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PROPERTIES AND INTRACELLULAR DISTRIBUTION OF CYTIDINE AND URIDINE DIPHOSPHOKINASES OF CUCUMBER COTYLEDONS

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Abstract—The properties and intracellular location of the CDP and UDP kinases (ATP:ribonucleoside diphosphate phosphotransferases, EC 2.7.4.6) of cucumber cotyledons have been investigated. The enzymes were characterized with respect to metal ion requirement, pH optima and kinetic constants. The results indicated that the $MgATP^{2-}$ complex was the true substrate for these enzymes and that they were markedly inhibited by free ATP^{4-}. Intracellular location studies indicated that most of the CDP and UDP kinases within the cell were located in the cytoplasmic and chloroplast fractions.

INTRODUCTION

THE NUCLEOSIDE monophosphokinases (ATP:ribonucleoside monophosphate phosphotransferases, EC 2.7.4.4) of healthy and virus-infected cucumber cotyledons have been investigated by Gilliland *et al.*[1] while Gilliland and Symons[2] have reported the partial purification of the CMP, CDP, UMP and UDP kinases isolated from these sources and also from *Nicotiana glutinosa*. The results indicated that the monophosphokinases and the diphosphokinases were separate enzymes and that the monophosphokinases were the rate-limiting step in the conversion of the monophosphate to the triphosphate.

This work has now been extended by the investigation of the properties of the CDP and UDP kinases in crude extracts of cucumber cotyledons and of the intracellular location of enzyme activity. Since column chromatography of enzyme extracts on Sephadex G-75 and on DEAE-cellulose resulted in multiple peaks of CMP, CDP, UMP and UDP kinase activity,[2] it was hoped that these present studies would also indicate whether or not these multiple peaks of kinase activity could be due to the existence of isoenzymes originating from different parts of the cell.

RESULTS

Validity of the Assay Procedure for CDP *and* UDP *Kinases*

A linear response of enzymic activity to protein concentration was obtained using crude extracts from cotyledons in the assay procedure provided the conversion of the labelled nucleotide was not greater than 5 per cent for CDP and 20 per cent for UDP. A linear response was also found with respect to the time of incubation up to 20 min or until the above per cent conversions were reached. Duplicate assays always agreed to within 5 per cent. In addition, the complete resolution of the labelled nucleotides by chromatography on the polyethyleneimine papers was shown by the absence of radioactivity outside the u.v. light absorbing areas of the nucleotides.

[1] J. M. GILLILAND, R. E. LANGMAN and R. H. SYMONS, *Virology* **30**, 716 (1966).
[2] J. M. GILLILAND and R. H. SYMONS, *Virology* **33**, 221 (1967).

Properties of the CDP *and* UDP *Kinases in Crude Cotyledon Extracts*

(*a*) *Apparent K_m values.* The apparent K_m values for CDP and UDP as substrates were estimated as 7·3 mM and 1·7 mM, respectively, by the method of Wilkinson[3] (see also Fig. 3). These values are 20–40 times greater than those of the corresponding monophosphates reported by Gilliland *et al.*[1] from studies on the CMP and UMP kinases from the same source. Apparent K_m values for $MgATP^{2-}$ as substrate (see below) were estimated as 2·1 mM in the presence of CDP and 3·7 mM in the presence of UDP.

These apparent K_m values indicated that a high concentration of nucleoside diphosphate and of $MgATP^{2-}$ would be needed in the assay medium for satisfactory enzyme assays. Although it was practical to work at four times the apparent K_m for UDP (8 mM), it became impractical to work at four times the apparent K_m of CDP (32 mM) due to overloading of the paper chromatogram and to the low conversion of CDP to CTP. Hence, both nucleotides have been used in the standard assay at a concentration of 8 mM.

