PHYSIOLOGY OF PEA FRUITS

VI.* CHANGES IN URIDINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE AND ADENOSINE DIPHOSPHATE GLUCOSE PYROPHOSPHORYLASE IN THE DEVELOPING SEED

By J. F. Turner†

[*Manuscript received March* 24, 1969]

Summary

Changes in UDPG pyrophosphorylase and ADPG pyrophosphorylase and a number of other constituents were followed during the growth of the pea seed. At all stages of development of the seed UDPG pyrophosphorylase activity was much higher than ADPG pyrophosphorylase activity. During the period of starch formation there was a similarity in the changes in starch and UDPG pyrophosphorylase and ADPG pyrophosphorylase. The maximum rate of starch synthesis coincided with the maximum activity of ADPG pyrophosphorylase. The results are consistent with the biosynthesis of starch from ADPG but do not preclude the participation of UDPG as substrate in this process. It is suggested that UDPG pyrophosphorylase may provide a source of glucose 1-phosphate from sucrose for ADPG formation.

I. Introduction

For some years it was assumed that starch phosphorylase played a dominant role in the synthesis of starch in plants. Starch phosphorylase (Hanes 1940*a*, 1940*b*) utilizes glucose 1-phosphate as substrate and transfers a glucosyl residue to a primer molecule (an α-1,4-glucan) according to reaction (1):

$$\text{glucose 1-phosphate} + \alpha\text{-1,4-glucan} \rightleftharpoons \alpha\text{-1,4-glucosylglucan} + P_i. \quad (1)$$

Subsequently the biosynthesis of glycogen from uridine diphosphate glucose (UDPG) by preparations from liver was demonstrated by Leloir and Cardini (1957) and later the synthesis of starch by a similar reaction (2):

$$\text{UDPG} + \alpha\text{-1,4-glucan} \rightleftharpoons \text{UDP} + \alpha\text{-1,4-glucosylglucan} \quad (2)$$

was reported by Fekete, Leloir, and Cardini (1960) and Leloir, Fekete, and Cardini (1961) using starch granule preparations from plant tissues. Further work (Recondo and Leloir 1961) showed that glucose was transferred at a faster rate from adenosine diphosphate glucose (ADPG) [reaction (3)] than from UDPG:

$$\text{ADPG} + \alpha\text{-1,4-glucan} \rightleftharpoons \text{ADP} + \alpha\text{-1,4-glucosylglucan}. \quad (3)$$

The formation of UDPG [reaction (4)] is catalysed by UDPG pyrophosphorylase (EC 2.7.7.9):

$$\text{UTP} + \text{glucose 1-phosphate} \rightleftharpoons \text{UDPG} + \text{pyrophosphate} \quad (4)$$

* Part V, *Aust. J. biol. Sci.*, 1957, **10**, 414–25.

† Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006.

and this enzyme is widely distributed in plants (Turner and Turner 1958). The synthesis of ADPG is by a similar reaction (5) catalysed by ADPG pyrophosphorylase:

$$\text{ATP} + \text{glucose 1-phosphate} \rightleftharpoons \text{ADPG} + \text{pyrophosphate} \qquad (5)$$

and the presence of this enzyme has been demonstrated in a number of plant tissues (Espada 1962; Ghosh and Preiss 1966; Sanwal and Preiss 1967).

By analogy with the synthesis and degradation of glycogen, Leloir (1964) suggested that the substrate for starch synthesis in plants is probably a nucleoside diphosphate sugar and the role of phosphorylase is starch degradation. Leloir considered that both UDPG and ADPG may be involved in starch synthesis *in vivo* and probably contribute equally because although UDPG reacts more slowly than ADPG (about one-tenth the rate) its concentration in plant tissues is higher (about 5–10 times). Some direct evidence supporting the synthesis of starch from ADPG was reported by Tsai and Nelson (1966). These authors found that a starch-deficient maize mutant, which synthesized only 25–30% as much starch as normal maize, lacked ADPG pyrophosphorylase although UDPG pyrophosphorylase was present.

