and the concentration and type of acceptor amine has been studied.

4. The manometric method has been used to demonstrate that heteropyrithiamine, quinilinothiamine and possibly oxythiamine are thiaminase substrates.

5. The effect of several structural analogues of thiamine on bracken thiaminase has been studied and it is shown that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride is a powerful but non-competitive inhibitor.

6. Conditions are described under which the rate of evolution of carbon dioxide is directly proportional to the amount of bracken-thiaminase preparation present. It is suggested that the manometric method could be used for estimating thiaminase activity.

REFERENCES

- Barnhurst, J. D. & Hennessy, D. J. (1952). *J. Amer. chem. Soc.* **74**, 353.
- Clarke, H. T. (1944). *J. Amer. chem. Soc.* **66**, 652.
- Cline, J. K., Williams, R. R. & Finkelstein, J. (1937). *J. Amer. chem. Soc.* **59**, 1052.
- Fujita, A., Nose, Y. & Kuratani, K. (1954). *J. Vitaminol., Japan*, **1**, 1.
- Fujita, A., Nose, Y., Kozuka, S., Tashiro, T., Ueda, K. & Sakamoto, S. (1952a). *J. biol. Chem.* **196**, 289.
- Fujita, A., Nose, Y., Uyeo, S. & Koizumi, J. (1952b). *J. biol. Chem.* **196**, 305.
- Fujita, A., Okamoto, T. & Nose, Y. (1955). *J. Vitaminol., Japan*, **1**, 24.
- Hall, N. F. & Sprinkle, M. R. (1932). *J. Amer. chem. Soc.* **54**, 3469.
- Kenten, R. H. (1957). *Biochem. J.* **67**, 25.
- Matsukawa, T. & Yurugi, S. (1954). *J. Vitaminol., Japan*, **1**, 27.
- Melnick, D. & Field, H. (1939). *J. biol. Chem.* **127**, 505.
- Rydon, H. N. (1951). *Biochem. J.* **48**, 383.
- Sealock, R. R. & Goodland, R. L. (1944). *J. Amer. chem. Soc.* **66**, 507.
- Sealock, R. R. & Livermore, A. H. (1944). *J. biol. Chem.* **156**, 379.
- Sealock, R. R. & Livermore, A. H. (1949). *J. biol. Chem.* **177**, 553.
- Williams, R. R. & Ruehle, A. E. (1935). *J. Amer. chem. Soc.* **57**, 1858.
- Woolley, D. W. (1953). *Nature, Lond.*, **171**, 323.

Uridine Diphosphoglucose Pyrophosphorylase of Pea Seeds

BY DONELLA H. TURNER AND J. F. TURNER

*Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O.,
Botany School, University of Sydney, Australia*

(Received 23 December 1957)

The reaction (1) catalysed by uridine diphosphoglucose (UDPG) pyrophosphorylase involves the pyrophosphorolytic cleavage of UDPG with the formation of uridine triphosphate (UTP) and glucose 1-phosphate (G 1-P):

The pyrophosphorolysis of UDPG was first reported by Munch-Petersen, Kalckar, Cutolo & Smith (1953) in *Zwischenferment* preparations. UDPG pyrophosphorylase has also been found in extracts from liver (Mills, Ondarza & Smith, 1954) and mammary gland (Smith & Mills, 1955). A more detailed investigation of the properties of the enzyme was carried out by Munch-Petersen (1955) with a preparation from yeast. Burma & Mortimer (1956) obtained evidence that UDPG pyrophosphorylase was present in homogenates of sugar-beet leaves and Neufeld, Ginsburg, Putman, Fanshier & Hassid (1957) have shown that the enzyme is present in extracts from mung bean and several other plant tissues.

Experiments on the synthesis of sucrose from G 1-P and fructose had suggested that UDPG pyrophosphorylase was active in pea extracts (Turner, 1953, 1954, 1957). In the present investigation UDPG pyrophosphorylase was found in pea-seed extracts and a preparation free from interfering enzymes was obtained. Some properties of the enzyme were studied and its presence in certain other plant tissue extracts was established. The equilibrium of the reaction was examined.

MATERIALS AND METHODS

Substrates. UTP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

UDPG was obtained from Pabst Laboratories and from Sigma Chemical Co., St Louis, Mo., U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill.

Sodium pyrophosphate was analytical-reagent grade (British Drug Houses Ltd.).