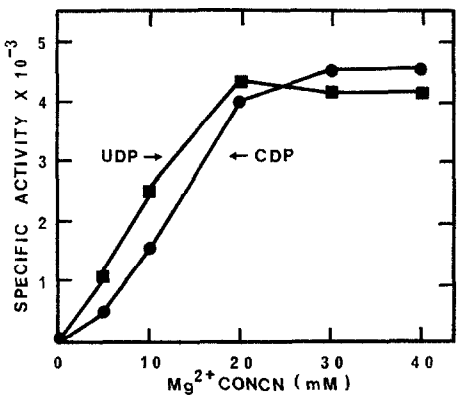

FIG. 1. THE EFFECT OF CONCENTRATION OF Mg^{2+} ON THE ACTIVITY OF CDP AND UDP KINASES IN CRUDE EXTRACTS OF CUCUMBER COTYLEDONS.

The concentration of Mg^{2+} in the assay medium was varied from 0–40 mM while the concentration of ATP^{4-} was constant at 20 mM.

(*b*) *The effect of* Mg^{2+} *and* ATP^{4-} *concentration on enzyme activity.* O'Sullivan and Perrin[4] have shown that the stability constant for $MgATP^{2-}$ is 73,000 M^{-1} in 0·1 M N-ethylmorpholine-HCl buffer, pH 8·0, and 18,000 M^{-1} in 0·1 M tris-HCl buffer, pH 8·0. Therefore if either Mg^{2+} or ATP^{4-} is added in excess of the other, the amount of the deficient ion would be insignificant. The effects of these two ions must therefore be considered relative to one another.

The effect of Mg^{2+} concentration at constant ATP^{4-} concentration on the activity of CDP and UDP kinases is shown in Fig. 1. The results show that a plateau of maximum activity was obtained when the Mg^{2+} concentration was at least the same as that of the ATP^{4-} (20 mM), which indicates that $MgATP^{2-}$ was most likely the true enzyme substrate. Conversely, the effect of ATP^{4-} on enzyme activity at constant Mg^{2+} concentration is shown in Fig. 2 where the ATP^{4-} and Mg^{2+} concentrations are plotted as a ratio. Two lots of assays are presented; one carried out as described in the Experimental section and the other with a

[3] G. N. WILKINSON, *Biochem. J.* **80**, 324 (1961).
[4] W. J. O'SULLIVAN and D. D. PERRIN, *Biochemistry* **3**, 18 (1964).

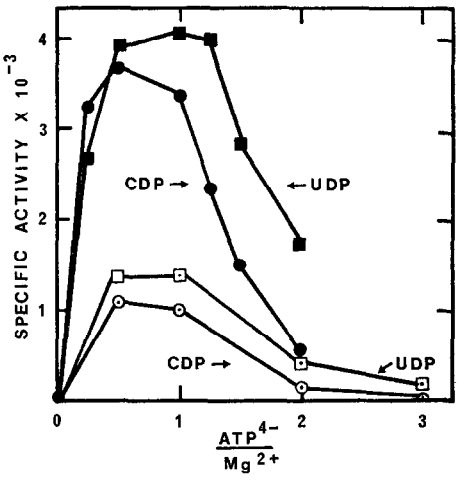

FIG. 2. THE EFFECT OF CONCENTRATION OF ATP^{4-} RELATIVE TO THAT OF Mg^{2+} ON THE ACTIVITY OF CDP AND UDP KINASES IN CRUDE EXTRACTS OF CUCUMBER COTYLEDONS.

The upper two curves give results obtained for assays of CDP and UDP kinases with a constant concentration of substrate of 8 mM and of Mg^{2+} of 20 mM in the assay medium while he ATP^{4-} concentration was varied from 0–40 mM. The lower two curves were for a constant substrate concentration of 0·5 mM and a Mg^{2+} concentration of 3·3 mM while the ATP concentration was varied from 0–10 mM.

