CHANGES IN NUCLEIC ACIDS AND OTHER PHOSPHORUS-CONTAINING COMPOUNDS OF DEVELOPING WHEAT GRAIN

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Summary

In both the developing endosperm and testa–pericarp there was a relationship between the content of inorganic phosphorus, acid-soluble acid-labile phosphorus, and acid-soluble organic phosphorus ("phytic acid"), and the water content. The times of restriction of supply of water to the endosperm and to the testa–pericarp fairly closely corresponded with the times of initiation of rapid synthesis of phytic acid and of a corresponding rapid decline in content of inorganic phosphorus and reactive phosphoryl groups.

The changes in DNA content of the endosperm indicate that rapid cell division occurred in the endosperm until about 14 days after flowering and further increase in size was due to cell expansion. The DNA content of the testa–pericarp indicates that the number of cells present was essentially constant throughout the period of development studied. In the endosperm, RNA increased rapidly during the period of cell division and thereafter the amount remained constant or declined slightly; the RNA content of the testa–pericarp showed little change during development, in agreement with the low rate of protein synthesis in this tissue.

The changes in lipid and lipid-phosphorus content probably reflected the expected increase in the amount of intracellular membranes.

I. INTRODUCTION

Since compounds of phosphorus have important functions in many phases of cellular metabolism, the amounts of selected compounds were measured to elucidate the changes which take place during development of wheat grain. Nucleic acids, phospholipids, phosphoproteins, acid-labile phosphates, and some other compounds were studied. Together with results reported previously (Jennings and Morton 1963) these findings enable improved understanding of the special biochemical functions of the morphological fractions of wheat grain.

II. MATERIALS AND METHODS

Endosperm, testa–pericarp, and embryo fractions were prepared from developing grain of three varieties (*Triticum vulgare* cv. Gabo and Insignia and *T. durum* cv. Dural) grown under field conditions in Adelaide in 1959 and 1960 as described previously (Jennings and Morton 1963).

(*a*) *Fractionation of Phosphates*

This was carried out according to Martin and Morton (1956). For analysis, the material was freeze-dried, finely ground, and dried to constant weight over phosphorus pentoxide under vacuum at room temperature.

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(*b*) *Estimation of Phosphates*

(i) *Total Phosphorus.*—The total phosphorus content of all extracts was determined with the following modification of published procedures (Martin and Doty 1949; Weil-Malherbe and Green 1951; Martin and Morton 1956). Suitable portions were digested by the procedure of Martin and Morton (1956) in 6 by $\frac{5}{8}$ in. test tubes; 0·5 ml water was added to each of the digests which were then boiled for about 10 min to hydrolyse the pyrophosphate formed during digestion. The digests were then neutralized with 2N NaOH solution (*p*-nitrophenol indicator) and then made just acid to the indicator. The volume of all digests was then adjusted with water to that of the digest with the greatest volume. After the addition of 6 ml isobutanol and 1 ml acid molybdate reagent (Weil-Malherbe and Green 1951) the tubes were subjected to vigorous "buzzing" for 15 sec (by holding the test tubes against a plastic-covered rubber bung which was eccentrically placed on the shaft of a fast electric motor). The tubes were centrifuged gently for 1 min to obtain separation of the phases; 5 ml of the isobutanol layer was added to 5 ml of freshly prepared acidified alcohol and mixed. Within 10 min subsequently, 0·2 ml of the 1% stannous chloride solution was added and mixed and the colours were allowed to develop for the required period (usually 10 min). Extinctions were always measured at 730 mμ within 15 min after the addition of the stannous chloride solution. A Unicam SP 600 spectrophotometer was used for these measurements; alcohol was used in the reference cuvette. There was a linear relation between extinction and phosphorus content, and results were very reproducible.

(ii) *Inorganic Phosphorus.*—An appropriate portion of extract was made to 4 ml, and 1 ml silicotungstic acid (Martin and Doty 1949) was added; hydrolysis was omitted. Otherwise, the procedure was as for total phosphates.