Preparation of uridine diphosphoglucose pyrophosphorylase from pea seeds. A crude extract was prepared from dried pea seeds (*Pisum sativum* L.) as described by Turner (1957), and centrifuged at 20 000 g for 20 min. at room

temperature. The supernatant was treated with neutralized saturated $(\text{NH}_4)_2\text{SO}_4$ at 4° and the fraction obtained between 60 and 70% saturation dissolved in 7 ml. of water and dialysed with rocking at 4° for 2.5 hr. against 25 mM-phosphate buffer $(\text{KH}_2\text{PO}_4\text{-NaOH})$, pH 7.

The clear, colourless enzyme preparation could be stored at -15° for 3 months with only slight loss of activity. The preparation was diluted 100-fold with water before use. Heating at 60° for 10 min. resulted in 75% loss of UDPG pyrophosphorylase activity. No interfering enzymic reactions involving any of the components of the UDPG pyrophosphorylase reaction were detected. In the presence of Mg^{2+} ions, UDPG underwent a non-enzymic decomposition leading to the formation of a substance which travelled the same distance as uridine monophosphate when applied to filter paper and developed with ethanol-ammonium acetate (M, pH 7.5; 7.5:3, v/v). This decomposition was suppressed in the presence of sodium pyrophosphate.

Preparation of enzyme extracts from other plant tissues. Seeds of maize, wheat, broad bean and pea were germinated and allowed to grow for 7–10 days in damp sand under light. The roots or shoots were removed, ground with 40 mM- NaHCO_3 and centrifuged. The supernatant was treated with $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation and the precipitate taken up in water and dialysed as described previously. Sugar-cane-stem cuttings were kept in the laboratory in damp sand for 10 days. The shoots and roots were then ground and treated in a similar manner.

Soaked wheat seeds and the laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) were blended with 40 mM- NaHCO_3 . Potato tubers were sliced, minced and the juice was extracted by squeezing through muslin. The extracts of these tissues were centrifuged and treated with saturated $(\text{NH}_4)_2\text{SO}_4$. The fraction collected between 50 and 80% saturation was suspended in water and dialysed.

Preparation of inorganic pyrophosphatase. Inorganic pyrophosphatase was prepared from baker's yeast by the method of Heppel & Hilmo (1951).

Enzyme digests. The enzyme digests were maintained at 30° . For the assay of UDPG pyrophosphorylase activity the following components were mixed: 0.5 μmole of UDPG, 2.5 μmoles of sodium pyrophosphate, 1.25 μmoles of MgCl_2 , 50 μmoles of 2-amino-2-hydroxymethylpropane-1,3-diol (tris)-HCl buffer, pH 7.9, 0.05 ml. of enzyme; total volume, 0.25 ml. The time of incubation was 15 min. The enzyme concentration was adjusted so that not more than 0.28 μmole of UTP (corresponding to 56% conversion of UDPG) was formed in 15 min. Under these conditions the reaction rate was approximately constant during the incubation period. To show the reverse reaction (the formation of UDPG from UTP) digests of the following composition were used: 0.5 μmole of UTP, 7.5 μmoles of G 1-P, 1.25 μmoles of MgCl_2 , 50 μmoles of tris-HCl buffer (pH 7.9), 0.05 ml. of inorganic pyrophosphatase, 0.05 ml. of enzyme; total volume, 0.25 ml.

In determining the equilibrium for the forward reaction enzymic digests of the following composition were used: 0.5 μmole of UDPG, 2.3 μmoles of sodium pyrophosphate, 6.7 μmoles of G 1-P, 1.25 μmoles of MgCl_2 , 50 μmoles of tris-HCl (or tris-acetate) buffer of the required pH, 0.05 ml. of enzyme; total volume, 0.25 ml. G 1-P was added so that the amount of UDPG remaining at equilibrium would be convenient for estimation. The equilibrium with the reverse reaction was determined with enzymic digests of the

following composition: 0.5 μmole of UTP, 6.7 μmoles of G 1-P, 2.3 μmoles of sodium pyrophosphate, 1.25 μmoles of MgCl_2 , 50 μmoles of tris-HCl buffer (pH 7.9), 0.05 ml. of enzyme; total volume 0.25 ml. Sodium pyrophosphate was added to prevent the non-enzymic decomposition of UDPG. For determination of the equilibrium, samples were taken from the enzymic digests at 120 and 150 min.

Digest samples were inactivated by the addition of 2 vol. of ethanol or 0.25 vol. of 5% (w/w) HClO_4 (followed by neutralization with dil. aq. NH_3 soln.) and centrifuged at 1000 g for 5 min.

Detection and estimation of uridine nucleotides. The uridine nucleotides were separated on paper chromatograms, eluted with water and estimated by their absorption at 262 m μ . Descending chromatography in all-glass apparatus maintained at 25° was employed (Hanes & Isherwood, 1949). Whatman no. 3MM papers, which had been washed as chromatograms with 2N-acetic acid and water, were used.