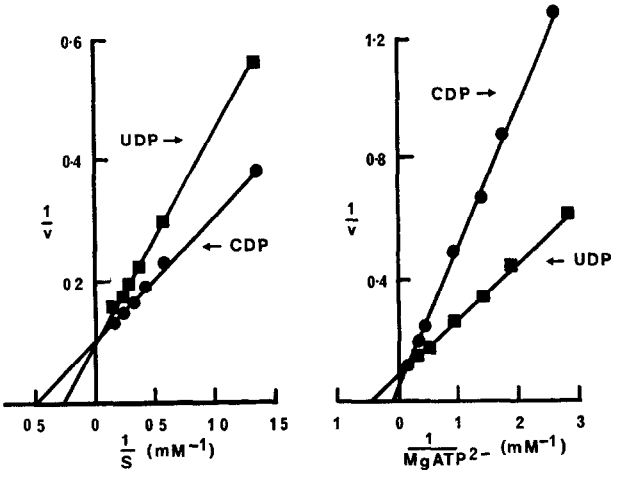

FIG. 3. LINEWEAVER-BURK DOUBLE RECIPROCAL PLOTS FOR CDP AND UDP KINASES.

Plots of the reciprocal of initial velocity (mμmoles of nucleoside diphosphate converted to the corresponding triphosphate per min) against the reciprocal of the concentration of CDP, UDP and $MgATP^{2-}$. When CDP and UDP were variable substrates, $MgATP^{2-}$ concentration was constant at 20 mM. When $MgATP^{2-}$ was the variable substrate, the concentration of CDP and UDP was constant at 8 mM. Experimental data were fitted to a computer programme for a rectangular hyperbola to obtain apparent K_m and V_{max} values.[3] The lines were drawn from the constants obtained.

low substrate concentration of 0·5 mM which allowed a higher ratio of ATP^{4-} to Mg^{2+} to be used without affecting the assay system. The results show that in both cases when ATP^{4-} was added in excess of Mg^{2+}, inhibition of enzyme activity occurred. At a ratio of ATP^{4-}: Mg^{2+} of 2:1, 80 per cent inhibition occurred and at a ratio of 5:1, 100 per cent inhibition. Hence, it would appear that free ATP^{4-} in excess of the $MgATP^{2-}$ complex was an effective inhibitor of both CDP and UDP kinases.

The Lineweaver-Burk double reciprocal plots for $MgATP^{2-}$, CDP and UDP as variable substrates are shown in Fig. 3. The linear plots obtained indicate than no homotropic co-operative effects[5] were occurring due to the enzyme interacting with more than one molecule of the same substrate. Further, the lack of stimulation of kinase activity by Mg^{2-} in excess of $MgATP^{2-}$ (Fig. 1) indicates no allosteric activation of the diphosphokinases by Mg^{2+} as has been reported for sheep-kidney pyruvate carboxylase.[6]

(*c*) *The effect of various ions on enzyme activity.* The results presented in Table 1 show that the activity of the CDP and UDP kinases was dependent on the presence of a divalent cation,

TABLE 1. EFFECT OF VARIOUS IONS ON THE SPECIFIC ACTIVITY OF CDP AND UDP KINASES IN EXTRACTS OF CUCUMBER COTYLEDONS

Salt added*	Specific activity	
	CDP kinase	UDP kinase
None	0	0
$MgSO_4$	4120	4740
$Mg(CH_3COO)_2$	4150	4750
$MgCl_2$	3600	4470
$MnCl_2$	3230	3860
$NiCl_2$	800	610
$ZnSO_4$	120	440
KCl	170	25

* ATP and salt added were both present at a concentration of 20 mM.

the monovalent K^+ having little stimulation of enzyme activity. The effectiveness of the divalent ions was in the order $Mg^{2+} > Mn^{2+} \gg Ni^{2+} > Zn^{2+}$. The ions Co^{2+} and Ca^{2+} caused formation of insoluble material in the assay system and were not used. The anions, sulphate, acetate and chloride had little effect on enzyme activity (Table 1).

(*d*) *Effect of* pH *of the assay medium on enzyme activity.* Both CDP and UDP kinases had a similar pH optimum of 8·0–8·5 and the activity greatly decreased below pH 7·0.