(iii) *Acid-labile Phosphates.*—The material was hydrolysed in 4 ml 1N HCl for 7 min at 100°C; after cooling, 6 ml isobutanol, 1 ml silicotungstic acid, and 1 ml neutral molybdate reagent (Weil-Malherbe and Green 1951) were added and inorganic phosphate was estimated as above.

(*c*) *Deoxyribose and Ribose Estimations*

(i) *Deoxyribose.*—This was estimated according to Webb and Levy (1955) except that the colour was developed with 2N NaOH saturated with trisodium phosphate. Extinctions were measured at exactly 2 min after addition of alkali. Calf thymus deoxyribonucleic acid was used as a standard.

(ii) *Ribose.*—This was estimated as described previously (Jennings and Morton 1963).

(*d*) *Estimation of Lipids*

Weighed amounts (about 150–200 mg) of the dried material was successively extracted with anhydrous organic solvents (methanol, methanol–chloroform (1 : 1 v/v), and n-butanol). The combined extracts were made to 25 ml with methanol. Portions of the extracts were dried to constant weight in vacuum over phosphorus pentoxide, calcium chloride, and paraffin chips at room temperature.

III. Results and Discussion

(a) *Changes in Acid-soluble Phosphorus Compounds*

Figure 1 shows that the total phosphorus content per grain increases in endosperm, testa–pericarp, and embryo throughout development. The relative concentration in the endosperm declines rapidly between day 5 and day 19, and thereafter remains constant. The rate of increase of total phosphorus per grain in the testa–pericarp parallels that of other major constituents, so that the relative concentration remains constant throughout development. There is a surprisingly large amount of total phosphorus in the embryo; relative to its dry weight this fraction contributes substantially to the total phosphorus content of whole grain.

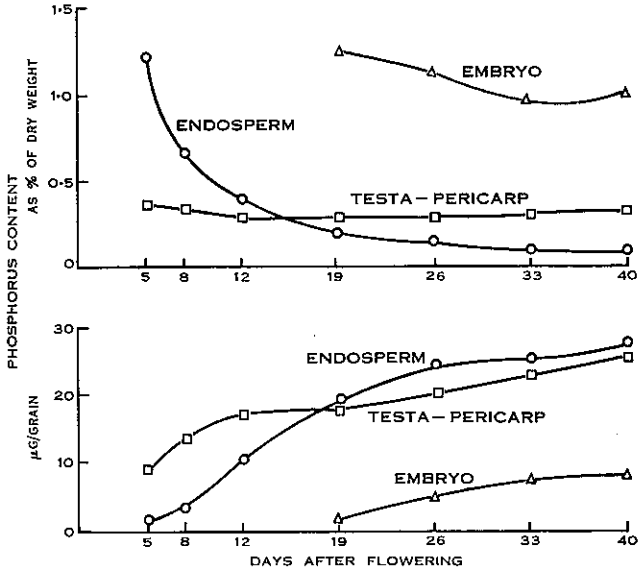

Fig. 1.—Changes in total phosphorus content for endosperm, testa–pericarp, and embryo of wheat grain (cv. Gabo, 1959 harvest) with time from flowering.

In general, the amounts of acid-soluble acid-labile phosphorus and of acid-soluble non-labile organic phosphorus respectively measure the amounts of reactive phosphoryl groups (in adenosine 5′-triphosphate and in uridine 5′-triphosphate, for example) and of phosphorylated intermediates (such as phytic acid and glucose 6-phosphate, for example). Both are related to the pool of inorganic phosphate (see Atkinson and Morton 1959). Figure 2 shows the changes in acid-labile, non-labile, and inorganic phosphorus, and in total acid-soluble phosphorus. In the endosperm, marked changes in the amount of each compound occur at about day 26. Whereas inorganic phosphorus previously increased in amount, at this time it begins to decline and continues to decline steeply until maturity. In contrast, both the acid-labile phosphorus and, more strikingly, the acid-stable organic phosphorus compounds, which were both previously present in small amounts, begin to increase

substantially at about day 26. Thus there are inverse changes in the amounts of inorganic phosphate and of acid-stable organic phosphate between day 26 and day 40.