Extracts of the enzyme digests were applied directly to the papers. A current of air was passed over the papers and up to seven applications of 24 μl . portions were made on each spot. The papers were developed for 16 hr. with the ethanol-ammonium acetate (M, pH 7.5; 7.5:3, v/v) solvent of Paladini & Leloir (1952). The nucleotides were detected by ultraviolet light with the procedure of Markham (1955) as modified by Rowan (1957), and eluted from the spot area with 3 ml. of water. The absorption was measured in a Beckman Model DU spectrophotometer and a molar absorptivity index of 10×10^3 at 262 m μ used for the uridine compounds (Ploeser & Loring, 1949). Appropriate areas of paper were taken for blank values. Individual estimations of nucleotides applied to papers, developed and extracted gave recoveries within 4–5% of the theoretical.

Determination of glucose. Glucose was estimated by the method of Turner, Turner & Lee (1957).

RESULTS

Uridine diphosphoglucose pyrophosphorylase activity

The pyrophosphorolysis of UDPG by the pea-seed enzyme is shown in Fig. 1. The reaction could be followed by estimating either the formation of UTP or the disappearance of UDPG; this was in agreement with preliminary experiments which showed that there was no significant change in any of the reactants (equation 1) when these were incubated individually with the enzyme preparation.

The reverse reaction (i.e. the formation of UDPG from UTP) was demonstrated by using an enzymic digest containing UTP, G 1-P and inorganic pyrophosphatase as described above. UDPG was isolated from a digest containing an excess of UTP over G 1-P by applying ethanolic extracts as 'streaks' to Whatman no. 3MM papers which were developed in the ethanol-ammonium acetate solvent. The UDPG was eluted, hydrolysed in HCl (pH 2) for 10 min. at 100° and the solution neutralized. The ratio of free glucose to uridine was found to be 1.

Equilibrium of the reaction. The equilibrium of the UDPG pyrophosphorylase reaction was in favour of UTP formation. In determining the apparent equilibrium constant (K^1) both UTP and UDPG were estimated and the corresponding changes in G 1-P and sodium pyrophosphate (PP) calculated. The value of K^1 , which is defined as

$$K^1 = \frac{[\text{UTP}] \cdot [\text{G 1-P}]}{[\text{UDPG}] \cdot [\text{PP}]}$$

was found to be 7.2 (mean of ten determinations) with enzymic digests of the composition described for the determination of the equilibrium with the forward reaction. There was no significant difference in the value of K^1 determined with the reverse reaction. Identical values were obtained after incubation for 120 and 150 min.

Variation in Mg^{2+} ion concentration affected the value of K^1 : with Mg^{2+} ion concentrations of 2.5, 5 and 10 mM the equilibrium constants were 8.4, 7.2 and 3.8 respectively at pH 7.9. Change in pH of the reaction mixture also affected the value of K^1 . In the presence of 2.5 mM- MgCl_2 , K^1 was 3.5 and 8.4 at pH 7.0 and 7.9 respectively.

Effect of Mg^{2+} ions. The effect of Mg^{2+} ion concentration on the pyrophosphorolysis of UDPG is shown in Fig. 2. The optimum concentration of Mg^{2+} ions under the conditions of the experiment was approximately 5 mM. Increase of Mg^{2+} ions beyond this concentration inhibited the reaction.

Mg^{2+} ions could be replaced by Mn^{2+} ions and an identical rate of UDPG pyrophosphorolysis was obtained. The optimum concentration of Mn^{2+} ions was approximately mM. Co^{3+} and Ni^{2+} ions at con-

centrations of 2.5 mM also stimulated UDPG pyrophosphorylase.

Effect of concentration of substrates. The effect of increasing sodium pyrophosphate concentration on the rate of reaction is shown in Fig. 3. The optimum concentration was approximately 10 mM and further increase depressed the rate of UDPG pyrophosphorolysis. At concentrations of sodium pyrophosphate less than 5 mM some non-enzymic breakdown of UDPG occurred. Increasing the concentration of UDPG from 2 to 4 mM did not

Fig. 2. Effect of MgCl_2 concentration on the UDPG pyrophosphorylase reaction. The enzymic digests were of the composition used for the assay of UDPG pyrophosphorylase. Concentration of MgCl_2 varied as shown.

Fig. 1. Pyrophosphorolysis of UDPG and the formation of UTP. $\square$, UDPG; $\circ$, UTP. The enzymic digest contained 0.9 μmole of UDPG, 5 μmoles of sodium pyrophosphate, 2.5 μmoles of MgCl_2 , 100 μmoles of tris-HCl buffer, pH 7.9, 0.1 ml. of enzyme, total volume 0.5 ml.