Intracellular Distribution of Nucleotide Kinases

The specific activities of the CDP and UDP kinases in extracts of mitochondria, of aqueous and non-aqueous prepared chloroplasts and in two supernatant fractions are shown in Table 2; results obtained for the CMP and UMP kinases have been included for comparison. It can be seen that the specific activities of the CMP, CDP and UDP kinases were similar to those of the 1000 *g* and 16,000 *g* supernatants prepared from a crude cotyledon homogenate but that of the UMP kinase was approximately one-half. By contrast, the negligible activities found in extracts of aqueous prepared chloroplasts indicated the almost complete loss of

[5] J. MONOD, J. WYMAN and J. P. CHANGEUX, *J. Molec. Biol.* **12**, 88 (1965).
[6] B. KEECH and G. J. BARRITT, *J. Biol. Chem.* **242**, 1983 (1967).

these enzymes during the preparation procedure. On the other hand, mitochondria, which were prepared by an aqueous method, gave specific activities for the CDP and UDP kinases similar to those found in extracts of non-aqueous chloroplasts and in the supernatant fractions but those of the CMP and UMP kinases were approximately one-half. It is considered that the results obtained for extracts of mitochondria and of non-aqueous prepared chloroplasts, especially those for the diphosphokinases, were not due to contamination by cytoplasmic enzymes since the specific activities in all cases were approximately the same.

TABLE 2. SPECIFIC ACTIVITIES OF NUCLEOTIDE KINASES IN EXTRACTS OF CHLOROPLASTS, MITOCHONDRIA ABD CYTOPLASM OF CUCUMBER COTYLEDONS

	Kinase specific activity			
Enzyme source*	CMP	UMP	CDP	UDP
Aqueous prepared chloroplasts	0·0	0·0	56	66
Non-aqueous prepared chloroplasts	24·9	4·7	3100	4000
Mitochondria	10·2	4·9	4690	3640
Homogenate				
1000 *g* supernatant	20·1	9·9	3660	4130
16000 *g* supernatant	23·6	11·9	3550	4520

* All enzyme extracts prepared as described in Experimental.

The calculation of the per cent distribution of the kinases between chloroplasts, mitochondria and cytoplasm from the data of Table 2 requires a knowledge of the distribution of total protein in these fractions. Mitochondria were found to contribute 6 per cent of the total protein as estimated by differential centrifugation of a crude cotyledon homogenate. From Table 2, therefore, it was calculated that mitochondria contributed 3–8 per cent of the total activity measured for each of the four kinases. To determine the relative distribution of the remaining 92–97 per cent of the kinase activity between chloroplasts and cytoplasm, the ratios of total protein to total chlorophyll in a crude homogenate and in aqueous and non-aqueous prepared chloroplasts were determined (Table 3). The closely similar ratios for the

TABLE 3. RATIOS OF PROTEIN TO CHLOROPHYLL IN VARIOUS PREPARATIONS OF CUCUMBER COTYLEDONS

	Total protein	Soluble protein†
Preparation	Total chlorophyll	Total chlorophyll
Crude cotyledon extract*	24·2	16·7
Aqueous prepared chloroplasts	6·3	3·5
Non-aqueous prepared chloroplasts	24·3	17·1

* Cotyledons were homogenized in sucrose extraction medium, filtered through Miracloth and centrifuged at 300 *g* for 3 min. The supernatant was then used for estimation of total chlorophyll, total protein and "soluble" protein remaining in supernatant after centrifugation at 10,000 *g* for 5 min.

† Chloroplasts, prepared as described in Experimental, were suspended in extraction buffer and total chlorophyll and total protein determined. After centrifugation at 10,000 *g*, the protein content of the supernatant was called soluble protein.

crude homogenate and the non-aqueous chloroplasts would indicate that all the protein of the homogenate originated from the chloroplasts. Since this is unlikely, the ratio for the non-aqueous chloroplasts was most probably high due to loss of chlorophyll relative to protein during chloroplast preparation. The low ratio of total protein to chlorophyll for aqueous prepared chloroplasts, on the other hand, indicates loss of protein during their preparation, a conclusion consistent with the results of Table 2.