Most of the acid-stable organic phosphorus in endosperm during this period is present as phytic acid (Knowles and Watkin 1922; Harris and Mosher 1934). The relative concentrations of inorganic phosphate and of acid-labile phosphorus decline

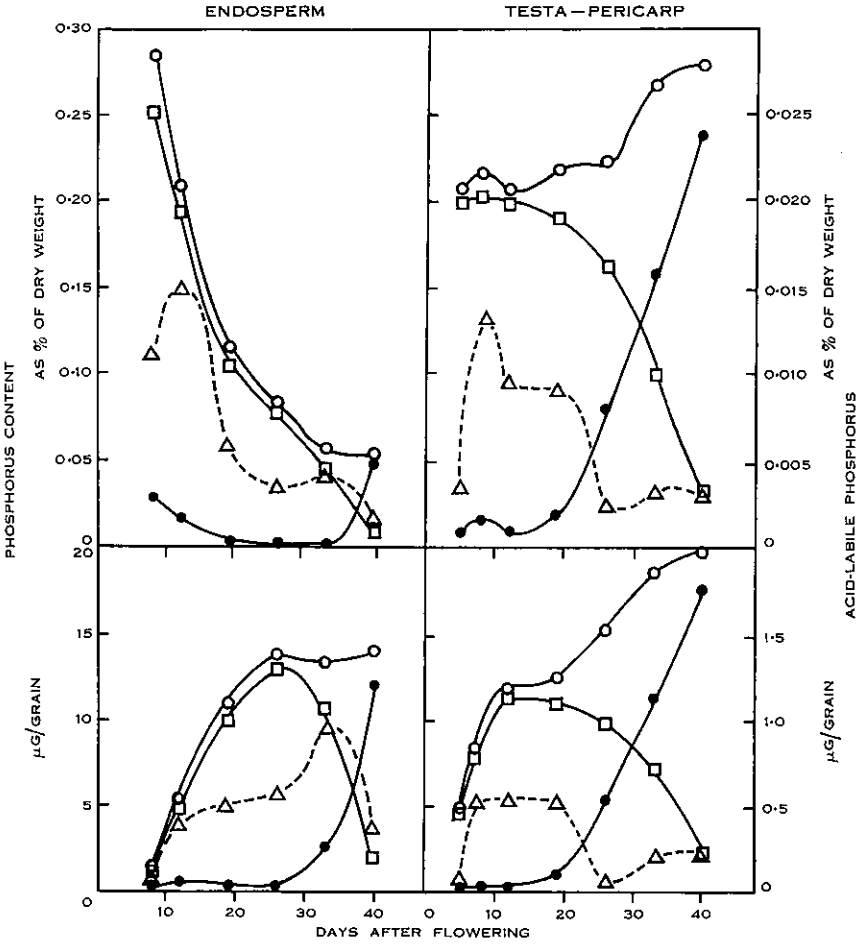

Fig. 2.—Changes in total acid-soluble phosphorus (○), inorganic phosphorus (□), non-labile organic phosphorus (●), and in acid-labile phosphorus (△), of endosperm and testa–pericarp (cv. Gabo, 1959 harvest) with time from flowering. The ordinate at the right-hand side refers to acid-labile phosphorus only.

throughout development. However, the relative concentration of acid-stable organic phosphorus declines to day 26 and then increases steeply, corresponding to the considerable increase in the amount of this fraction per grain. Similar changes are seen in the testa–pericarp fraction (Fig. 2), except that the decline in amount of inorganic phosphate and increase in amount of acid-stable organic phosphorus occurs at about day 12.

