PHYSIOLOGY OF PEA FRUITS

III. CHANGES IN STARCH AND STARCH PHOSPHORYLASE IN THE DEVELOPING SEED

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[*Manuscript received March* 18, 1957]

Summary

During the growth of pea seeds there is a period in which the starch content rises rapidly and the sucrose content falls. This paper describes a study of the control of this starch synthesis. A method was developed for the assay of starch phosphorylase in peas and the changes in enzyme activity during development of the pea seed were followed. Over most of the period of development a linear relationship existed between the rate of starch synthesis and starch phosphorylase activity. It is suggested that the activity of starch phosphorylase may be a major factor in controlling the rate of starch formation in the pea. The rate of starch synthesis began to decrease when the pea ceased to gain water and the linear relationship then no longer held. The bearing of these observations on sugar–starch relationships and on the general problems of growth is discussed.

I. INTRODUCTION

It is well recognized that starch and sugars (especially sucrose) are closely related in many plant tissues. Advances in knowledge of the enzyme systems concerned in starch (Hanes 1940) and sucrose metabolism (Turner 1953, 1954; Cardini, Leloir, and Chiriboga 1955; Leloir and Cardini 1953, 1955) have indicated the probable mechanisms of carbohydrate changes in plant tissues. There is, however, little information on the control of the direction and extent of the starch and sugar syntheses and of starch–sugar transformations.

It was considered that the developing pea might provide a suitable material for the study of changes in carbohydrate metabolism during growth and of the factors controlling starch synthesis. During development of the pea seed there is a rapid rise in the starch content (Bisson and Jones 1932; McKee, Robertson, and Lee 1955) and this is accompanied by a fall in sugars. The change from a sweet, succulent pea to a seed of high starch content is of considerable commercial importance. The content of alcohol-insoluble solids (of which starch is a major component) has been used as a measure of maturity in peas (Kertesz 1935) and correlates well with physical determinations of hardness (Lynch and Mitchell 1950).

The present experiments are concerned with the changes in starch content and starch phosphorylase activity during the growth of pea seeds. Changes in certain other constituents were followed simultaneously.

II. MATERIALS AND METHODS

(*a*) *Sampling of Peas*

Peas (*Pisum sativum* L., var. Canner's Perfection) were taken from crops at Hawkesbury Agricultural College, Richmond, N.S.W. In order to provide pods

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of the same age, fully opened flowers were tagged on one day in each season. The dates of tagging were October 14, 1954, and September 25, 1955. Subsequently on specified days at 7 a.m. a minimum of 150 tagged pods was picked at random. Two hours later the peas were cut from the hulls, mixed, and samples taken for analysis.

(*b*) *Analytical Methods*

Fresh weight, dry weight, and moisture content were determined by the methods described by Turner (1949). Starch was determined by the methods of Nielsen and Gleason (1945) and Pucher, Leavenworth, and Vickery (1948). These methods gave similar results but difficulty was encountered in hydrolysing the starch by the method of Pucher *et al.* The values reported here were obtained by the method of Nielsen and Gleason. Sucrose was determined by a method to be described (Turner, Turner, and Lee, unpublished data).

(*c*) *Assay of Starch Phosphorylase*

The assay was based on the method of Green and Stumpf (1942), with the addition of molybdate to inhibit phosphatase as suggested by Bailey, Thomas, and Whelan (1951). Phosphoglucomutase did not interfere during the assay period. The plant extract was incubated with glucose 1-phosphate, a starch primer, and ammonium molybdate. The inorganic orthophosphate liberated after a standard time was estimated and taken as a measure of starch phosphorylase activity.

(i) *Reagents.*— Glucose 1-phosphate was prepared by an unpublished method of Professor C. S. Hanes and Dr. R. Hill. A commercial soluble starch was used as primer for the reaction. The soluble starch could be replaced by potato or pea starch.