During the development of the pea seed there is a rapid rise in the starch content (Bisson and Jones 1932; McKee, Robertson, and Lee 1955; Turner and Turner 1957). In a previous study of the metabolism of starch and sugars Turner and Turner (1957) found that over most of the period of development of the pea the changes in starch and starch phosphorylase activity were similar. Recent developments and hypotheses concerning starch synthesis prompted a further study of changes in the developing pea seed. The present investigation is concerned with the activities of UDPG pyrophosphorylase and ADPG pyrophosphorylase during the growth of pea seeds. Changes in starch and sugars were followed simultaneously.

II. Materials and Methods

(*a*) *Sampling of Peas*

Peas (*Pisum sativum* L., cv. High Market Freezer) were taken from crops grown at the University of Sydney Agricultural Research Station, Castle Hill, N.S.W. To obtain pods of the same age, fully opened flowers were tagged on one day for each crop. The dates of tagging were September 27, 1966, and November 3, 1966. Subsequently on specified days at 7 a.m. a minimum of 60 tagged pods was picked at random. Two hours later the peas were cut from the hulls, mixed, and samples taken for analysis and enzyme assay.

(*b*) *Analytical Methods*

Fresh weight, dry weight, and water content were determined by the methods described by Turner (1949).

(i) *Estimation of Sugars.*—To a 10-g portion of the mixed sample of peas 20 ml absolute ethanol was added and the mixture stored at −18°C. There was no detectable change in the sugars over a period of several months in storage (Turner, Turner, and Lee 1957). When sugar analyses were to be carried out the mixture was finely ground and centrifuged. Portions of the supernatant were treated with barium hydroxide and zinc sulphate (Somogyi 1945) and centrifuged. The clear colourless supernatant was used for sugar determinations.

Reducing sugars were determined by the method of Nelson (1944). Samples of the supernatant fraction were also heated in the presence of 0·05N hydrochloric acid at 100°C for 10 min and reducing sugars estimated. The increase in reducing sugars after hydrolysis is termed "sucrose". Turner, Turner, and Lee (1957) showed that sucrose is the predominant sugar present in the pea seed and there are smaller amounts of fructose, glucose, and galactose.

(ii) *Estimation of Starch.*—The centrifuged residue after ethanolic extraction of the pea seeds was washed three times with 67% (v/v) ethanol, twice with acetone, and once with ether. The residue was dried at 70°C and then *in vacuo*. The starch content of this fraction was determined by a modification of the method of Pucher, Leavenworth, and Vickery (1948). A portion (25–30 mg) of the dried extracted residue was boiled in a centrifuge tube with 4 ml water. After cooling in ice, 3 ml 70% perchloric acid and a small quantity of acid-washed sand were added and the mixture triturated for 10 min and centrifuged. The supernatant was collected and the residue ground again with water (4 ml) and 70% perchloric acid (3 ml) and centrifuged. The extraction was repeated and sodium chloride (1 g) and 2% iodine–potassium iodide solution (2 ml) were added to the combined supernatants. After centrifuging, 10 ml of 1N sulphuric acid was added to the residue, the mixture heated in a boiling water-bath with occasional stirring until the starch–iodine complex dissolved, and then heated for a further 60 min. After cooling the solution was neutralized with sodium hydroxide, diluted to 100 ml, and reducing sugars determined as described previously.

(c) *Assay of UDPG Pyrophosphorylase and ADPG Pyrophosphorylase*

The assays were based on the procedure used by Espada (1962) for ADPG pyrophosphorylase which was similar to the method employed by Munch-Petersen (1955) for UDPG pyrophosphorylase. UDPG pyrophosphorylase was measured by continuously determining the formation of glucose 1-phosphate produced during the incubation of UDPG and inorganic pyrophosphate with pea seed enzyme preparations. Glucose 1-phosphate production was monitored by incorporating phosphoglucomutase and glucose 6-phosphate dehydrogenase in the reaction mixtures and following the reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) spectrophotometrically. In the assay of ADPG pyrophosphorylase, UDPG was replaced by ADPG.

(i) *Reagents.*—UDPG, ADPG, phosphoglucomutase, glucose 6-phosphate dehydrogenase, bovine serum albumin, and $NADP^+$ were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Before use, phosphoglucomutase and glucose 6-phosphate dehydrogenase were dialysed for 2 hr at 2°C against 0·1M Tris–HCl buffer, pH 7·9, containing 0·1 ml thioglycollic acid per litre.

