Nucleoside Diphosphokinase of Pea Seeds

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The nucleoside diphosphokinase reaction may be formulated as involving a phosphate transfer from adenosine triphosphate to uridine diphosphate with the formation of uridine triphosphate and adenosine diphosphate:

Adenosine triphosphate + uridine diphosphate ⇌ adenosine diphosphate + uridine triphosphate (1)

Nucleoside diphosphokinase was demonstrated by Berg & Joklik (1953) in yeast extracts and these authors also observed that phosphate transfer occurred between adenosine triphosphate and inosine diphosphate. Krebs & Hems (1953) reported a reaction between adenosine triphosphate and inosine diphosphate giving adenosine diphosphate and inosine triphosphate in extracts from pigeon-breast muscle and rat intestinal mucosa. Berg & Joklik (1954) investigated in more detail the properties of the enzyme from yeast and rabbit muscle.

Indications of nucleoside diphosphokinase activity in higher plants were obtained by Burma & Mortimer (1956) in homogenates of sugar-beet leaves, and the presence of the enzyme in pea-seed extracts was suggested by Turner (1957) after investigations of sucrose synthesis. Nucleoside diphosphokinase activity has recently been observed in crude homogenates of leaves of *Impatiens holstii* (Ganguli, 1958) and in extracts of wheat-seedling scutella (Keys, 1958).

It is desirable that more detailed information should be available on the mechanisms of uridine triphosphate formation in higher plants. In the present investigation nucleoside diphosphokinase was found in pea-seed extracts and a preparation free from interfering enzyme reactions was obtained. Some properties of the enzyme and the equilibrium of the reaction were studied. Nucleoside diphosphokinase activity was also demonstrated in extracts from a number of other plant materials.

MATERIALS AND METHODS

Substrates. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine 5′-monophosphate (AMP), uridine triphosphate, uridine 5′-monophosphate, inosine triphosphate and inosine monophosphate were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

Uridine diphosphate, guanosine triphosphate and cytidine triphosphate were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of nucleoside diphosphokinase from pea seeds. Crude extracts were prepared from dried pea seeds (*Pisum sativum* L.) as described previously (Turner, 1957) and centrifuged at 20 000 *g* for 20 min. at room temperature. The supernatant was treated with saturated $(NH_4)_2SO_4$, pH 7, at 4°, and the fraction precipitating between 45 and 55% saturation was dissolved in 7 ml. of water and dialysed with rocking at 4° for 2 hr. against 25 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7.

The nucleoside diphosphokinase preparation was clear and colourless and could be stored at −15° for 6 months with only slight loss of activity. The enzyme extract possessed high activity and was normally diluted 1000–1400-fold with 0·5 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7, before use. No interfering enzyme reactions involving any of the components of the nucleoside diphosphokinase reaction were observed. Adenosine triphosphatase activity was not detected in the extract. Heating at 60°, 70° and 80° for 10 min. resulted in 6, 65 and 95% loss respectively of nucleoside diphosphokinase activity.

Preparation of enzyme extracts from other plant tissues. Seeds of wheat, barley, broad bean and pea were germinated and grown for 8–10 days in damp sand in light. The roots or shoots were excised, ground in 50 mM-$NaHCO_3$, and the extract was centrifuged and the supernatant treated with saturated $(NH_4)_2SO_4$. For wheat and barley shoots, fractions were collected between 40 and 55% saturation, and for pea shoots and roots and broad-bean shoots and roots fractions were taken between 45 and 55% saturation. The precipitates were suspended in water and dialysed as described previously.

Sugar-cane-stem cuttings were grown in damp sand at 30° for 12 days. The shoots and roots were blended with 50 mM-$NaHCO_3$. The fractions obtained between 35 and 70% saturation with $(NH_4)_2SO_4$ were taken up in water and dialysed.

Soaked wheat seeds, laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) and potato tubers were blended with 50 mM-$NaHCO_3$. The centrifuged extracts from silver-beet leaves and potato tubers were treated with saturated $(NH_4)_2SO_4$ and the fraction precipitating between 45 and 55% saturation was suspended in water and dialysed. The fraction collected from wheat seeds was that precipitating between 45 and 60% saturation.

