CHANGES IN CARBOHYDRATE, PROTEIN, AND NON-PROTEIN NITROGENOUS COMPOUNDS OF DEVELOPING WHEAT GRAIN

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Summary

Analyses were carried out on the main morphological parts of grain of *Triticum vulgare* and of *T. durum* during development from a few days after flowering to maturity. The low molecular weight constituents (non-protein nitrogenous compounds, sucrose, reducing sugars, and α-keto acids) decreased in concentration but the amounts per grain remained relatively constant during development. However, the macromolecular materials (starch, pentosans, and protein) increased in concentration and in amount per grain. Metabolism of developing endosperm is discussed in relation to these observations.

I. INTRODUCTION

Various aspects of the chemistry of the developing wheat grain have been studied by previous workers (see, for example, Woodman and Engledow 1924; McCalla 1938; Koblet 1940); the results provide some information concerning changes of a number of grain constituents during development. However, as a basis for more detailed biochemical investigations, information was required not only concerning changes in individual components but also of changes in several components relative to one another and in each of the morphological fractions of the grain.

This paper describes the changes in some selected carbohydrate and nitrogenous components during development of the grain of three wheat varieties grown under field conditions.

II. MATERIALS

Two varieties of bread wheat, *Triticum vulgare* cv. Gabo and *T. vulgare* cv. Insignia, of good and poor baking quality respectively, and one macaroni wheat, *T. durum* cv. Dural, were studied. General conditions for growth during the three years 1958–1960 varied from drought (1959) to good conditions (1960).

The plants were grown in field plots at the Waite Institute, Adelaide. At each harvest, the required number of ears (not less than c. 1000) were collected by hand from randomized sites. Edge effects were eliminated, and sampling errors were reduced as far as possible by use of standard statistical procedures. The ears were transferred to a room at 2°C, mixed, and stored at −15°C.

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III. Methods

(*a*) *Separation of Morphological Fractions of Grain*

The grain was separated from the ears while frozen, either by hand or by a mechanical thresher in a room at −15°C. Embryo, endosperm (including the aleurone layer), and testa–pericarp fractions were separated by hand from the frozen grain. The grain was dissected on a stainless steel plate secured to the top of a brick encased in "Coolite" insulating material, and pre-cooled to −15°C so that manipulation could be carried out at room temperature. The fractions were stored at −15°C. Homogenates were prepared at 0°C in a mechanically driven glass homogenizer with a "Teflon" pestle (Arthur H. Thomas and Co. Ltd.). Analyses were carried out on this material.

(*b*) *Large-scale Isolation of Endosperm*

Until about 20 days after flowering grain may be squeezed or rolled in aqueous medium when, on rupture, the endosperm and testa–pericarp are obtained intact. For some studies, therefore, endosperm was obtained as follows: A mill made up of a round plastic bottle (*c.* 500-ml capacity) filled with sand and inserted into a round glass jar (of about 3-l. capacity) was loaded with up to 50 g (fresh weight) of grain, a predetermined volume of aqueous medium added (usually about 100 ml), and the mill rotated at about 100 r.p.m. on a set of mechanical rollers for about 5 min. The correct volume of liquid must be determined experimentally; if too small, the endosperm is excessively damaged, and if too large, the grain is not drawn between the surfaces of the two bottles and little rupture of grain occurs. The liquid used is chosen according to experimental requirements; for many purposes, 0·5M sucrose is suitable. The milled grain is collected on a sieve and endosperm is collected by suction into a receiving vessel. Some loss of low molecular weight components of endosperm may occur.

(*c*) *Chemical Analyses*

The moisture content of the samples was determined by drying at 103°C for 16 hr. Nitrogen contents were determined by either macro- or microKjeldahl procedures. The amide nitrogen content was determined by a micromodification of the method of Leach and Parkhill (1956). The non-protein nitrogen fraction was obtained by dialysis of homogenized material (prepared at 0–2°C to reduce proteolytic activity to a minimum) against distilled water for several hours at 2°C. No other methods for the separation of the non-protein nitrogen fraction were satisfactory. Portions of homogenates placed in a boiling water-bath for 5 min before dialysis gave the same value for the dialysable (or non-protein) nitrogen content as homogenates dialysed without prior treatment, indicating that no significant amount of proteolysis occurred under the conditions used.