The results suggest that there is a very active enzymic system, present in both endosperm and testa–pericarp cells, which catalyses conversion of inorganic phosphate to phytic acid (predominant in the fraction containing acid-soluble, acid-stable organic phosphorus). This system probably involves the intermediary formation of a compound with reactive phosphoryl groups (such as adenosine triphosphate). The amount per grain of acid-labile phosphate declines in both endosperm and in testa–pericarp during the phase of rapid synthesis of phytic acid (i.e. between day 26 and day 40 for endosperm, and between day 19 and day 40 for testa–pericarp). As yet little information is available concerning the enzymic synthesis of phytic acid (see Courtois 1951). Hoffmann-Ostenhof, Jungwirth, and Dawid (1958) showed that crude preparations of yeast hexokinase contain an enzyme which catalyses phosphorylation of inositol from adenosine 5′-triphosphate; this enzyme is probably distinct from hexokinase (Atkinson, Morton, and Simons, unpublished data, 1959).

TABLE 1

RELEVANT EXTINCTION RATIOS FOR EXTRACTS OF NUCLEIC ACIDS FROM WHEAT GRAIN (CV. GABO, 1959 HARVEST)

E_{240}, E_{260}, and E_{300} refer to extinction values at wavelengths 240, 260, and 300 mμ respectively

Days after Flowering	Endosperm			Testa–Pericarp		
	$\frac{E_{240}}{E_{260}}$	$\frac{E_{280}}{E_{260}}$	$\frac{E_{300}}{E_{260}}$	$\frac{E_{240}}{E_{260}}$	$\frac{E_{280}}{E_{260}}$	$\frac{E_{300}}{E_{260}}$
5				0·67	0·68	0·15
8	0·59	0·63	0·09	0·68	0·68	0·17
12	0·55	0·65	0·18	0·44	0·71	0·18
19	0·75	0·64	0·22	0·94	0·64	0·23
26	1·02	0·62	0·23	0·91	0·76	0·37
33	0·85	0·78	0·54	1·03	0·81	0·45
40	1·13	0·81	0·57	1·02	0·81	0·46

There is a striking similarity in the changes of amount of inorganic phosphate per grain (Fig. 2), and of amount of water per grain (Jennings and Morton 1963, Fig. 2), in both endosperm and testa–pericarp. There appears to be a relationship between the supply of water to the developing grain and the movement of inorganic phosphate from the soil and from other parts of the plant into the endosperm and the testa–pericarp. The times of restriction of supply of water to the endosperm and to the testa–pericarp fairly closely correspond with the times of initiation of rapid synthesis of phytic acid. The amount (and the concentration) of inorganic phosphate then declines, whereas the amount of total acid-soluble phosphate subsequently either remains fairly constant (as in endosperm) or increases (as in testa–pericarp). The relationship between the water status of the cell and the initiation of enzymic reactions involving phosphorylated compounds in developing cereals warrants further study.

(*b*) *Changes in Nucleic Acids*

Nucleic acids were extracted with buffered 10% potassium chloride as used by Martin and Morton (1956) who found that several other procedures were unsatisfactory with plant tissues (see also Smillie and Krotkov 1960). At early stages of development, the extracts had absorption spectra typical of solutions rich in nucleic acid, whereas at later stages the extracts contained a higher proportion of protein (Table 1). In addition, the presence of dispersed starch probably contributed to the general extinction of the solutions.

Deoxyribonucleic acid was estimated by determination of deoxyribose by the method of Webb and Levy (1955). Unsatisfactory results were obtained with Ceriotti's (1952) procedure, possibly due to interference by arabinose. As estimated by spectrophotometry, the absolute values of nucleic acid determined from absorption spectra were too high as compared with estimates based on determination of phosphorus, which set an upper limit for the amount of nucleic acid. The amount of RNA was therefore estimated as the difference between the total nucleic acid, as calculated from the phosphorus content, and the deoxyribonucleic acid, as calculated from the deoxyribose content of the extract in potassium chloride. It was assumed that the DNA contained 9·9%, and the RNA 9·4% of phosphorus (Leslie 1955). Figure 3 shows that the amount of DNA per endosperm increases between days 8 and 19 and then remains constant; there is little change after day 14. By assuming that the amount of DNA per nucleus is essentially constant, this result indicates that cell division has ceased by day 19, or that the rate of formation of new cells is then equal to the rate of cell disintegration. These results are consistent with the earlier conclusions of Gordon (1922) and of Sandstedt (1946), based on cytological observations. These workers showed that endosperm cells are derived by formation of cell walls between the nuclei of the multinucleate cell which comprises the endosperm immediately after fertilization, and by division of the peripheral cells which later become the aleurone layer. The rate of cell division declined by about 14 days after pollination, although in the aleurone layer some cell division continued very slowly (Sandstedt 1946). Clearly, after about day 19, the endosperm increases in size by cell expansion and not by cell division.