Reaction mixtures. The reaction mixtures were maintained at 30°. For the assay of nucleoside diphosphokinase

activity the following components were mixed: 0·3 (approx.) μmole of ATP, 0·3 (approx.) μmole of uridine diphosphate, 0·38 μmole of $MgCl_2$, 7·5 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)-HCl buffer, pH 8, 0·05 ml. of enzyme (containing approx. 0·3 μg. of protein); total volume 0·15 ml. The reaction mixtures were incubated for 15 min. The enzyme concentration was adjusted so that not more than 30% of ATP was converted into ADP in 15 min.: under these conditions the rate of reaction was approximately constant during the incubation period.

Reaction mixtures of similar composition were employed for the determination of the equilibrium except that a more concentrated enzyme (equivalent to 2·1 μg. of protein/reaction mixture) was used. Samples were taken after incubation for 120 and 150 min.

Digest samples were inactivated by the addition of 2 vol. of ethanol.

Detection and estimation of nucleotides. The nucleotides were separated on paper chromatograms, eluted and estimated spectrophotometrically, substantially as previously described (Turner & Turner, 1958). The papers were developed for 18 hr. with *iso*butyric acid–aq. NH_3 soln.–water (66:1:33, by vol.) as solvent (Pabst Laboratories, 1956). Because of a small amount of residual *iso*butyric acid remaining on the chromatograms, the spot areas were eluted with 3 ml. of 50 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7, rather than with water. A molar extinction coefficient of $15{\cdot}4 \times 10^3$ at 260 mμ was used for the adenosine compounds (Bock, Ling, Morell & Lipton, 1956) and 10×10^3 at 262 mμ for the uridine compounds (Ploeser & Loring, 1949).

Determination of protein. The protein content of the enzyme extracts was estimated by the spectrophotometric method of Warburg & Christian (1941).

RESULTS

Nucleoside diphosphokinase activity

Preliminary experiments established that there was no change in ATP, ADP, uridine tri- or diphosphate when these were incubated individually with the enzyme preparation. The reaction could be followed by determining the change in any one of the reactants (equation 1). Nucleoside diphosphokinase activity of the pea-seed enzyme as estimated by formation of ADP and also disappearance of ATP is shown in Fig. 1. In assays of nucleoside diphosphokinase activity, changes in the adenosine nucleotides were followed because of their greater molar extinction coefficient.

The reverse reaction (i.e. the formation of ATP and uridine diphosphate from ADP and uridine triphosphate) was shown by using mixtures of the following composition: 1·21 μmoles of ADP, 1·20 μmoles of uridine triphosphate, 1·52 μmoles of $MgCl_2$, 30·0 μmoles of tris-HCl buffer, pH 8, 0·2 ml. of enzyme (containing 1·8 μg. of protein); total volume 0·6 ml. Results analogous to those illustrated in Fig. 1 were obtained.

Equilibrium of the reaction. All four reactants (ADP, ATP, uridine tri- and di-phosphate) were estimated in determining the apparent equilibrium constant (K^1). The value of K^1, which is defined as

$$K^1 = \frac{[\text{Uridine triphosphate}].[\text{ADP}]}{[\text{Uridine diphosphate}].[\text{ATP}]},$$

was found to be 0·91 (mean of eight determinations) at pH 8, and with 2·5 mM-$MgCl_2$.

Variation of pH had only slight effect on the value of K^1. In the presence of 2·5 mM-$MgCl_2$, K^1

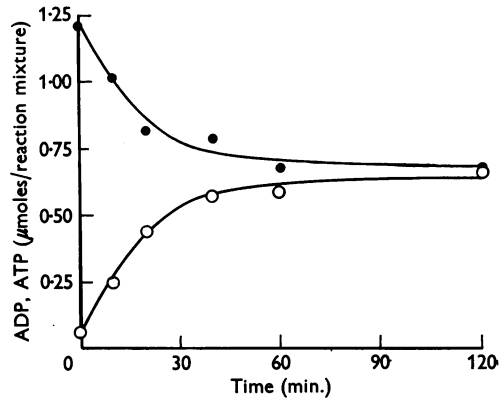

Fig. 1. Nucleoside diphosphokinase activity as measured by formation of ADP (○) and loss of ATP (●). The reaction mixture contained 1·27 μmoles of ATP, 1·24 μmoles of uridine diphosphate, 1·52 μmoles of $MgCl_2$, 30 μmoles of tris-HCl buffer, pH 8, 0·2 ml. of enzyme (containing 1·8 μg. of protein); total volume 0·6 ml. Temp. 30°.

