch content presented by other workers such as

![Graph](image)

**Fig. 7. Plot of frequency of maxima of Fig. 6a.**
MacGregor et al. (21) and Cerning and Guilbot (17) show points deviating from smooth curves, but we cannot determine the rates with sufficient precision.

The determinations of amylases that we have already presented (2) show fluctuations similar to those we are discussing and of similar periodicity. This is illustrated for Hilgendorf of 1972 in Fig. 6, b and c, and we have similarly observed the coincidence for the three cultivars in the years 1970 and 1971. The experimental points for blue values of Fig. 11 can also be fitted by more complex curves showing periodicities. It is important to our argument that the maxima and minima for these various analyses do not coincide on calendar time scale but require “phase” shifts of a few days for coincidence. We hope to explore these time relationships in a future publication.

The stable amylase concentrations of wheat show an abrupt decline to negligible activity part way through development (2). The transition of starch concentration from steep rise to level is also abrupt (Fig. 9) and coincides with the abrupt decline of stable amylase activity (Table II). Generally this transition point occurs some 10 to 15 days before ripeness (defined as first reaching 20%, dry basis, ear moisture) and at between 60 and 120% moisture content. Beyond the transition point, kernel weight continues to increase, indicating that dry matter continues to accumulate in the kernel, and the level concentration of starch suggests that other dry matter is also accumulating at a constant rate. We have observed that total protein concentration is constant also in this period. The transition point does not coincide with the decline of green pigment (Fig. 9).

The level concentration attained by starch is not constant but shows
Fig. 9. Plot of starch concentration in developing grains, cv. Aotea, 1970, compared with dry kernel weight, ear moisture, green pigment, and stable amylase activity. The full-line curves are drawn precisely through experimental points that lie at the same intervals as the starch concentration points.

<table>
<thead>
<tr>
<th></th>
<th>Amylase Decline</th>
<th>Starch Break</th>
<th>Starch Level</th>
<th>Protein %</th>
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<tr>
<td></td>
<td>A&lt;sup&gt;1&lt;/sup&gt;</td>
<td>B&lt;sup&gt;1&lt;/sup&gt;</td>
<td>C&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Hilgendorf</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1970</td>
<td>51</td>
<td>49</td>
<td>63</td>
<td>14.9</td>
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<tr>
<td>1971</td>
<td>52</td>
<td>50</td>
<td>64</td>
<td>15.5</td>
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<tr>
<td>Aotea</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1970</td>
<td>42</td>
<td>41</td>
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<td>1971</td>
<td>46</td>
<td>47</td>
<td>67</td>
<td>11.7</td>
</tr>
<tr>
<td>Cappelle Desprez</td>
<td></td>
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<td></td>
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<tr>
<td>1970</td>
<td>49</td>
<td>47</td>
<td>67</td>
<td>10.8</td>
</tr>
<tr>
<td>1971</td>
<td>53</td>
<td>49</td>
<td>68</td>
<td>12.0</td>
</tr>
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</table>

<sup>1</sup>The letters refer to Fig. 9. A is the time at which stable amylase activity approaches zero. B is the time at which the percentage starch ceases to rapidly increase. C is the average level percentage of starch. Protein has been determined in a ripe sample actually having starch concentration C.
Fig. 10. Rate of starch deposition in developing grains compared in two seasons. Composite curves for three cultivars.

Fig. 11. Blue values of starches of developing grains, 1970 season, interpreted as percentage amylose. The simplest curves have been drawn through the points to show only the general trend; more complex curves can be drawn that show periodicities.
fluctuations. It is conceivable that the fluctuations reflect a competition between starch and other compounds for available metabolites; protein is the obvious competitor. The few analyses we have, of 1969 material, show that the rate of protein deposition has a second maximum in this period before ripeness.

The actual level attained by starch concentration is roughly inversely related to the protein content of the ripe material, as shown in Table II. The main increase in starch concentration is at the rate of about 2% per day. Differences between cultivars have been observed, the rise of starch concentration being more curvilinear for cv. Cappelle Desprez than for the other two cultivars.

Starch deposition is closely reflected in increased kernel weight. Fluctuations of the rate of change of kernel weight closely correspond to activities of stable and labile amylases (Fig. 6, b and c), suggesting that these enzymes are connected with starch synthesis and deposition.

Our working hypothesis is that the stable amylase is associated with linear polymerization and that the labile amylase is associated with branching of the linear polymer. While we have no conclusive evidence for this interpretation, it so far is in accord with all our observations. The time shifts necessary to produce correspondence in Fig. 6 suggest that the labile (branching) enzyme acts after the stable (linear) enzyme. We further hypothesize that β-amylase is a “trimming”

Fig. 12. Electron micrograph of large (type A) starch granule, cv. Hilgendorf, 1971. 24 days after emergence, fixed in 2% aqueous potassium permanganate for 2 hr. at room temperature to show shell structure, groove reticulum (GR), and amyloplast envelope (AE) with interior thylakoid (T).
enzyme concerned with the uniformity of the outer branches of the polymer.