(ii) *Preparation of Enzyme Extracts.*—The standard assay enzyme was prepared as follows:

Peas (10 g) were finely ground with 12 ml water and acid-washed sand. The ground material was centrifuged at 1000 *g*, the supernatant filtered through cotton wool, and centrifuged at 20,000 *g* for 20 min at 0°C. To 10 ml supernatant was added 40 ml cold saturated ammonium sulphate, pH 6·0. After standing for 1 hr at 0°C the precipitate was collected by centrifuging at 20,000 *g* for 7 min at 0°C, taken up in water, and diluted to the required volume.

Partially purified starch phosphorylase was obtained by repeated fractional ammonium sulphate precipitation of pea extracts.

(iii) *Measurement of Enzyme Activity.*—A mixture of 0·25 ml 0·6M citrate buffer (citric acid–NaOH), pH 6·0, 0·1 ml 0·018M ammonium molybdate, and 1·0 ml enzyme extract was incubated at 30°C. After 15 min, 0·25 ml 5 per cent. (w/v) soluble starch and 0·2 ml 0·26M glucose 1-phosphate, pH 6·0, were added and samples taken for analysis at 0 and 20 min. The rate of reaction was constant during the 0–20-min period. Assays for starch phosphorylase on replicate samples of peas agreed within 5 per cent.

Inorganic phosphate was determined on trichloroacetic acid extracts of the enzyme digests by the method of Allen (1940). Maltose and glucose were detected

by running trichloroacetic acid extracts on paper chromatograms using *n*-propanol–ammonia–water (80 : 15 : 5 v/v) as solvent and the silver spray of Trevelyan, Procter, and Harrison (1950).

One unit of enzyme is defined as the amount which catalyses the liberation of 0·05 mg inorganic phosphorus per 1·8 ml digest in 20 min at 30°C. This represents 3·1 per cent. of the glucose 1-phosphate supplied and digests containing 1–4 units of enzyme were used. Within this range the activity of the partially purified enzyme preparations and of the standard assay preparations was directly proportional to the concentration of enzyme.

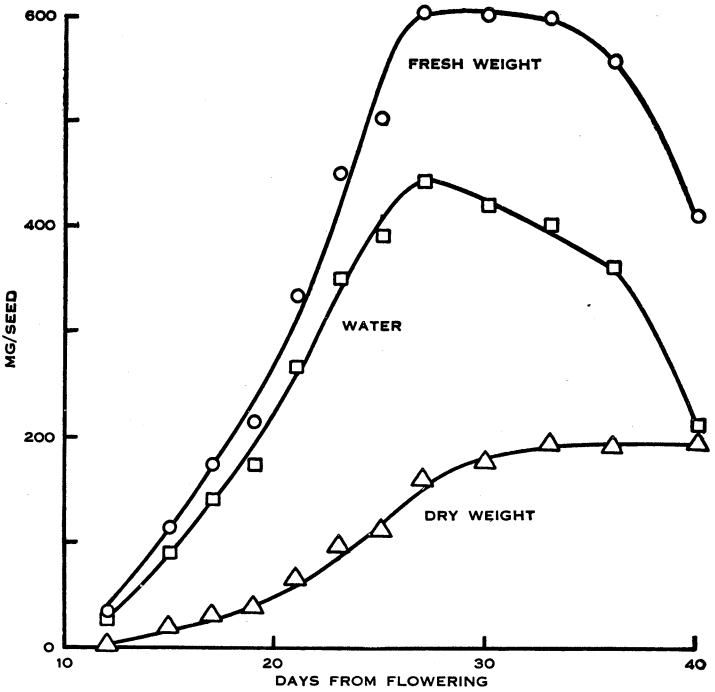

Fig. 1.—Changes in fresh weight, dry weight, and water in pea seeds with time from flowering.