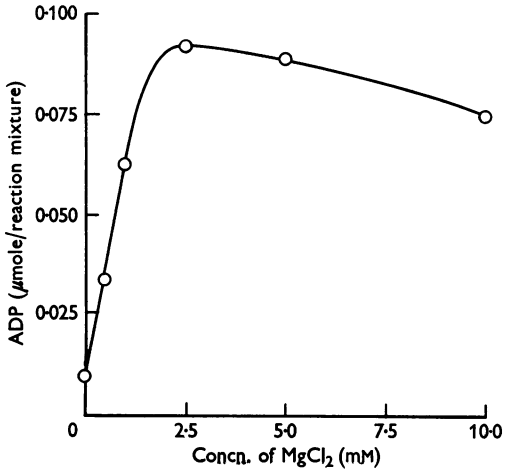

Fig. 2. Effect of $MgCl_2$ concentration on nucleoside diphosphokinase activity. The reaction mixtures contained 0·34 μmole of ATP, 0·36 μmole of uridine diphosphate, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·23 μg. of protein); total volume 0·15 ml. Concentration of $MgCl_2$ was varied as shown. Time of incubation, 15 min. Temp. 30°.

was 1·2, 0·91 and 0·81 at pH 7, 8 and 9 respectively. The value of K^1 was not significantly affected by variation of Mg^{2+} ion concentration. The values at pH 8 and with $MgCl_2$ concentrations 1·0, 2·5 and 5·0 mM were 0·96, 0·91 and 0·98 respectively.

Cofactor requirements. The enzyme was largely inactive in the absence of a suitable bivalent metal ion. Fig. 2 shows the effect of Mg^{2+} ion concentration on nucleoside diphosphokinase activity. The reaction rate reached a maximum at a concentration of 2·5 mM-$MgCl_2$; at concentrations greater than 5 mM, the rate decreased.

Mg^{2+} ions could be replaced by Mn^{2+}, Co^{2+} and Ca^{2+} ions and to a lesser extent by Zn^{2+} and Ni^{2+} ions (Table 1).

Effect of pH. The activity of the enzyme was studied in a series of buffers containing tris (final concentration 0·2 M) and acetic acid over the range pH 6–9 (Fig. 3). The optimum pH was approximately 8. In separate experiments with glycine (final concn. 0·2 M)–NaOH buffers the activity was observed to decrease by 10% when the pH was increased from 9 to 10.

Effect of inhibitors. Ethylenediaminetetra-acetate (EDTA) (10 mM) completely inhibited the reaction and mM-EDTA inhibited nucleoside diphosphokinase activity by 50%. Arsenate (10 mM) inhibited the reaction by 70%. Fluoride, iodoacetate or inorganic pyrophosphate at 10 mM-concentration, *p*-chloromercuribenzoate or molybdate at mM-concentration, or Hg^{2+} ions at 0·1 mM-concentration did not affect the activity.

Table 1. *Effect of bivalent metal ions on nucleoside diphosphokinase activity*

The reaction mixtures contained: 0·30 μmole of ATP, 0·31 μmole of uridine diphosphate, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·23 μg. of protein); total volume 0·15 ml. Salts of bivalent metals were added as shown. Time of incubation, 15 min. Temp. 30°.