The ease with which protein can be lost from chloroplasts is also shown in Table 3 by the ratios of "soluble" protein (determined after centrifugation of fractions in low ionic strength buffer) to total chlorophyll. Again the ratios for the crude cotyledon extract and the non-aqueous chloroplasts were similar and they indicate that 69 per cent ($16{\cdot}7/24{\cdot}2 \times 100$) of the protein in the former and 70 per cent ($17{\cdot}1/24{\cdot}3 \times 100$) of protein in the latter was "soluble" in low ionic strength buffer. There was also a loss of 56 per cent ($3{\cdot}5/6{\cdot}3 \times 100$) of the protein from the already depleted aqueous chloroplasts under similar conditions.

It is considered, therefore, that the chloroplast values of Table 3 provide maximum and minimum estimates of total protein:chlorophyll ratios. On this basis, the correct value for chloroplast protein as per cent of total protein in a crude homogenate lies between 26 per cent ($6{\cdot}3/24{\cdot}2 \times 100$) and 100 per cent ($24{\cdot}3/24{\cdot}2 \times 100$). However, if we assume that 70 per cent of protein in a crude homogenate originated from the chloroplasts (see Refs. 7–9), 6 per cent from the mitochondria (see above) and hence 24 per cent from the cytoplasm (determined by difference), then it can be calculated from the results of Table 2 that the chloroplasts contained 30–80 per cent of the nucleotide kinases studied, the cytoplasm 20–75 per cent and the mitochondria 3–8 per cent.

DISCUSSION

The results presented here have extended the observations reported previously[1,2] on the nucleotide kinases in virus-infected and healthy cucumber cotyledons. The high specific activities of the CDP and UDP kinases relative to the CMP and UMP kinases confirm the earlier conclusions that the monophosphokinases provide a rate-limiting step in the conversion of monophosphates to triphosphates. The high activity of diphosphokinases relative to monophosphokinases has also been noted for enzymes isolated from a variety of sources.[10]

The results have also indicated that $MgATP^{2-}$ was a substrate for the CDP and UDP kinases and that these enzymes were markedly inhibited by free ATP^{4-}. A similar effect has recently been reported for sheep-kidney pyruvate carboxylase,[6] but, in contrast to the diphosphokinases, this enzyme also showed allosteric activation by Mg^{2+} and $MgATP^{2-}$. It is conceivable that variation of the relative concentrations of ATP^{4-} and Mg^{2+} could control the activity of the CDP and UDP kinases in the plant cell.

The finding of nucleotide kinase activity in extracts of non-aqueous prepared chloroplasts and of negligible activity in extracts of aqueous prepared chloroplasts has indicated an almost complete loss of these enzymes into the aqueous extraction medium, an effect found to occur also with other chloroplast enzymes.[7,9,11] Because of this effect, it has not

[7] U. HEBER and E. TYSZKIEWICZ, *J. Exptl Botany* **13**, 185 (1962).
[8] C. R. STOCKING and A. ONGUN, *Am. J. Botany* **49**, 284 (1962).
[9] M. ZUCKER and H. T. STINSON, *Arch. Biochem. Biophys.* **96**, 637 (1962).
[10] H. NAKAMURA and Y. SUGINO, *J. Biol. Chem.* **241**, 4917 (1966).
[11] R. M. SMILLIE, *Can. J. Botany* **41**, 123 (1963).

been possible to obtain an accurate assessment of the proportion of total kinase activity located in the chloroplasts. However, the chloroplasts and cytoplasm were the major source of the kinases studied as only 3–8 per cent of the total were found associated with the mitochondria. As only one nucleus is present per cell compared to about 100 chloroplasts and 1000 mitochondria,[12] nuclei should contribute a negligible proportion of the total kinase activity of a cotyledon extract. The observed distribution of most of the kinase activity between chloroplasts and cytoplasm suggests that the multiple peaks of enzyme activity obtained by Gilliland and Symons[2] during column chromatography of enzyme extracts could be due to chloroplast and cytoplasmic isoenzymes.

EXPERIMENTAL

Materials

Unlabelled nucleotides were purchased from Schwarz BioResearch Inc., New York, and from Sigma Chemical Co., Missouri. ^{32}P-Ribonucleoside 5′-monophosphates were prepared as described by Symons.[13,14] α-^{32}P-Ribonucleoside 5′-diphosphates were prepared from the labelled monophosphates by the method of Hoard and Ott[15] and β-^{32}P-ribonucleoside 5′-diphosphates by the method of Moffatt.[16]

Plants

Cucumber seedlings (*Cucumis sativus L.* var. Polaris) were grown under artificial light as described by Gilliland *et al.*[1] Cotyledons were used from plants which had no primary leaves present.