The soluble sugars were separated by repeated extraction with 80% ethanol at 75°C; the ethanol extract was then evaporated to near dryness and the residue dissolved in water. The reducing sugars were determined by the procedure of Nelson (1944) after clarification of the extracts (Somogyi 1945) both before inversion (giving "reducing sugars") and after inversion (giving "sucrose" by difference) by the A.O.A.C. (1955) procedure 29·26(*c*). The insoluble residues from the ethanol extraction were

hydrolysed with 1·0N HCl for 6 hr in a boiling water-bath. After clarification of the hydrolysates, the content of reducing sugar ("starch" plus "pentosans") was determined as above, and the pentose content (giving "pentosans") was determined by the methods of Dische and Schwartz (1937), Dische (1953), and of Dische *et al.* (1953); see also Ashwell (1957). It was found necessary to include suitable control hydrolysates of starch and ribose in each batch of determinations.

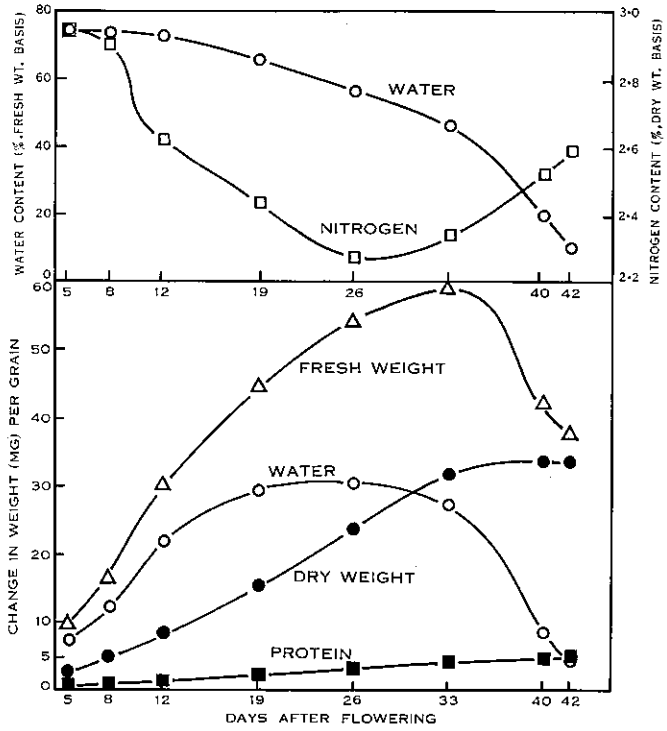

Fig. 1.—Changes in water content, nitrogen, protein (total nitrogen × 5·7), dry weight, and fresh weight of whole wheat grain (cv. Gabo, 1959 harvest) with time from flowering.

Keto acids were estimated according to Friedemann and co-workers (see Friedemann 1957), but modified so that the samples were mixed by "buzzing" (see Jennings and Morton 1963) and the colour of the sodium carbonate layer was read at 370 mμ (Bonting 1955).

(*d*) *Proteins*

Fractions were obtained by successive extraction with 0·01M sodium pyrophosphate, pH 7·5, and 0·05N acetic acid, essentially as described by Graham, Morton, and Simmonds (1963). Amino acids were determined according to Moore and Stein (1954) by using the automatic apparatus described by Simmonds and Rowlands (1960).

All fractionations were carried out in replicate and all determinations were also carried out in replicate. Results given are means of the several determinations, which generally agreed within 5%.

IV. Results and Discussion

The developing grain consists mostly of testa–pericarp during early stages, and of endosperm at later stages of development. Analyses were therefore carried out on these distinctive morphological fractions throughout development; whole grain was also analysed for some components. To enable comparisons, the results presented below are mostly for cv. Gabo, 1959 harvest. Results for other varieties and for other years differ in some detail but generally support the conclusions drawn from these studies on cv. Gabo.