Fig. 3. Effect of concentration of sodium pyrophosphate on the UDPG pyrophosphorylase reaction. Enzymic digests of the composition described for the assay of UDPG pyrophosphorylase were used. Concentration of sodium pyrophosphate varied as shown.

affect the rate of reaction; in these experiments the initial concentration of sodium pyrophosphate was 10 mM.

Effect of pH. There was little variation in enzyme activity over the range pH 7.5–9.0. In Fig. 4 the results obtained with a series of buffers containing tris (final concentration 0.2M) and acetic acid are shown. In separate experiments with glycine (final concentration 0.2M)–NaOH buffers it was observed that the activity decreased by 30% when the pH was raised from 9 to 10.

Effect of inhibitors. The following did not significantly affect the activity: fluoride, inorganic orthophosphate, fluoride plus inorganic orthophosphate, iodoacetate, or arsenate at 10 mM-concentration; *p*-chloromercuribenzoate at mM-concentration, or Hg^{2+} ions at 0.1 mM-concentration. Ethylenediaminetetra-acetate (10 mM) inhibited the reaction by 90%.

Distribution of uridine diphosphoglucose pyrophosphorylase

Active preparations of the enzyme were obtained from all plant tissues examined. These were wheat shoots, maize shoots, broad-bean shoots, sugar-cane shoots, pea roots, sugar-cane roots, wheat seeds, silver-beet leaves and potato tubers. A phosphatase which acted on UTP to give uridine diphosphate and uridine monophosphate was present in crude extracts of pea seeds and in the extracts prepared from potato tubers, silver-beet leaves, wheat seeds and shoots, broad-bean shoots and maize shoots.

Fig. 4. Effect of pH on the UDPG pyrophosphorylase reaction. Composition of the enzymic digests was that described for the assay of UDPG pyrophosphorylase. The reaction was studied in tris (0.2M)–acetate buffers of pH indicated.

DISCUSSION

The present investigation shows that active preparations of UDPG pyrophosphorylase free from interfering enzyme reactions may be readily obtained from pea seeds.

Under all the experimental conditions used, the equilibrium of the reaction favoured the breakdown rather than the synthesis of UDPG. With the optimum concentration (5 mM) of Mg^{2+} ions and at pH 7.9 and 30°, K^1 was 7.2. Increase in Mg^{2+} ion concentration and decrease in pH of the reaction mixture each depressed the value of K^1 . Effects of Mg^{2+} ion concentration and pH on the apparent equilibrium constant have been observed with other enzymic reactions involving phosphoric compounds and Mg^{2+} ions, e.g. the adenosine triphosphate–creatine transphosphorylase reaction (Noda, Kuby & Lardy, 1954). The K^1 values reported in the present paper are not thermodynamic equilibrium constants but represent the total concentration of each component (ionic and magnesium complexes) present at equilibrium. To determine a true thermodynamic equilibrium constant it would be necessary to know the nature of the components which actually participate in the reaction. Adequate information to calculate the concentrations of the possible ‘reacting’ components under the experimental conditions is not available.

The values of K^1 reported here differ from the figure of approximately 1 obtained by Munch-Petersen (1955) with yeast UDPG pyrophosphorylase at pH 7.4 and unspecified temperature and Mg^{2+} ion concentration (possibly approximately 5 mM). The concentrations of UDPG and sodium pyrophosphate used differed from those in the present investigation.

From the results of the present investigation it appears likely that the thermodynamic equilibrium of the UDPG pyrophosphorylase reaction lies in the direction of UTP formation. This may support the suggestion of Kalckar (1954) that the glucose-phosphate linkage in UDPG has a higher bond energy than in free G 1-P. This hypothesis has been supported by investigations on sucrose synthesis. The equilibrium constant of the reaction

was found to be approximately 5 (Cardini, Leloir & Chiriboga, 1955; Leloir, 1956), whereas Doudoroff (1943) obtained an equilibrium constant of 0.05 for the reaction

The depressing effect of high Mg^{2+} ion concentrations on the pyrophosphorolysis of UDPG may

have been due to complex formation with sodium pyrophosphate. It was observed that precipitates formed in enzymic digests where the molar concentration of Mg^{2+} ions was equal to or greater than the concentration of sodium pyrophosphate. The optimum concentration of 5 mM for Mg^{2+} ions found in the present investigation is in reasonable agreement with the value of 2 mM- Mg^{2+} ions observed by Munch-Petersen (1955) for yeast UDPG pyrophosphorylase. The ability of Mn^{2+} , Ni^{2+} and Co^{2+} ions to replace Mg^{2+} ions as cofactor for pea UDPG pyrophosphorylase suggests that the metal requirement may be for a suitable bivalent cation. This agrees with the observations of Neufeld *et al.* (1957), who found that the enzyme from mung-bean seedlings required Mg^{2+} , Mn^{2+} or Co^{2+} ions.