(ii) *Preparation of Enzyme Extracts.*—The assay enzyme was prepared as follows: Peas (10–15 g) were finely ground with 12–50 ml 0·1M Tris–HCl buffer, pH 7·9, containing 0·1 ml thioglycollic acid per litre. The mixture was centrifuged at 1000 *g*, the supernatant filtered through cotton wool, and centrifuged at 37,000 *g* for 30 min. To the supernatant was added 4 vol. saturated $(NH_4)_2SO_4$, pH 7, and, after standing for 30 min, the precipitate was collected by centrifuging at 37,000 *g* for 10 min and suspended in Tris–HCl buffer. The suspension was centrifuged at 37,000 *g* for 20 min and the supernatant dialysed for 1·5 hr against the Tris–HCl buffer. All operations were carried out at 0–2°C. The dialysed extract was used for the assay of UDPG pyrophosphorylase and ADPG pyrophosphorylase.

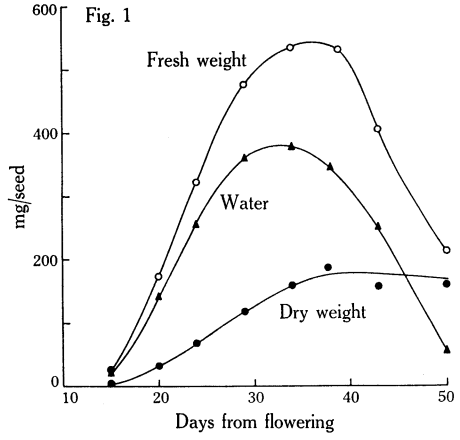
(iii) *Determination of UDPG Pyrophosphorylase Activity.*—Enzyme activity was determined by following the rate of increase in optical density at 340 nm in a Beckman model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder. For the assay the dialysed extract was diluted with 0·1M Tris–HCl buffer, pH 7·9, containing 0·1 ml thioglycollic acid per litre. The reaction mixtures, which were maintained at 30°C, contained the following components: 0·5 μmole UDPG, 2·5 μmoles sodium pyrophosphate, 0·5 μmole $NADP^+$, 7·5 μmoles $MgSO_4$, 100 μmoles Tris–HCl buffer (pH 7·9), 3 μg phosphoglucomutase, 8 μg glucose 6-phosphate dehydrogenase, 0·05–0·20 ml diluted, dialysed pea enzyme extract; total volume 1·5 ml. The reaction was started by the addition of pyrophosphate and the change in optical density recorded. Dilutions of the enzyme preparation were such that optical density changes of 0·015–0·050 per minute were obtained. Within this range the enzyme activity observed was proportional to the amount of dialysed pea extract used. The phosphorolysis of UDPG (Carminatti and Cabib 1961; Dankert, Goncalves, and Recondo 1964) could also lead to the production of glucose 1-phosphate which would interfere with the assay procedure. Accordingly, enzyme reaction mixtures containing 3 μmoles of disodium orthophosphate in place of the sodium pyrophosphate were followed. With reaction mixtures containing large amounts of the enzyme preparation phosphorolysis of

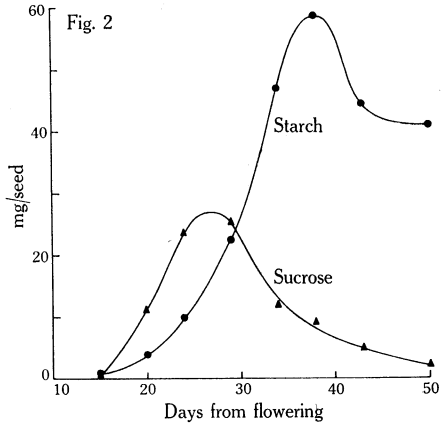
UDPG was observed but was not significant with the dilutions employed for the pyrophosphorylase assays. One unit of UDPG pyrophosphorylase is defined as the amount which catalyses the production of 1 μmole of glucose 1-phosphate per minute at 30°C in reaction mixtures of the composition described for the assay.