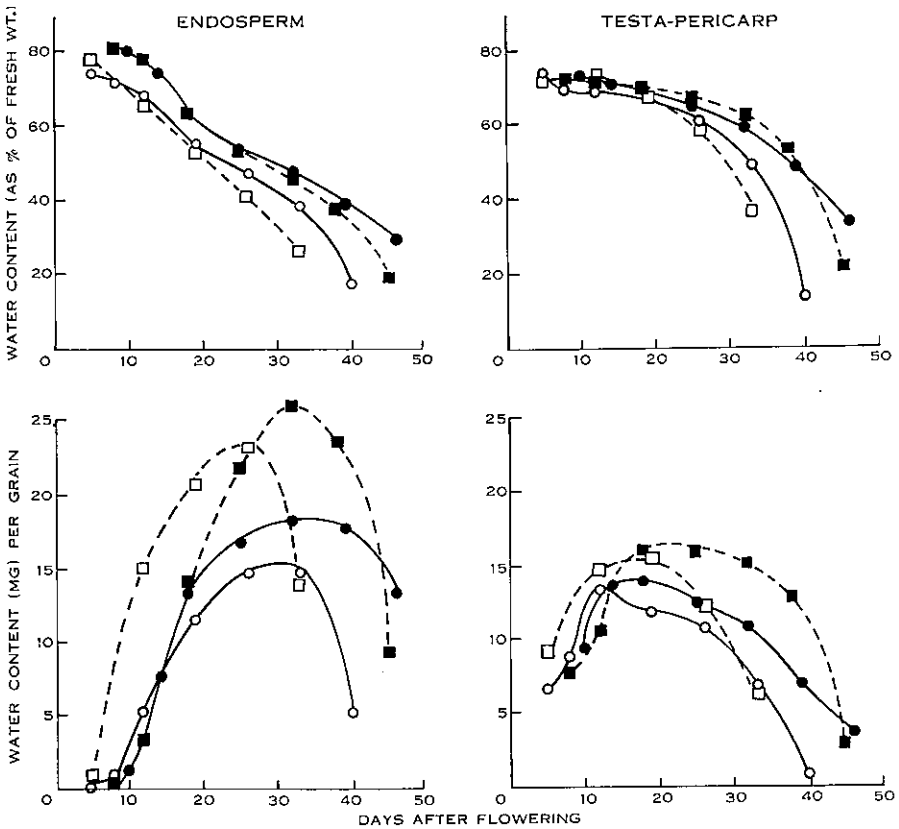

Fig. 2.—Changes in water content of endosperm and of testa–pericarp of wheat grain in 1959 (drought conditions) and in 1960 (good conditions) for cv. Gabo, 1959 (○) and 1960 (●) harvests, and for cv. Dural, 1959 (□) and 1960 (■) harvests, with time from flowering.

(*a*) *Changes in Water Content*

The changes in water, fresh weight, dry weight, and nitrogen of grain during development is shown in Figure 1. Both fresh and dry weight per grain increase until about 35 days after flowering. The decline in water content which then occurs is accompanied by a marked decline in the rate of increase of dry weight.

As shown by Figure 2, the change in water content of the endosperm was essentially similar to that of the whole grain, increasing until about day 30 and declining rapidly from about day 35. In the testa–pericarp, however, the water content reached a maximum at about day 12, declined slowly until about day 26, and then declined rapidly until maturity (Fig. 2).

There are marked differences between cv. Gabo and cv. Dural in rapidity of development and maturation of the grain; correspondingly, there are distinct differences in the changes of water content during development (Fig. 2). Rapid loss of water corresponds with the initiation of the maturation phase. The time of initiation of this phase is not only dependent on the variety, but also on the water

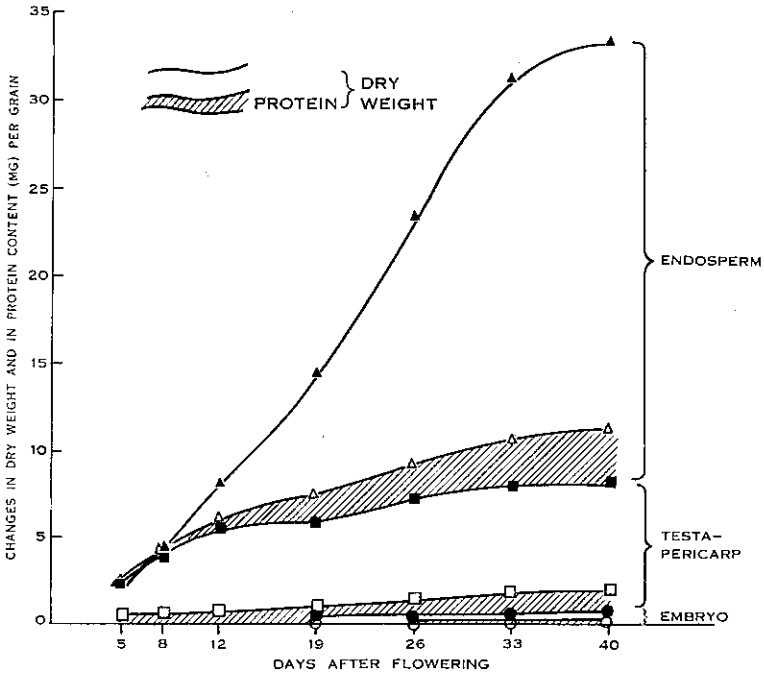

Fig. 3.—Changes in dry weight and in protein (total nitrogen × 5·7) of embryo, testa–pericarp, and endosperm of wheat grain (cv. Gabo, 1959 harvest) with time from flowering. The protein contribution to the dry weight of each fraction is indicated by the hatching.

availability, as is seen by comparison of results (Fig. 2) for Gabo and Dural in 1959 (low rainfall) and in 1960 (adequate rainfall). It appears likely that changes in the cells of the tissue connecting the grain itself to the rachilla may modify availability of water and of nutrients for endosperm growth and thus initiate grain maturation. Woodman and Engledow (1924) earlier observed distinctive changes in water content of grain during development. Whereas such changes affect the time scale of development, they do not modify the general relationships between low molecular weight compounds and macromolecular synthetic products.