(iv) *The Presence of Phosphatase and Amylases.*—When pea enzyme extracts were allowed to act on glucose 1-phosphate there was some phosphatase action which was detected by the production of glucose. The phosphatase was completely inhibited by 1×10^{-3}M ammonium molybdate. This concentration of molybdate had no effect on the starch phosphorylase activity of either the partially purified or standard assay enzyme preparations. A figure for phosphatase activity was obtained from the difference in phosphate production between digests with and without molybdate. These values must be considered as approximate as Porter (1953) has observed that phosphatase activity may be depressed by high phosphorylase levels.

A change in iodine colour which was observed in digests containing starch and the standard enzyme assay extract suggested that α-amylase was present. Some

maltose was produced in similar digests indicating β-amylase activity. β-Amylase is absent from most starch-bearing leguminous seeds (Peat 1951). The use of mercuric chloride as an inhibitor of amylase activity was unsatisfactory as a routine procedure as the protein content of the assay extracts varied with the age of the peas. The concentration of mercuric chloride (1×10^{-4}M) required to inhibit the amylases also caused some inhibition of starch phosphorylase. Porter (1950) has shown that β-amylase is an inhibitor of starch phosphorylase. The values quoted for starch phosphorylase may have been affected by the presence of β-amylase and should be

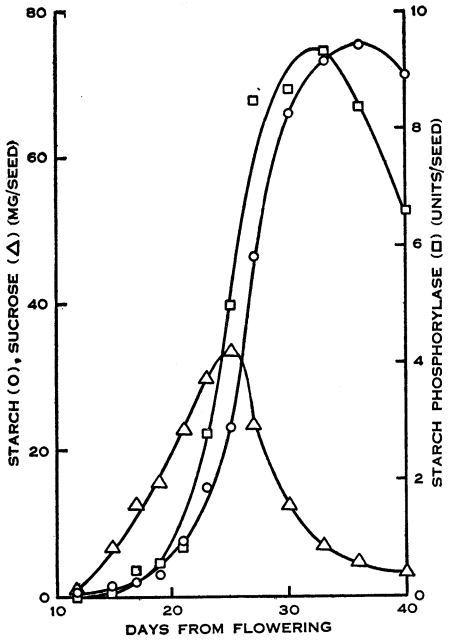

Fig. 2

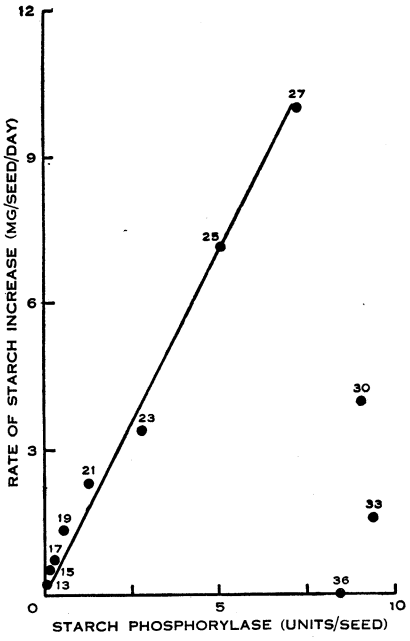

Fig. 3

Fig. 2.—Changes in starch, starch phosphorylase, and sucrose in pea seeds with time from flowering.

Fig. 3.—Relation between the rate of starch increase and starch phosphorylase activity at various times. The days from flowering are shown by the number adjacent to each point. The line was fitted visually.

considered as "apparent starch phosphorylase". β-Amylase was very approximately estimated by following the rate of maltose formation in digests containing starch and the standard assay enzyme. Extracts of the enzyme digests were examined by paper chromatography and the maltose estimated visually.

III. Results

Experiments were carried out in two successive seasons (1954 and 1955). The results were essentially the same in both seasons even though the maximum fresh weight per seed in 1955 was 50 per cent. above that of 1954. The activity of starch phosphorylase and also the starch content per unit fresh weight were similar in each

season. More samples were taken in 1955 and the results of this season are reported here.