(iv) *Determination of ADPG Pyrophosphorylase Activity.*—The method for the determination of ADPG pyrophosphorylase activity was analogous to that used for UDPG pyrophosphorylase. For the assay of ADPG pyrophosphorylase the dialysed extract was diluted with a medium containing 0·1M Tris–HCl buffer, pH 7·9, 2 mM 3-phosphoglycerate (Ghosh and Preiss 1966; Sanwal and Preiss 1967), 4 mM cysteine, and 0·2 mg bovine serum albumin per millilitre. The reaction mixtures contained the following components: 0·5 μmole ADPG, 2·5 μmoles sodium pyrophosphate, 0·5 μmole $NADP^+$, 7·5 μmoles $MgSO_4$, 100 μmoles Tris–HCl buffer (pH 7·9), 3 μg phosphoglucomutase, 8 μg glucose 6-phosphate dehydrogenase, 3 μmoles 3-phosphoglycerate, 6 μmoles cysteine, 0·3 mg bovine serum albumin, 0·05–0·20 ml diluted, dialysed pea enzyme extract; total volume 1·5 ml. In other respects the procedure was similar to that described for the determination of UDPG pyrophosphorylase. One unit of ADPG pyrophosphorylase is defined as the amount which catalyses the production of 1 μmole of glucose 1-phosphate per minute at 30°C in reaction mixtures of the composition described for the assay.

III. Results

Two crops in 1966 were used in the experiments. More samples were taken in the first crop (date of tagging: September 27, 1966) and the results for this are reported here. The results are expressed on a per seed basis. McKee, Robertson, and Lee (1955) showed that cell division in the pea seed was complete before 15 days from flowering: the results on a per seed basis therefore bear a constant relationship to those on a per cell basis.





Figs. 1 and 2.—Changes in fresh weight, dry weight, and water (Fig. 1) and in sucrose and starch (Fig. 2) in pea seeds during development.

Fresh weight per seed increased from the time of the first sampling (15 days from flowering) until 34 days and decreased after 38 days (Fig. 1). Dry weight per seed increased until approximately 38 days from flowering and there was little change subsequently. The water content per seed increased from the first pick, reached a maximum approximately 33 days after flowering, and thereafter decreased until the last sampling. The water content per seed at the conclusion of the experiment was only 14% of the value observed at 34 days after flowering.

The sucrose content per seed increased from the first sampling until approximately 27 days from flowering and then decreased (Fig. 2). Reducing sugars, which are not graphed, were present in low amount relative to sucrose and changed in a manner similar to that described by Turner, Turner, and Lee (1957). The reducing sugars increased from 15 days after flowering, reached a maximum of 0·30 mg per seed at 27 days after flowering, and subsequently decreased. The starch content per seed was very low at 15 days from flowering and increased slowly until between 20 and 24 days from flowering (Fig. 2). A period of rapid starch synthesis then ensued and continued until after 34 days from flowering. Starch content per seed subsequently decreased until 43 days from flowering after which there was little change.

The changes in UDPG pyrophosphorylase activity and ADPG pyrophosphorylase activity per seed are shown in Figure 3. UDPG pyrophosphorylase per seed was very low at 15 days but after 20 days from flowering increased rapidly to a broad maximum and then declined until the experiment was concluded. ADPG pyrophosphorylase, after a rapid increase, reached a sharp maximum at 34 days from flowering and subsequently decreased markedly.

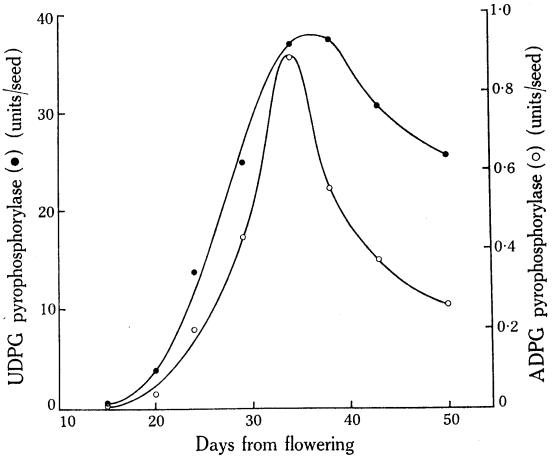


Fig. 3.—Changes in UDPG pyrophosphorylase and ADPG pyrophosphorylase in pea seeds during development.