Addition	Final concn. (mM)	ADP formed (μmole/reaction mixture)
None	—	0·003
$MgCl_2$	2·5	0·084
$MnCl_2$	0·5	0·043
	1·0	0·068
	2·5	0·064
$CoCl_2$	1·0	0·027
	2·5	0·061
$CaCl_2$	1·0	0·028
	2·5	0·053
$ZnSO_4$	1·0	0·023
	2·5	0·022
$NiCl_2$	2·5	0·022

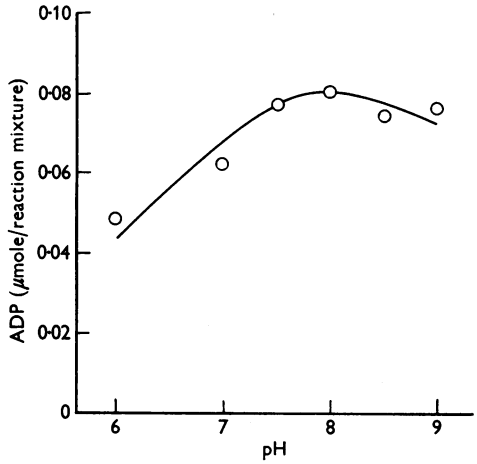

Fig. 3. Effect of pH on nucleoside diphosphokinase activity. The reaction mixtures contained 0·28 μmole of ATP, 0·26 μmole of uridine diphosphate, 0·38 μmole of $MgCl_2$, 7·5 μmoles of tris–acetate buffer of required pH, 0·05 ml. of enzyme (containing 0·35 μg. of protein); total volume 0·15 ml. Time of incubation, 15 min. Temp. 30°.

Specificity of the pea-seed nucleoside diphosphokinase preparation

When ADP was incubated with inosine triphosphate, guanosine triphosphate or cytidine triphosphate, ATP was formed together with the corresponding nucleoside diphosphate. The rates of reaction of these nucleoside triphosphates and that of uridine triphosphate are compared in Table 2. The reaction with inosine triphosphate was more rapid than with uridine triphosphate, whereas the rates with guanosine triphosphate and cytidine triphosphate were lower. In separate experiments it was found that there was no reaction when ATP was incubated with uridine 5′-monophosphate or inosine monophosphate.

Distribution of nucleoside diphosphokinase

All plant tissues examined yielded extracts containing nucleoside diphosphokinase activity. The tissues were wheat seeds and shoots, pea roots and

Table 2. *Specificity of the nucleoside diphosphokinase preparation*

The reaction mixtures contained 0·29 μmole of ADP, 0·38 μmole of $MgCl_2$, 7·5 μmoles of tris-HCl buffer, pH 8, 0·05 ml. of enzyme (containing 0·32 μg. of protein); total volume 0·15 ml. Nucleoside triphosphates were added as shown. Time of incubation, 20 min. Temp. 30°.

Nucleoside triphosphate	Amount added (μmole)	ATP formed (μmole/reaction mixture)
Uridine	0·30	0·052
Inosine	0·30	0·082
Guanosine	0·32	0·032
Cytidine	0·27	0·025

shoots, broad-bean roots and shoots, sugar-cane roots and shoots, barley shoots, silver-beet leaves and potato tubers. Extracts possessing very high nucleoside diphosphokinase activity were obtained from potato tubers.

DISCUSSION

The present study has shown that active nucleoside diphosphokinase preparations free from interfering enzymes may be readily obtained from pea seeds.

At the equilibrium of the reaction approximately equal amounts of ADP, ATP and uridine di- and tri-phosphate were present. The value of K^1 found was 0·91 at the optimum concentration (2·5 mM) of Mg^{2+} ions and at pH 8 and 30°. This confirms the conclusion of Berg & Joklik (1954) that the free energy of hydrolysis of the terminal pyrophosphate bond of uridine triphosphate is probably approximately equivalent to that of ATP. As pointed out by Noda, Kuby & Lardy (1954) and Turner & Turner (1958), values of K^1 in reactions of this type are not true thermodynamic-equilibrium constants but represent the total concentration of each substance (as ions and magnesium complexes) present at equilibrium. To calculate the true thermodynamic-equilibrium constant it would be necessary to determine the nature and amount of the components which actually participate in the reaction. The value of K^1 found in the present investigation for the nucleoside diphosphokinase reaction involving ADP, ATP and uridine di- and tri-phosphate is in agreement with the observation of Berg & Joklik (1954) that the equilibrium constant was approximately 1 with the enzyme from yeast.