Since the amount of DNA per grain in the testa–pericarp fraction is essentially constant throughout development after day 5 (Fig. 3), the number of testa–pericarp cells is essentially constant, in agreement with the cytological studies of Percival (1921).

The testa–pericarp surrounds the endosperm and hence cell expansion may be even more vigorous in this tissue than in endosperm, consistent with the relatively rapid synthesis of pentosans during development of testa–pericarp (see Jennings and Morton 1963, Fig. 5).

Figure 3 also shows that the amount of total RNA per grain increases in the endosperm until day 19; thereafter it declines. The relative concentration declines throughout development. Cellular RNA consists of nucleolar RNA and extranuclear ribosomal (template) RNA, transport (small) RNA, and messenger RNA (see Morton 1961). In other tissues, nucleolar RNA and extranuclear ribosomal RNA account

for over 70% of the total RNA, and it is assumed that a similar distribution would apply to endosperm and testa–pericarp of wheat grain. The various types of RNA are all related to protein synthesis. It would appear that, in the endosperm, RNA increases rapidly during the period of cell division, and thereafter the amount is maintained or slightly declines. The synthesis of pyrophosphate-soluble proteins also increases rapidly until day 19 and thereafter declines somewhat, whereas increase of acetic acid-soluble protein is particularly rapid after day 19 (Jennings and Morton

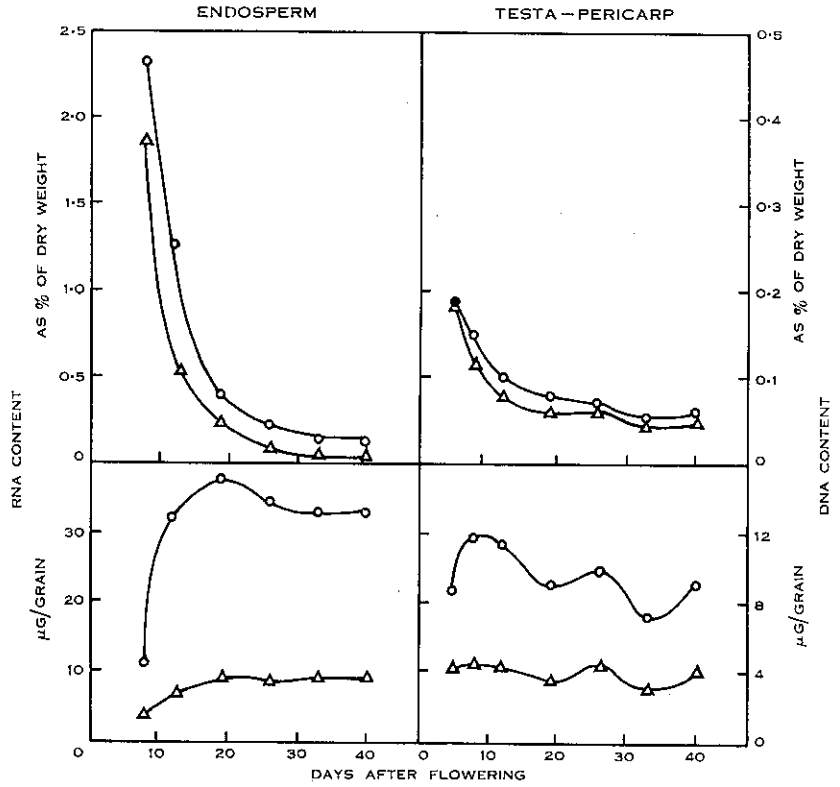

Fig. 3.—Changes in total RNA (○) and DNA (△) of endosperm and testa–pericarp (cv. Gabo, 1959 harvest) with time from flowering. The ordinate at the right-hand side refers to DNA only.