Blue values determined on the isolated starches have been calculated to percentages of amyllose in the starch. This proportion of linear polymer (Fig. 11) increases until ripeness, with similar fluctuations of rate for each cultivar in the 1 year for which we have made analyses. After ripeness the curves for the three cultivars diverged, the eventual level for cv. Hilgendorf being notably lower than for the other two. The levels attained, around 20%, are in accord with accepted data for mature wheat starch. It may be pertinent that cv. Hilgendorf 61 contains appreciable concentration of free glucose at maturity and beyond, whereas cv. Cappelle Desprez and Aotea do not (9).

We have calculated rates of deposition for amyllose and for amylopectin separately and compared them with the rate of deposition of starch as a whole to determine which kind of polymer, linear or branched, is concerned with the fluctuations. During the development stages the fluctuations of starch deposition correlated closely with fluctuations of branched polymer but not with linear polymer. The results were not clear-cut for the post-ripe situation and further work on this point is required.
Our results are in accord with the blue values and iodine absorptions of starches of developing grains determined by Harris and MacWilliam (22), Merritt and Walker (20), Wolf et al. (23), and Bice et al. (18). Merritt and Walker (20) concluded that high amylase content is associated with smaller granule size and one is tempted to speculate that the increased proportion of amylase during grain development may be due in some part to the formation of the smaller type B starch granules. Their conclusion that amylase of Glacier (Pentlandfield) barley was still increasing at ripeness is scarcely supported by the experimental points and is not supported by their total starch data.

**Structural Considerations**

We have examined starch granules during the stages of kernel development by both optical and transmission electron microscopy. The well-known shell structure (24,25) can be clearly seen in Fig. 12 for a large (type A) starch granule at an early stage of its development and in Fig. 13 for two small (type B) starch granules within one plastid at a later stage of kernel development.

Buttrose (26) was able to demonstrate shells in his material only after Lintnerization followed by permanganate staining, whereas we have been successful in showing them after normal aqueous permanganate fixation without Lintnerization. With glutaraldehyde-osmium fixation, shell structures are not revealed and the whole starch granule appears electron transparent. It may be
noted that starch granules of amyloplasts of wheat roots of the same varieties fixed in the same way do not show shells though they do show a distinct core and a distinct outer layer.

Many young plastids exhibit a few profiles of thylakoids (membranes) between the starch granule and the plastid envelope. The presence of a reticulum of tubules lying within the groove of the starch granule is confirmed. This system of tubes (connected, according to Buttrose (26), with the inner plastid membrane) presents a large surface area of membraneous material within a minimum space. Furthermore, particularly in young plastids, the envelope often bulges out opposite the groove of the granule. It is tempting to suggest from the structure of the groove reticulum that this is the site of active starch formation in the plastid. Sections fixed in glutaraldehyde-osmium show a distinct zone adjacent to the groove reticulum in young plastids (Fig. 14). This could represent the first stage of polymerization. We confirm the finding of Buttrose (26) that the tubules of the groove reticulum connect with the inner plastid membrane though not, in our material, with the frequency suggested by his diagram.

In spite of Buttrose's (27) finding that in his experiments the shells were of diurnal origin, it is tempting to propose that each shell corresponds to one of the periods of starch deposition that we have shown. It is also possible that the shells do not result from periodic deposition. In our material the outermost layer of the granule usually bears a constant relationship to the shell bands. That is, the boundary of the granule is always just beyond the edge of a dark band. If the shells are of diurnal origin, this constancy would be a result of our harvestings being at a constant time of day—sampling was performed about 4 hr. after sunrise. It is even conceivable that the bands are not in fixed positions but are mobile, being a result of diffusion effects. The chemical properties have not been established but it is possible that staining is due to long-chain polyprenol

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**Fig. 15.** Relationship between "pericarp" amylase activity (solid lines) and percentage total soluble fructose (dashed lines) in developing grains of Aotea 1971. Left-hand curves on same time scale; right-hand curves, adjusted time scales to give best coincidence.
intermediates remaining from the polymerization of glucose via a dolichol phosphate mediated type of reaction.

**General**

The flow of precursors into the grain and through the separate parts of the grain must be time-consuming and rate-limited (28) processes; so also must be diffusion processes between and within the endosperm cells. It is likely that the earlier stages of glucose activation and polymerization occur in the groove-reticulum, whereas the final stages must be at the surface of the granule where deposition occurs, again a spatial separation requiring a diffusion or transport phase. Burton (29) in 1939 suggested that “overshoot” in biological steady-state systems results from diffusion processes rather than the kinetics of the reactions. Hess and Boiteux (30) have recently emphasized that oscillations are a normal property of complex dynamic systems and must be expected to occur in all biological systems. There is a considerable theoretical and observational literature for oscillating biological systems. An important point made by Bünning (31) is that endogenous rhythms frequently appear diurnal or have this character impressed upon them.

Not all fluctuations that we have observed will fit into the simple periodicity that we have proposed. This is to be expected since some events, either chemical or anatomical, will be dependent on others. We finally present an example, in Fig. 15, of the relationship between “pericarp” amylase activity and total soluble fructose concentration. The complex periodicities of these two determinations can be made to coincide only when their time scales are adjusted by both translation and expansion. Thus the changes in fructose concentration initially lag behind the changes in amylase activity by 2 or 3 days and, with the passing of time, the lag between the two increases to about 10 days. We suggest that an anatomic delay exists in the relation between these two quantities and that the main changes reflect the degradation of the pericarp tissues.

**Acknowledgments**

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