The slight inhibitory effect of high Mg^{2+} ion concentration on the nucleoside diphosphokinase reaction may have been due to increased magnesium–nucleotide-complex formation. The optimum concentration of Mg^{2+} ions, 2·5 mM, is in reasonable agreement with that of 5 mM found by Berg & Joklik (1954) with the enzyme from yeast and rabbit-muscle extracts. These workers reported that Mn^{2+} and Ca^{2+} ions could replace Mg^{2+} ions and this observation is in accord with the present results. These results, together with the stimulation by Co^{2+}, Zn^{2+} and Ni^{2+} ions, suggest that the co-factor requirement of the enzyme from pea seeds is for a suitable bivalent cation.

The nucleoside diphosphokinase preparation from pea seeds catalysed a reaction between ADP and uridine triphosphate, inosine triphosphate, cytidine triphosphate or guanosine triphosphate. The reaction was most rapid with inosine triphosphate and uridine triphosphate; with cytidine triphosphate and guanosine triphosphate the rate of reaction was slower. There was no detectable reaction between ATP and uridine 5′-monophosphate or inosine monophosphate. These results support the suggestion of Berg & Joklik (1954) that the reaction is specific for a nucleoside triphosphate as phosphate donor and a nucleoside diphosphate as phosphate acceptor. As mentioned previously, Berg & Joklik reported a reaction between ADP and uridine triphosphate or inosine triphosphate with nucleoside diphosphokinase from yeast or rabbit muscle. Sanadi, Gibson, Ayengar & Jacob (1956) observed a reaction between ADP and guanosine triphosphate in extracts from pig-kidney cortex and ox-heart muscle.

The presence of nucleoside diphosphokinase in extracts of all plant tissues examined suggests a wide distribution for this enzyme. The enzymic formation of uridine triphosphate is of interest in relation to the carbohydrate metabolism of plants, as uridine diphosphate glucose may be formed from uridine triphosphate and glucose 1-phosphate by uridine diphosphate glucose pyrophosphorylase, which is also widely distributed in higher plants (Turner & Turner, 1958). It is known that uridine diphosphate glucose is the probable precursor of sucrose in plants (Cardini, Leloir & Chiriboga, 1955; Leloir & Cardini, 1953, 1955; Turner, 1954, 1957). The high nucleoside diphosphokinase activity of pea-seed extracts may be significant in relation to the rapid sucrose–starch conversions which occur in the developing pea seed (Turner & Turner, 1957; Turner, Turner & Lee, 1957). Similarly, the very active nucleoside diphosphokinase preparations obtained from potato tubers are consistent with the rapid sugar–starch transformations observed when potatoes are transferred from 0–1° to higher temperatures (Barker, 1936).

SUMMARY

1. Nucleoside diphosphokinase, which catalyses the reaction

$$\text{adenosine triphosphate} + \text{uridine diphosphate} \rightleftharpoons \text{adenosine diphosphate} + \text{uridine triphosphate}$$

was found in pea-seed extracts. Preparations free from interfering enzyme reactions were readily obtained.

2. The apparent equilibrium constant ([adenosine diphosphate] [uridine triphosphate]/[adenosine triphosphate] [uridine diphosphate]) was determined. Increase in Mg^{2+} ion concentration and variation in pH had little effect on the apparent equilibrium constant.

3. Mg^{2+} ions were necessary for the reaction. Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+} and Ni^{2+} ions could partially replace Mg^{2+} ions as cofactor for the enzyme.

4. The effects of Mg^{2+} ion concentration, pH and inhibitors on nucleoside diphosphokinase activity were studied.

5. A rapid reaction occurred between adenosine diphosphate and inosine triphosphate or uridine triphosphate, whereas adenosine diphosphate and guanosine triphosphate or cytidine triphosphate reacted more slowly.

6. Extracts were prepared from a number of plant tissues and all yielded preparations containing nucleoside diphosphokinase activity.

7. The possible significance of the nucleoside diphosphokinase reaction in the carbohydrate metabolism of plants has been discussed.

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