From the time of the first sampling (12 days from flowering) fresh weight per seed increased steadily until 27 days (Fig. 1) and remained constant until 33 days. Fresh weight then decreased. The dry weight per seed increased until 30 days from flowering and thereafter changed very little; the dry weight did not fall at any stage in the experiment. Water per seed increased until 27 days from flowering and then decreased until 40 days when the water content was less than half the value attained at 27 days. McKee *et al.* (1955) also observed that the pea seed failed to accumulate water after a certain stage in development.

The changes in starch content per seed (Fig. 2) are in general agreement with results previously reported by Bisson and Jones (1932) and McKee *et al.* (1955). The starch content was very low at 12 days from flowering and increased slowly over the next 9 days. About 21 days after flowering a phase of rapid starch synthesis commenced and continued until 30 days. Subsequent changes in starch content were small. The changes in starch phosphorylase activity (Fig. 2) were approximately parallel to those in starch content.

By fitting tangents to the smoothed curve shown in Figure 2, the daily rates of starch synthesis were calculated. In Figure 3, these rates are plotted against the starch phosphorylase activities on the corresponding days, read from the smoothed curve in Figure 2. A linear relationship was obtained until 27 days from flowering after which time the remaining points were not linear. This departure from linearity coincides with the cessation of water increase.

The sucrose content per seed rose from the first sampling until 25 days after flowering and subsequently decreased rapidly (Fig. 2). Changes in sugars will be considered in detail in a subsequent publication.

Phosphatase activity rose until 23 days from flowering and then fell to a low level. β-Amylase activity appeared to be relatively high in the initial period to 23 days but subsequently decreased markedly.

IV. Discussion

It is generally considered that starch phosphorylase is responsible for starch synthesis in plants. In some plant tissues, however, starch is formed when the ratio of inorganic phosphate to glucose 1-phosphate appears to be unfavourable for the production of starch. On the basis of this evidence Ewart, Siminovitch, and Briggs (1954) concluded that the phosphorylase reaction was not mediating starch synthesis. This conclusion is doubtful as the results of gross analyses of tissues may bear little relation to the actual concentration of reactants at the site of synthesis within the cell. This aspect has been discussed by Trevelyan, Mann, and Harrison (1954).

Sucrose is probably the main carbohydrate transported into the pea and therefore the main raw material for starch formation. It has been found in this Laboratory (Turner *et al.*, unpublished data) that the concentration of hexoses is not more than 5 per cent. of the sucrose concentration during the main part of the growing period of the pea. From the data of McKee *et al.* (1955) it can be calculated that the total phosphorus in the pea seed at 40 days after flowering is only 3·8 per cent. of

that which would be produced (as inorganic orthophosphate) by the observed formation of starch by the phosphorylase reaction. This indicates either that practically all the phosphorus entering the seed is re-exported or, more likely, that the amounts of hexose phosphates transported into the pea are too low to make a significant contribution to starch formation.

The rapid rise in starch content and corresponding fall in sucrose are the main points taken for consideration in the present discussion. The sucrose content increased from the first sample until 25 days from flowering. Starch was then increasing and, although sucrose was probably still being imported at approximately the same rate, starch synthesis was so intense that the level of sucrose fell. These trends continued until 30 days from flowering after which time there was little increase in dry weight of the seed.