IV. Discussion

The results of this investigation for the changes in fresh weight, water, dry weight, starch, and sucrose are similar in form to those reported previously (McKee, Robertson, and Lee 1955; Turner and Turner 1957; Turner, Turner, and Lee 1957). The changes were delayed in time presumably due to the use of a different pea variety and to the crop being grown in a different district.

Both UDPG and ADPG pyrophosphorylases were present throughout development of the pea seed. The UDPG pyrophosphorylase activity was at all times much higher than the ADPG pyrophosphorylase activity. At 34 days from flowering, when the rate of starch synthesis reached a maximum, UDPG pyrophosphorylase activity was 36·8 units per seed whereas ADPG pyrophosphorylase activity was 0·89 units per seed. At 50 days from flowering, when the experiment was concluded, UDPG and ADPG pyrophosphorylase activities were 25·5 and 0·26 respectively. These results are in agreement with other recent observations. Unpublished work in

this laboratory has shown that UDPG pyrophosphorylase is also much more active than ADPG pyrophosphorylase in extracts from wheat grains at all stages of development. Nomura *et al.* (1967) found the activity of UDPG pyrophosphorylase was much higher than ADPG pyrophosphorylase activity in preparations from both bean and rice leaves. Vidra and Loerch (1968) reported UDPG pyrophosphorylase was more active than ADPG pyrophosphorylase in enzyme preparations from maize endosperm.

Figures 2 and 3 show that, during the period of starch synthesis, there was a similarity in the changes in starch and UDPG pyrophosphorylase and ADPG pyrophosphorylase. The maximum for UDPG pyrophosphorylase was broader and later than the maximum for ADPG pyrophosphorylase. It may be especially significant that the maximum *rate* of starch synthesis was at 34 days from flowering and this coincided with the maximum activity of ADPG pyrophosphorylase. The rate of starch synthesis declined after this time and the ADPG pyrophosphorylase fell sharply.

The results are consistent with the biosynthesis of starch from ADPG but do not preclude the participation of UDPG as substrate in this process. An important role for ADPG is suggested by the parallel increases in starch and ADPG pyrophosphorylase and by the simultaneous attainment of the maximum rate of starch synthesis and the maximum activity of ADPG pyrophosphorylase. If ADPG is the predominant substrate for starch synthesis, a function of UDPG pyrophosphorylase may be the production of glucose 1-phosphate from sucrose for subsequent ADPG formation. Sucrose is probably the main carbohydrate transported into the pea and is therefore the main raw material for starch formation (Turner and Turner 1957). The extent to which sucrose is being continually synthesized and degraded in the pea seed is unknown but it is clear from the data in Figure 2 that sucrose reaches a maximum and then declines: this decline coincides with the phase of rapid starch synthesis. It appears that at least part of the substrate for this starch synthesis arises from sucrose already present in the pea seed supplemented by sucrose which continues to be transported into the seed. Sucrose may be broken down by the following reaction:

$$\text{sucrose} + \text{UDP} \rightleftharpoons \text{UDPG} + \text{fructose}, \qquad (6)$$

and the enzyme catalysing this reaction, sucrose synthetase, is present in pea seeds (Turner 1953, 1954, 1957; Cardini, Leloir, and Chiriboga 1955). The UDPG so formed may be degraded by UDPG pyrophosphorylase to yield glucose 1-phosphate [reaction (4)]. The equilibrium of this reaction is in favour of UTP and glucose 1-phosphate formation (Turner and Turner 1958). The glucose 1-phosphate produced would be available for ADPG formation by ADPG pyrophosphorylase. Nucleoside diphosphokinase, which catalyses the reversible phosphate transfer between ATP and UTP, is present in high activity in pea seeds (Kirkland and Turner 1959). ADPG can replace UDPG in reaction (6) although it is believed that UDPG is the natural substrate for sucrose biosynthesis (Cardini and Recondo 1962). Because of the lower affinity of sucrose synthetase for ADP than UDP and the strong inhibition of the reaction by uridine nucleotides, Fekete and Cardini (1964) concluded that the formation of ADPG *in vivo* by the reaction of ADP with sucrose is improbable.