(*b*) *Changes in Carbohydrates*

Figure 3 shows the changes in dry weight and of protein (total nitrogen × 5·7) in endosperm, testa–pericarp, and embryo, and Figure 4 shows the relative contributions of the three morphological components to the total dry weight and to the nitrogen content of the grain. Synthesis of storage protein and of starch in the endosperm largely accounts for the rapid increase of dry weight of the developing grain after fertilization.

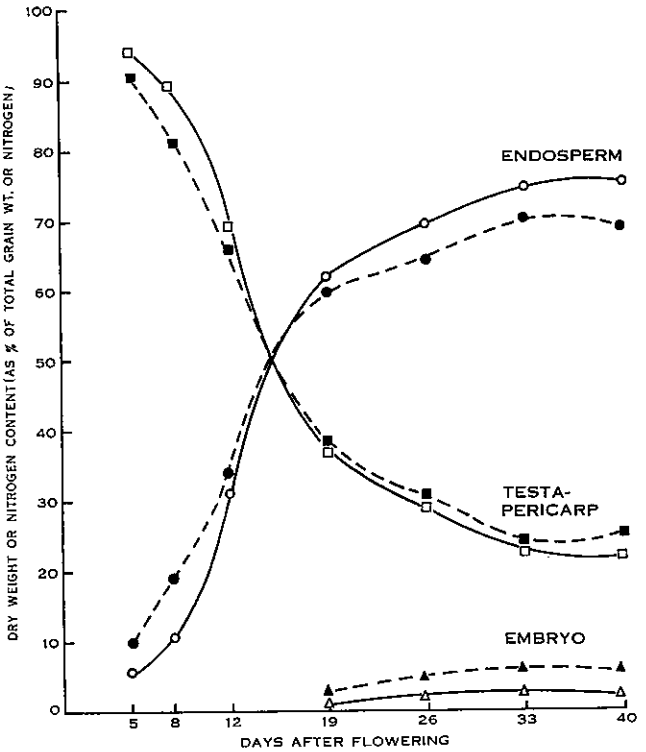

Fig. 4.—Relative contributions to the dry weight and nitrogen content of the whole grain (cv. Gabo, 1959 harvest) of the dry weight (————) and of the nitrogen content (— — —) of the endosperm, testa–pericarp, and embryo.

To elucidate the relationship between starch, reducing sugars, sucrose, and pentosans, the changes in each of these were measured. The endosperm starch content (Fig. 5), as the amount per endosperm, increases rapidly and almost linearly from about day 12 to day 35, and, as a percentage of endosperm dry weight, also reaches a maximum value about this time. Reducing sugars and sucrose make a significant contribution to the dry weight of the endosperm; the amounts per grain increase between days 8 and 20, but in the same period decline markedly as a percentage of the dry weight (Fig. 5). From day 20 to maturity the reducing sugars and sucrose form a relatively constant proportion of the dry weight. The initial marked decline when rapid starch synthesis is initiated indicates that starch synthesis depletes the pool

of precursor compounds; subsequently, the pool is maintained at a relatively constant level necessary for continued starch synthesis. Reducing sugars almost completely disappear during the maturation phase, i.e. after day 35. These relationships are consistent with starch synthesis from reducing sugars which arise from sucrose translocated to the endosperm from elsewhere in the plant. Wood (1960) has also shown that sucrose which is translocated to the wheat grain is a precursor of part