The enzyme was found in all plant materials examined, suggesting that its distribution may be fairly general. As UDPG is the probable precursor of sucrose in plants, the enzymic transformations of UDPG are of considerable interest. The enzyme may be of major significance in plant tissues exhibiting pronounced sugar-starch changes, where G 1-P may be incorporated into either starch or sucrose. Thus the high UDPG pyrophosphorylase activity in the pea extracts is consistent with the rapid transformations of sugar into starch observed in the developing pea seed by Turner & Turner (1957) and Turner *et al.* (1957). The present investigation supports previous suggestions (Turner, 1954, 1957) that synthesis of sucrose from G 1-P and fructose in pea extracts takes place through the intermediate formation of UDPG formed from UTP and G 1-P. Evidence that the pea extracts contained a functional glycolytic system (which could lead to the production of UTP) has been recently confirmed (Hatch & Turner, 1958).

SUMMARY

1. Uridine diphosphoglucose pyrophosphorylase, which catalyses the reaction

was found in pea-seed extracts. A purified preparation free from interfering enzymes was obtained.

2. The apparent equilibrium of the reaction was in favour of uridine triphosphate formation. The apparent equilibrium constant ([uridine triphosphate] [glucose 1-phosphate]/[uridine diphosphoglucose] [inorganic pyrophosphate]) was affected by Mg^{2+} ion concentration and pH.

3. Mg^{2+} ions, which could be replaced by Mn^{2+} , Co^{2+} or Ni^{2+} ions, were essential for the reaction.

4. The effects of Mg^{2+} ion concentration, concen-

tration of substrates, pH and inhibitors on the pyrophosphorolysis of uridine diphosphoglucose were studied.

5. Active preparations of the enzyme were obtained from a number of plant tissues.

6. The possible relationship of the reaction catalysed by the enzyme to the carbohydrate metabolism of plants has been discussed.

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 Miss S. K. Harris and Mr J. B. Lee for technical assistance; 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.

REFERENCES

- Burma, D. P. & Mortimer, D. C. (1956). *Arch. Biochem. Biophys.* **62**, 16.
 Cardini, C. E., Leloir, L. F. & Chiriboga, J. (1955). *J. biol. Chem.* **214**, 149.
 Doudoroff, M. (1943). *J. biol. Chem.* **151**, 351.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Hatch, M. D. & Turner, J. F. (1958). **69**, 495.
 Heppel, L. A. & Hilmo, R. J. (1951). *J. biol. Chem.* **192**, 87.
 Kalckar, H. M. (1954). In *The Mechanism of Enzyme Action*, p. 675. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins Press.
 Leloir, L. F. (1956). In *Currents in Biochemical Research*, p. 585. Ed. by Green, D. E. New York: Interscience Publishers Inc.
 Markham, R. (1955). In *Modern Methods of Plant Analysis*, vol. 4. Ed. by Paech, K. & Tracey, M. V. Berlin: Springer-Verlag.
 Mills, G. T., Ondarza, R. & Smith, E. E. B. (1954). *Biochim. biophys. Acta*, **14**, 159.
 Munch-Petersen, A. (1955). *Acta chem. scand.* **9**, 1523.
 Munch-Petersen, A., Kalckar, H. M., Cutolo, E. & Smith, E. E. B. (1953). *Nature, Lond.*, **172**, 1036.
 Neufeld, E. F., Ginsburg, V., Putman, E. W., Fanshier, D. & Hassid, W. Z. (1957). *Arch. Biochem. Biophys.* **69**, 602.
 Noda, L., Kuby, S. A. & Lardy, H. A. (1954). *J. biol. Chem.* **210**, 83.
 Paladini, A. C. & Leloir, L. F. (1952). *Biochem. J.* **51**, 426.
 Ploeser, J. M. & Loring, H. S. (1949). *J. biol. Chem.* **178**, 431.
 Rowan, K. S. (1957). *J. exp. Bot.* **8**, 256.
 Smith, E. E. B. & Mills, G. T. (1955). *Biochim. biophys. Acta*, **18**, 152.
 Turner, D. H. & Turner, J. F. (1957). *Aust. J. biol. Sci.* **10**, 302.
 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. & Lee, J. B. (1957). *Aust. J. biol. Sci.* **10**, 407.