Preparation of Enzyme Extracts

All operations were carried out at 4°.

(*a*) *Crude enzyme extract.* Homogenates of cotyledons were prepared by grinding in a mortar and pestle with 10 ml/g fresh weight of the following extraction buffer: 0·02 M 2-mercaptoethanol, 0·05 M tris-HCl buffer, pH 8·0. The homogenates were strained through cheese-cloth and centrifuged at 10,000 *g* for 10 min and the supernatant used for the determination of enzymic properties.

(*b*) *Mitochondrial and chloroplast enzyme extracts.* Chloroplast and mitochondrial fractions were prepared in aqueous media by the method of James and Das[17] but the sucrose extraction medium was modified to 0·3 M sucrose, 0·02 M 2-mercaptoethanol, 4 mM $MgSO_4$, 0·067 M sodium phosphate buffer, pH 7·3. Chloroplasts were also prepared by the non-aqueous method of Stocking[18] which involved fractionation on the basis of density of freeze-dried and ground cotyledons in hexane–CCl_4 mixtures.

Enzyme extracts were prepared from mitochondria and from aqueous and non-aqueous prepared chloroplasts suspended in extraction buffer by sonicating at 0° using a Dawe Type 1130A Soniprobe at full power and a cumulative time of 25 sec. The supernatant, obtained after centrifuging the sonicate at 16,000 *g* for 10 min, was used as enzyme source in the intra-cellular location experiments (Table 2).

Assay of Ribonucleotide Kinases

Nucleoside monophosphokinases were assayed as described by Gilliland *et al.*[1] For nucleoside diphosphokinase assays, the assay medium contained in a volume of 0·15 ml; 0·02 M ATP, 0·03 M $MgSO_4$, 6 mM 2-mercaptoethanol, 0·08 M tris-HCl buffer, pH 8·0, 8 mM α- or β-^{32}P-ribonucleoside 5′-diphosphate (specific activity 1–2 $\times$ 10^5 counts/min/μmole) and 0·05 ml of the crude enzyme extract previously diluted 40 times in extraction buffer. CDP kinase assays were incubated at 37° for 5 min and UDP kinase assays for 10 min. The reaction was stopped by the addition of 0·04 ml of 5 N formic acid followed by cooling in an ice bath. The labelled nucleotides were then separated by chromatography on polyethyleneimine papers as described by Gilliland *et al.*[1] except that only a monophosphate marker was added. The separated nucleotides were located under u.v. light, cut out and counted in a Packard liquid scintillation spectrometer.

[12] J. Bonner and J. E. Varner, *Plant Biochemistry*, p. 4. Academic Press, New York (1965).
[13] R. H. Symons, *Biochem. Biophys. Res. Commun.* **24**, 872 (1966).
[14] R. H. Symons, *Biochim. Biophys. Acta* **155**, 609 (1968).
[15] D. E. Hoard and D. G. Ott, *J. Am. Chem. Soc.* **87**, 1785 (1965).
[16] J. G. Moffatt, *Can. J. Chem.* **42**, 599 (1964).
[17] W. O. James and V. S. R. Das, *New Phytologist* **56**, 325 (1957).
[18] C. R. Stocking, *Plant Physiol.* **34**, 56 (1959).

Protein and Chlorophyll Estimation

Protein was determined by the method of Lowry *et al.*[19] using bovine serum albumin as standard and chlorophyll by the method of Arnon.[20]

Units of Enzyme Activity

A unit of nucleoside mono- or diphosphokinase activity is defined as one mμmole of ^{32}P-nucleoside mono- or diphosphate converted per min to the corresponding di- and/or triphosphate under the specified assay conditions. A unit of specific activity is defined as one unit of enzyme activity per mg of protein.

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[19] O. H. LOWRY, N. J. ROSEBROUGH, A. D. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
[20] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).