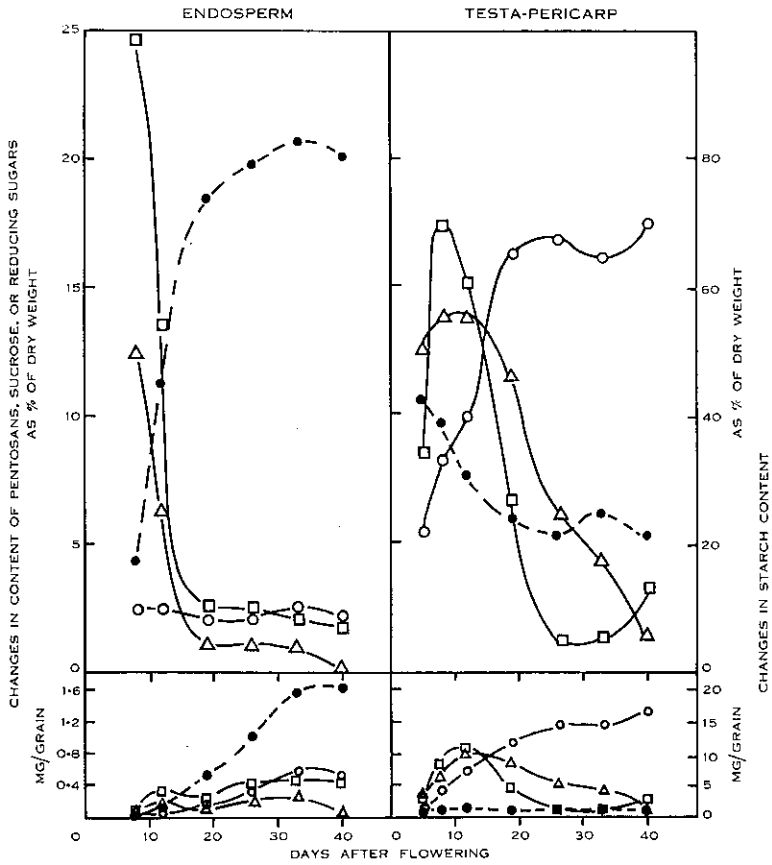

Fig. 5.—Changes in pentosans (○), sucrose (□), reducing sugars (△), and starch (●) in the endosperm and testa–pericarp of wheat grain (cv. Gabo, 1959 harvest) with time from flowering. The ordinate at the right-hand side refers to starch only.

of the starch. It is probable that phosphorolysis of sucrose in the endosperm forms glucose 1-phosphate, the precursor of uridine diphosphate glucose from which amylose arises by glucosylyl transfer (Leloir, Rongine de Fekete, and Cardini 1961; see also Atkinson and Morton 1959). Until about day 20, the developing grain is green due to chlorophyll which occurs in the innermost layer of the parenchyma of the pericarp. Chloroplasts have been observed in these cells by electron microscopy (Jennings, Morton, and Palk 1963). Photosynthesis in these cells could possibly contribute to

the sucrose pool and hence to starch synthesis in the endosperm. This tissue could also have some function in removal of carbon dioxide formed in the actively synthesizing endosperm cells.

Several workers (Ford and Peat 1941; Perlin 1951; Gilles and Smith 1956; Montgomery and Smith 1956; Ewald and Perlin 1959) have shown that wheat flour contains water-soluble pentosans of xylose and arabinose, with galactose and traces of glucose, and have proposed structures for the pentosans. The amount of pentosans per grain increases in the endosperm throughout development and represents a fairly constant proportion of the dry weight (Fig. 5). Since new cell formation probably ceases by about day 14 (see Jennings and Morton 1963) the increase in pentosan is very probably due to increased synthesis of new cell walls associated with the enlargement of endosperm cells to accommodate the newly formed storage materials (starch and storage proteins).

In the testa–pericarp, the amount of starch per grain is relatively constant and the percentage of the dry weight declines during development (Fig. 5). However, pentosans increase both in amount per grain and as a percentage of the dry weight (Fig. 5). The changes in reducing sugars, sucrose, and pentosans suggest that, in testa–pericarp, sucrose and reducing sugars are predominantly precursors of pentosans and that pentosan synthesis is more active than is starch synthesis. As with endosperm tissue, the pentosan synthesis is almost certainly associated with enlargement of testa–pericarp cells during development.

These results (cf. Figs. 3 and 5) and those of McCalla (1938), Koblet (1940) and Bilinski and McConnell (1958) are supported by cytological observations on the time of initiation and sites of starch synthesis. By light microscopy Sandstedt (1946) observed that starch synthesis in wheat endosperm commenced at about 5 days after fertilization, when new cell walls are being formed, whereas starch synthesis in pericarp declined within a few days after fertilization. Light microscopy and electron microscopy (Jennings, Morton, and Palk 1963) on the tissues used in this study have confirmed that starch synthesis proceeds rapidly in endosperm cells soon after fertilization and precedes accumulation of storage protein in protein bodies.

(c) *Changes in Keto Acids, Amino Acids, and Proteins*

Figures 3 and 4 show that the rapid synthesis of protein in the developing grain occurs mainly in the endosperm. Although the amount of protein nitrogen per grain increases almost linearly from about day 12 to maturity, owing to the more rapid synthesis of starch initially the total protein nitrogen (expressed as a percentage of the dry weight) declines somewhat between day 8 and day 19 and then remains relatively constant (Fig. 6).