1963, Fig. 7). The acetic acid-soluble proteins may be removed rapidly from the sites of synthesis (involving nucleic acids) and they may accumulate in special compartments in the endosperm. It has now been shown by electron microscopy that vacuolar structures exist in endosperm cells and that proteins accumulate in these as protein bodies (Graham *et al.* 1962; Jennings, Morton, and Palk 1963). In the testa–pericarp, the amount of total RNA per grain shows some fluctuation during development but there is little difference between the initial period of flowering and maturity (Fig. 3). In this tissue, there is only slight change in the amount of protein per grain during development (Jennings and Morton 1963, Fig. 6).

(c) *Changes in Protein-bound Phosphorus*

Figure 4 shows that endosperm also contains protein-bound phosphorus which increases in amount throughout development; there is little comparable change in the testa–pericarp. After an initial decline until about day 12, the relative concentration of the protein-bound phosphorus remains about constant during development (Fig. 4). The phosphorus/nitrogen ratio in this fraction also remains approximately the same after day 12. The nature of the protein-bound phosphorus is not known.

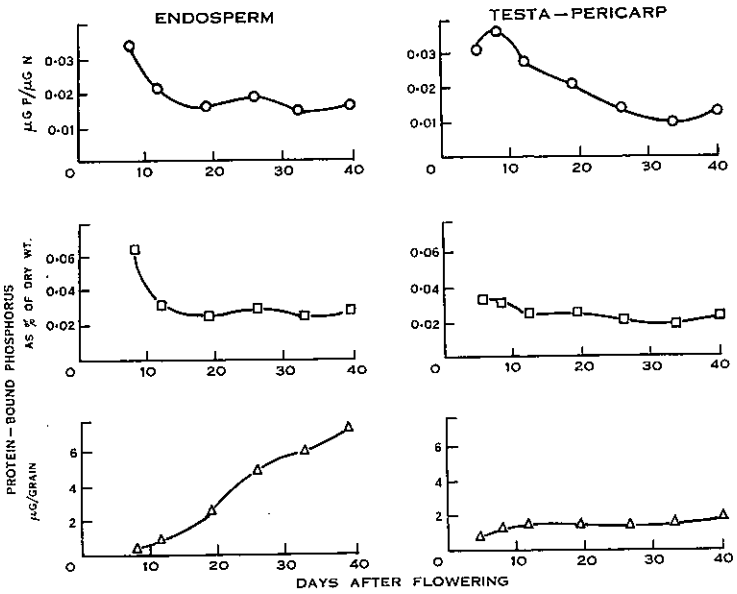

Fig. 4.—Changes in amount (△) and percentage content (□) of the protein-bound phosphorus and in the ratio of protein-bound phosphorus to protein nitrogen (○) of endosperm and testa–pericarp (cv. Gabo, 1959 harvest) with time from flowering.

(d) *Changes in Lipids and in Lipid Phosphorus*

As shown in Figure 5, the amount of total lipid per grain in endosperm increases between day 8 and day 26, and then remains relatively constant; expressed as a proportion of the dry weight, lipid declines between day 8 and day 26, so that the rate of synthesis is much lower than that of starch and of protein. Lipid phosphorus showed essentially the same trends as did the total lipid; as a proportion of the weight of lipid, the phosphorus content was relatively constant throughout development. Lipoproteins containing phospholipids constitute an important part of all cell membranes and of intracellular membranes. The increase in amount of lipid and of lipid phosphorus (Fig. 5) therefore probably reflects an increase in the amount of intracellular membranes, which are abundant in endosperm cells as observed by electron microscopy (Jennings, Morton, and Palk 1963). In the testa–pericarp

tissue, synthesis of total lipids and of lipid phosphorus is relatively rapid until about day 19; this probably corresponds with the period of rapid cell enlargement.