There is evidence (Turner 1953, 1954; Cardini *et al.* 1955) that sucrose may be synthesized in pea extracts from uridine diphosphoglucose (UDPG) and fructose by reaction (**1**):

$$\text{UDPG} + \text{fructose} \rightleftharpoons \text{sucrose} + \text{uridine diphosphate.} \quad \ldots\ldots(1)$$

UDPG may be formed by reaction (**2**) which has been observed in extracts from yeast and animal tissues (Munch-Petersen *et al.* 1953; Mills, Ondarza, and Smith 1954):

$$\text{Uridine triphosphate} + \text{glucose 1-phosphate} \rightleftharpoons \text{UDPG} + \text{pyrophosphate.} \quad \ldots\ldots(2)$$

The degradation of sucrose by a reversal of these reactions would yield glucose 1-phosphate and fructose. An active fructokinase which catalyses the phosphorylation of fructose to fructose 6-phosphate has been observed in pea extracts in this Laboratory and by Medina and Sols (1956). Both phosphohexoisomerase and phosphoglucomutase occur in peas (Hanes 1940) and these enzymes make possible the conversion of fructose 6-phosphate to glucose 1-phosphate. Thus sucrose may be completely converted to glucose 1-phosphate, the substrate for starch phosphorylase.

In the initial period of growth of the pea (up to 21 days from flowering) the level of starch phosphorylase was low and there was little starch synthesis. Both phosphatase and β-amylase activities were relatively high up to 23 days from flowering. Phosphatase may have depressed starch synthesis by removing part of the available glucose 1-phosphate and β-amylase may have inhibited starch phosphorylase (Porter 1950). There was no evidence that the amylases degraded starch in the developing pea seed. Ono (1955) has observed that a decrease in β-amylase activity in the radish coincides with the period of starch formation.

After 21 days from flowering the starch phosphorylase activity increased rapidly and an acceleration of starch synthesis ensued. Over most of the period of development of the pea there was a linear relationship between the rate of starch formation and the starch phosphorylase activity. This suggests that the level of starch phosphorylase may be a major factor controlling starch synthesis in the peas under investigation.

The lack of any direct relationship between starch and starch phosphorylase after 27 days from flowering is interesting. The departure from linearity coincides with the cessation of net water uptake by the seed and may be closely related to this phenomenon. After 27 days the water content of the seed decreased and the transformation to the dry seed began. It is apparent that a point must be reached when starch synthesis ceases, perhaps ultimately due to substrate limitation as well as to the desiccation of the regions of synthesis; at this stage considerable amounts of starch phosphorylase are still present. Starch phosphorylase may be readily demonstrated in dried pea seeds (Hanes 1940).

The concentration of glucose 1-phosphate necessary to maintain the observed rate of starch synthesis in the pea may be approximately estimated. Rowan (unpublished data) found that the peak concentration of hexose monophosphates during the period of active starch synthesis in the peas under investigation was approximately 2 μmoles/g fresh weight. Glucose 1-phosphate is unlikely to comprise more than 5–10 per cent. of the hexose monophosphate fraction. From the enzyme data obtained in this investigation and assuming a mean growing temperature for the peas of 15°C (with a Q_{10} of 2 for starch phosphorylase) it can be calculated that the glucose 1-phosphate concentration needed for the observed rate of starch formation must be of the order of 5 μmoles/g fresh weight. This value is minimal as it is derived from initial velocities in the enzyme assays and assumes an inorganic phosphate concentration approximating to zero. It appears, therefore, that the concentration of glucose 1-phosphate at the site of starch synthesis is considerably higher than the content found by analysis of the whole tissue, and that there are regions of high concentration within the cells.

There is little information on the factors which may control or influence the absolute level of starch phosphorylase. The possibility of adaptive enzyme formation in response to the presence of glucose 1-phosphate cannot be excluded. The change may also be the response to some hormone variation or genetic characteristic which is part of the normal growth pattern. The results obtained may have some interesting implications regarding the general problems of growth in plants and the extent to which the activity of enzyme systems may control syntheses and metabolism as a whole.

V. Acknowledgments

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation and Transport, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Mr. J. B. Lee and Mr. J. Smydzuk for carrying out some of the analyses; to Mr. L. J. Lynch, Mr. R. S. Mitchell, and Mr. G. G. Coote for their helpful advice; to Hawkesbury Agricultural College for the supply of peas; to Dr. R. N. Robertson for his interest during the course of the investigation; and to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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