Non-protein nitrogen forms a high proportion (about 48%) of the total nitrogen initially (at day 8) and the proportion declines markedly to about 15% at day 19, and then more slowly to about 3% at maturity. The amount of non-protein nitrogen (mostly due to free amino acids) per grain is almost constant during development (Fig. 6). During development of the endosperm, the pattern of the relative changes in total protein and in non-protein nitrogen (Fig. 6) resembles the pattern of relative

changes in starch and in reducing sugars (Fig. 5) and is consistent with a precursor pool–product relationship.

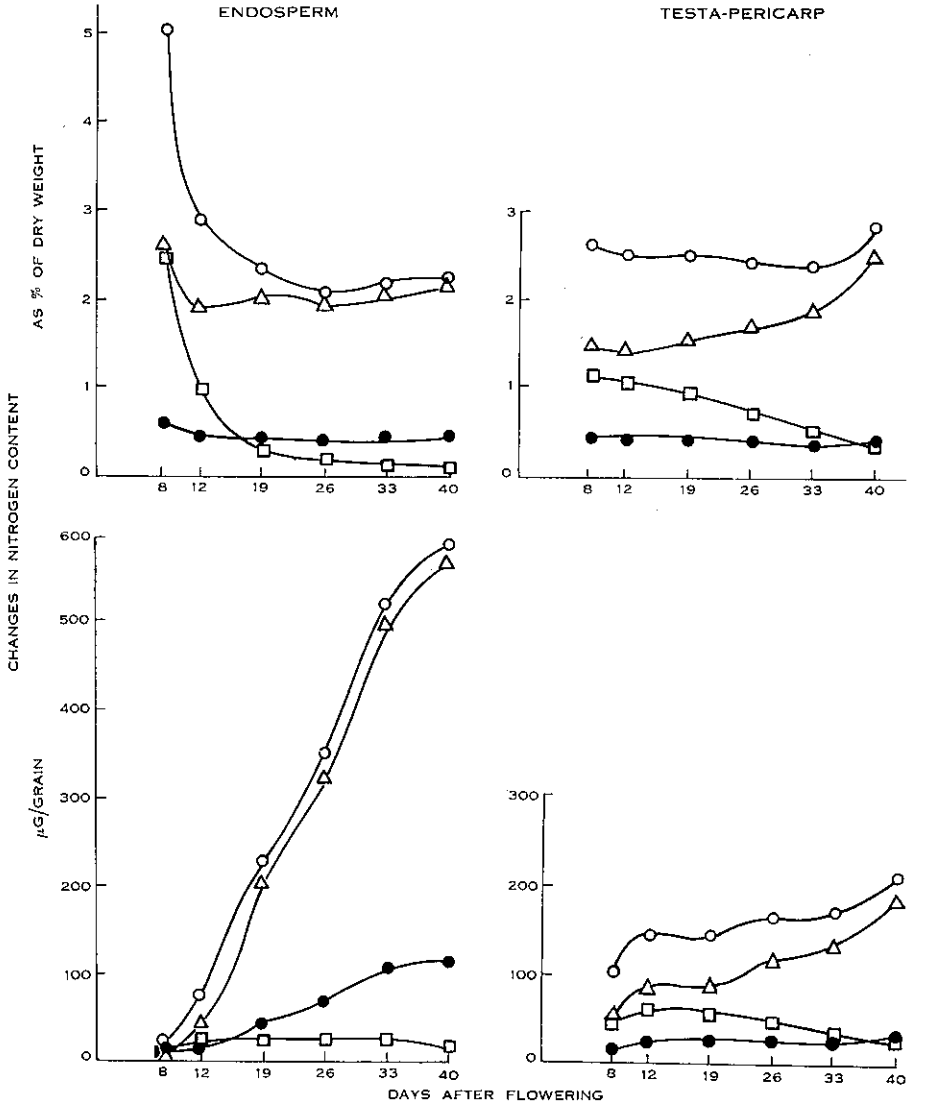

Fig. 6.—Changes in total nitrogen (○), protein nitrogen (△), non-protein nitrogen (□), and amide nitrogen (●) in the endosperm and testa–pericarp of wheat grain (cv. Gabo, 1959 harvest) with time from flowering.

In the testa–pericarp (Figs. 2 and 3) there is much less synthesis of protein, but the relationships between total protein nitrogen, amide nitrogen, and non-protein nitrogen (Fig. 5) are essentially the same as discussed for endosperm.

Characteristically, proteins of wheat flour have a relatively high proportion of residues of glutamine and of proline. Figure 6 shows that the amount of amide

nitrogen per grain increases almost linearly during development of the endosperm; the proportion of the total nitrogen as amide nitrogen increases from about 10% initially (at day 8) to about 20% at day 20 and then remains relatively constant to maturity.