If this situation obtains in the pea seed, the source of ADPG would be synthesis from ATP and glucose 1-phosphate by ADPG pyrophosphorylase. Fructose produced in reaction (6) may be converted to glucose 1-phosphate as outlined by Turner and Turner (1957). This suggested mechanism is an extension of that put forward previously (Turner and Turner 1957) and is essentially similar to that of Fekete and Cardini (1964).

V. Acknowledgments

The author wishes to thank Mr. E. S. Blanch for technical assistance and to express his indebtedness to Dr. N. K. Matheson; to Mr. M. O'Reilly of Arthur Yates and Company Pty. Ltd. for the supply of pea seed; and to Professor I. A. Watson and Mr. J. D. Oates. This work was supported by the Australian Research Grants Committee.

VI. References

Bisson, C. S., and Jones, H. A. (1932).—*Pl. Physiol., Lancaster* **7**, 91.
Cardini, C. E., Leloir, L. F., and Chiriboga, J. (1955).—*J. biol. Chem.* **214**, 149.
Cardini, C. E., and Recondo, E. (1962).—*Pl. Cell Physiol., Tokyo* **3**, 313.
Carminatti, H., and Cabib, E. (1961).—*Biochim. biophys. Acta* **53**, 417.
Dankert, M., Goncalves, R. J., and Recondo, E. (1964).—*Biochim. biophys. Acta* **81**, 78.
Espada, J. (1962).—*J. biol. Chem.* **237**, 3577.
Fekete, M. A. R. de, and Cardini, C. E. (1964).—*Archs Biochem. Biophys.* **104**, 173.
Fekete, M. A. R. de, Leloir, L. F., and Cardini, C. E. (1960).—*Nature, Lond.* **187**, 918.
Ghosh, H. P., and Preiss, J. (1966).—*J. biol. Chem.* **241**, 4491.
Hanes, C. S. (1940*a*).—*Proc. R. Soc.* B **128**, 421.
Hanes, C. S. (1940*b*).—*Proc. R. Soc.* B **129**, 174.
Kirkland, R. J. A., and Turner, J. F. (1959).—*Biochem. J.* **72**, 716.
Leloir, L. F. (1964).—*Biochem. J.* **91**, 1.
Leloir, L. F., and Cardini, C. E. (1957).—*J. Am. Chem. Soc.* **79**, 6340.
Leloir, L. F., Fekete, M. A. R. de, and Cardini, C. E. (1961).—*J. biol. Chem.* **236**, 636.
McKee, H. S., Robertson, R. N., and Lee, J. B. (1955).—*Aust. J. biol. Sci.* **8**, 137.
Munch-Petersen, A. (1955).—*Acta chem. scand.* **9**, 1523.
Nelson, N. (1944).—*J. biol. Chem.* **153**, 375.
Nomura, T., Nakayama, N., Murata, T., and Akazawa, T. (1967).—*Pl. Physiol., Lancaster* **42**, 327.
Pucher, G. W., Leavenworth, C. S., and Vickery, H. B. (1948).—*Analyt. Chem.* **20**, 850.
Recondo, E., and Leloir, L. F. (1961).—*Biochem. biophys. Res. Commun.* **6**, 85.
Sanwal, G. G., and Preiss, J. (1967).—*Archs Biochem. Biophys.* **119**, 454.
Somogyi, M. (1945).—*J. biol. Chem.* **160**, 69.
Tsai, C. Y., and Nelson, O. E. (1966).—*Science, N.Y.* **151**, 341.
Turner, D. H., and Turner, J. F. (1957).—*Aust. J. biol. Sci.* **10**, 414.
Turner, D. H., and Turner, J. F. (1958).—*Biochem. J.* **69**, 448.
Turner, J. F. (1949).—*Aust. J. sci. Res.* B 2, 138.
Turner, J. F. (1953).—*Nature, Lond.* **172**, 1149.
Turner, J. F. (1954).—*Nature, Lond.* **174**, 692.
Turner, J. F. (1957).—*Biochem. J.* **67**, 450.
Turner, J. F., Turner, D. H., and Lee, J. B. (1957).—*Aust. J. biol. Sci.* **10**, 407.
Vidra, J. D., and Loerch, J. D. (1968).—*Biochim. biophys. Acta* **159**, 551.