(e) *Synthetic Processes in Cells of Wheat Grains*

The results given here and in the previous paper (Jennings and Morton 1963) show that the developing wheat grain, and especially the endosperm tissue, are actively synthesizing a large number of compounds. Starch and storage protein

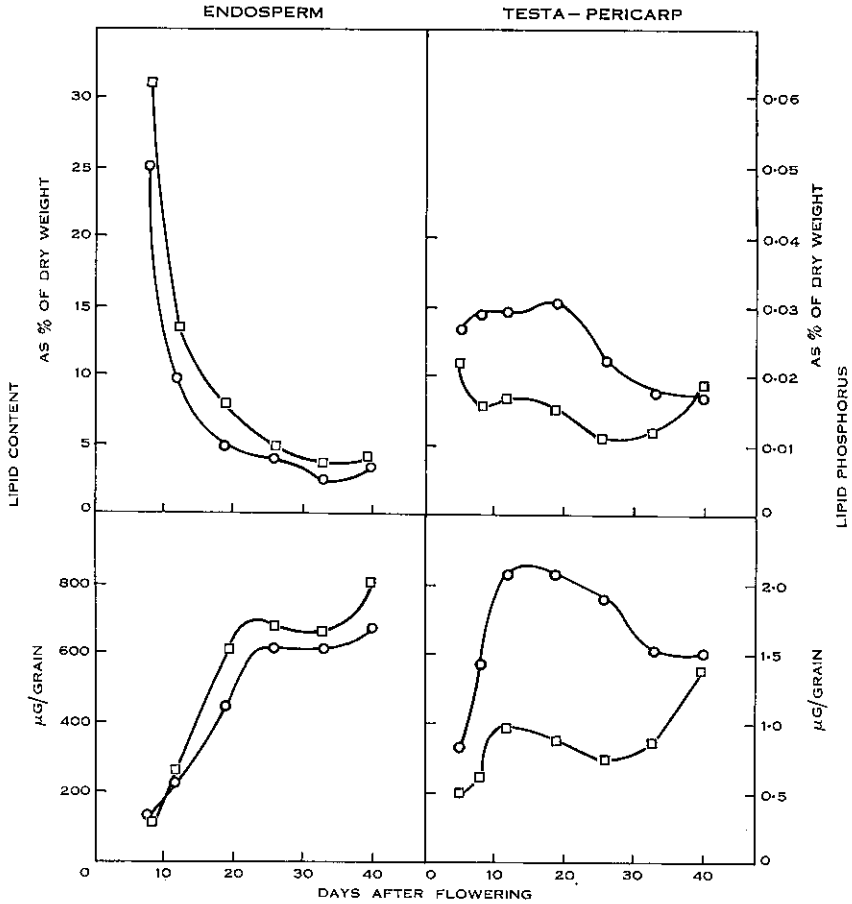

Fig. 5.—Changes in lipid (□) and lipid phosphorus (○) of endosperm and testa–pericarp (cv. Gabo, 1959 harvest) with time from flowering. The ordinate at the right-hand side refers to lipid phosphorus only.

appear to be removed from the sites of synthesis by a process of internal secretion and thus accumulate without interfering with synthetic processes. These syntheses are reflected in changes in a number of compounds, especially of nucleic acids and of phytic acid. It seems likely that the latter acts as a source of potentially reactive phosphoryl groups for synthetic work during germination of the embryo. Rowan and Turner (1957) have reported changes in phosphorus-containing fractions related

to starch synthesis in ripening peas; they also observed an increase in an organic phosphorus fraction (possibly phytic acid) in the late stages of development.

The changes described here are the chemical expression of cytological changes observed with the electron microscope (Jennings, Morton, and Palk 1963). This chemical and cytological information has provided a basis for studies on protein synthesis in developing wheat grain (see Graham *et al.* 1962).

IV. Acknowledgment

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