TABLE 1

AMINO ACID RESIDUES AND KETO ACIDS OF ENDOSPERM OF WHEAT GRAIN, CV. GABO, 1959 HARVEST

All analyses were adjusted to 95% recovery of nitrogen applied to ion-exchange columns; the actual recoveries of nitrogen applied varied from 75 to 100%, and in most cases were about 90%. Most of the replicates agreed within 10%. Analytical values are expressed as μg amino or keto acid per grain

	Whole Endosperm	Non-protein Nitrogenous Compounds		Protein				Flour from Mature Grain§
Days after flowering:	8*	12	19	12†	19†	26‡	33‡	
Amino acid								
Aspartic acid	11	19	22	18	39	95	161	175
Threonine	5·1	4·4	4·9	8·2	30	52	88	86
Serine	5·4	6·7	9·8	11	38	78	113	129
Glutamic acid	29	42	32	35	346	604	898	1039
Glycine	5·3	4·6	6·6	11	30	48	97	106
Alanine	8·6	12	17	13	49	99	87	107
Valine	8·4	4·7	3·3	21	59	78	142	239
Isoleucine	4·8	3·2	5·2	13	34	67	116	126
Leucine	9·4	3·7	3·6	21	75	121	187	242
Tyrosine	3·5	1·5	1·0	10	41	45	64	44
Phenylalanine	5·8	2·3	2·9	12	63	94	161	114
Lysine	9·8	5·8	5·7	18	36	63	77	66
Histidine	3·5	2·2	1·8	8·1	32	53	67	78
Amide nitrogen	3·3	5·7	4·7	7·9	42	76	111	122
Arginine	8·1	2·8	3·9	17	44	74	107	108
Proline	7·5	15	7·9	24	139	214	367	432
Total		135	132					
Keto acid‖								
Pyruvic	0·063			0·11	0·14	0·17	0·83	0·81
α-Ketoglutaric	0·067			0·19	0·32	0·84	1·06	0·17

* Non-protein nitrogen 48% of total nitrogen.

† Calculated from difference between analyses for whole endosperm and for non-protein nitrogen.

‡ Analyses for whole endosperm; non-protein nitrogen less than 7% of total nitrogen.

§ Non-protein nitrogen less than 3% of total nitrogen.

‖ Total monocarboxylic and dicarboxylic keto acids are expressed as pyruvic and α-ketoglutaric acids respectively.

Tables 1 and 2 show the changes during endosperm development in the amino acids (except for cysteine, cystine, methionine, and tryptophan, which form a very

small proportion of the amino acids of wheat proteins). Since the non-protein nitrogen forms a high proportion of total nitrogen up to about day 20, the amino acids of the dialysable fraction after hydrolysis are shown together with the amino acids of the proteins, for day 12 and day 19. Hydrolysis of the dialysable fraction did not change significantly the amounts of amino acids, except for a marked increase of amide and of glutamic acid and a lesser increase of aspartic acid due to hydrolysis

TABLE 2

AMINO ACID RESIDUES OF ENDOSPERM OF WHEAT GRAIN, CV. GABO, 1959 HARVEST

All analyses were adjusted to 95% recovery of nitrogen applied to ion-exchange columns; the actual recoveries of nitrogen applied varied from 75 to 100%, and in most cases were about 90%. Most of the replicates agreed within 10%. Amino acid nitrogen expressed as a percentage of total nitrogen

	Whole Endosperm	Non-protein Nitrogenous Compounds		Protein				Flour from Mature Grain§
Days after flowering:	8*	12	19	12†	19†	26‡	33‡	
Amino acid								
Aspartic acid	5·4	9·0	10·9	4·6	2·4	3·3	3·8	3·6
Threonine	2·9	2·4	2·7	2·3	2·1	2·1	2·3	2·0
Serine	3·6	4·2	6·2	3·6	3·0	3·6	3·5	3·5
Glutamic acid	12·9	17·9	13·8	7·8	18·7	18·9	18·8	19·3
Glycine	5·4	4·5	6·6	5·4	3·6	3·4	4·6	4·5
Alanine	7·0	9·6	13·1	5·2	4·8	5·6	3·3	3·6
Valine	5·0	2·6	1·9	6·0	4·1	3·2	3·9	5·8
Isoleucine	2·5	1·6	2·6	3·2	2·1	2·4	2·8	2·7
Leucine	4·8	1·8	1·8	5·4	4·6	4·4	4·5	5·1
Tyrosine	1·3	0·51	0·35	1·8	1·7	1·1	1·1	0·65
Phenylalanine	2·3	0·87	1·1	2·4	3·0	2·6	3·0	1·9
Lysine	9·0	5·0	5·0	8·0	4·0	4·0	3·3	2·5
Histidine	4·5	2·7	2·2	5·1	4·9	4·7	4·0	4·1
Amide nitrogen	11·9	19·8	16·6	14·2	18·3	19·3	18·8	18·4
Arginine	12·0	4·0	5·6	12·8	7·8	7·6	7·4	6·6
Proline	4·5	8·4	4·6	7·0	10·0	8·9	10·2	10·7

* Non-protein nitrogen 48% of total nitrogen.

† Calculated from difference between analyses for whole endosperm and for non-protein nitrogen.

‡ Analyses for whole endosperm; non-protein nitrogen less than 7% of total nitrogen.

§ Non-protein nitrogen less than 3% of total nitrogen.

of glutamine and asparagine. The dialysable fraction contained little if any peptides, indicating that no significant amount of proteolysis occurred during dialysis.

Although the amount of non-protein nitrogen per endosperm remained constant between day 12 and day 19 (Fig. 6 and Table 1), the amounts per endosperm of several amino acids increased and that of others decreased (Table 1). These variations probably reflect the extent to which the rate of supply of an amino acid to the pool

(of protein precursors) was greater than or less than the rate of incorporation into protein. Thus the amounts per endosperm of free glutamic acid and of proline decline during the period when the protein-bound residues of these amino acids show a marked increase (Table 1); these relationships are also seen from the changes in percentage composition of the non-protein and protein fractions (Table 2). The free amino acids of the endosperm may be formed by transamination of keto acids, or they may arise by translocation from other parts of the plant. Table 1 shows that the amounts per endosperm of the mono- and dicarboxylic α-keto acids (pyruvic and α-ketoglutaric acids) increase throughout development to day 33. The amounts of these keto acids are extremely small in relation to the amounts of the corresponding

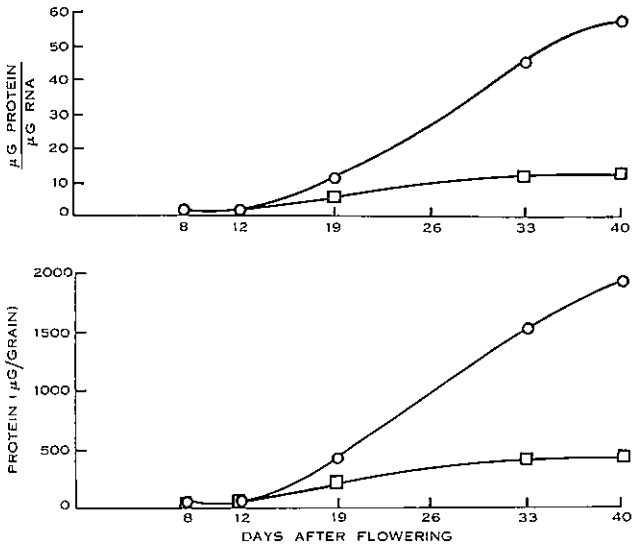

Fig. 7.—Changes in the pyrophosphate-soluble protein (□) and acetic acid-soluble protein (○) of wheat endosperm (cv. Gabo, 1959 harvest) and in the ratio of the amounts of these to the amounts of total ribonucleic acid present with time from flowering.

amino acids (alanine and glutamic acid respectively); thus transamination and glutamic acid formation, catalysed by glutamate dehydrogenase, are probably of considerable significance in developing endosperm.

Marked change in the amino acid composition of the endosperm protein occurs between day 12 and day 19 whereas it remains relatively constant thereafter (Table 2), during a period of considerable accumulation of protein-bound amino acids (Table 1). Some fractionation of the groups of proteins of developing endosperm is obtained by successive extraction with 0·01M sodium pyrophosphate (pH 7·4) and 0·05N acetic acid (Graham, Morton, and Simmonds 1963; Graham and Morton 1963). Figure 7 shows that, at about day 19 and thereafter there is a rapid increase in the amount of acetic acid-soluble protein as compared with pyrophosphate-soluble

protein. The relative constancy of the amino acid composition between day 19 and maturity reflects this rapid accumulation of storage protein (extracted by acetic acid).

The biosynthesis of proteins is related to the biosynthesis of ribonucleic acid (see Harris 1960; Chantrenne 1961). Figure 7 shows that the ratio protein/total ribonucleic acid (see Jennings and Morton 1963) during endosperm development increases markedly for acetic acid-soluble proteins, whereas there is relatively little change for pyrophosphate-soluble protein. These relationships suggest that the pyrophosphate extract may largely contain proteins which are intermediates in the formation of the storage proteins.

V. Acknowledgments

We are grateful to Mr. H. R. Lovelock for his valuable assistance in planting, harvesting, and dissecting arrangements for this and later work concerned with developing wheat endosperm.

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