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A Uridine Diphosphatase from Sugar Cane

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(Received 8 February 1963)

The metabolic role of non-specific phosphatases is difficult to assess but there is some information about the function of specific phosphatases. For instance, fructose 1,6-diphosphatase of liver (Mokrasch, Davidson & McGilvery, 1956) and photosynthetic tissue (Racker & Schroeder, 1958; Smillie, 1960) is probably essential for reversal of the glycolytic pathway associated with the processes of glycogenesis and photosynthesis.

During a study of uridine diphosphate glucose-fructose transglucosylase from sugar-cane storage tissue (Hatch, Sacher & Glasziou, 1963) a phosphatase that rapidly hydrolysed UDP but not UTP or UMP was found. With extracts containing both these enzymes, UDP-glucose and fructose were converted quantitatively into sucrose, UMP being the only other detectable product. The present paper describes the partial purification and properties of the uridine diphosphatase.

EXPERIMENTAL

Preparation of uridine diphosphatase

From sugar-cane storage tissue. Tissue from the mature internodes of sugar-cane stalks (hybrid variety, Pindar) was used as a source of enzyme. Juice was obtained by passing strips of the rind-free tissue through a laboratory roller-mill. After filtration, 100 ml. of the juice was cooled to 0°, its pH was adjusted to 7.0 with 1N-KOH, and then saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 7.1, 0°) was added to give 70% saturation. The saturated $(\text{NH}_4)_2\text{SO}_4$ was neutralized with aq. NH_3 and its pH was measured after 1 ml. had been diluted with 10 ml. of water. After standing for 1 hr. the

precipitated protein was obtained by centrifuging at 10 000 g for 10 min. The sedimented protein was dissolved in 10 ml. of 5 mM-tris-HCl buffer, pH 7.5, and dialysed against 2 l. of the same buffer for 16 hr. at 0°. When this preparation was frozen and thawed, approx. 25% of the protein was precipitated without loss of uridine-diphosphatase activity. The precipitated protein was removed by centrifuging and the supernatant was adjusted to pH 4.8 with 0.1N-HCl. After standing for 5 min. at 3° the precipitated protein was removed by centrifuging at 10 000 g for 5 min. and the supernatant was adjusted to pH 7.5 with 0.1N-KOH. A portion (10 ml.) of this supernatant was added to the top of a column containing 0.4 g. of diethylaminoethylcellulose (DEAE-cellulose; Cellex-D, Bio-Rad Laboratories, Richmond, Calif., U.S.A.) that had been thoroughly washed with 5 mM-tris-HCl buffer, pH 7.5, and then packed into the column under pressure. The column was washed with 10 ml. of 0.02M-KCl in 5 mM-tris-HCl buffer, pH 7.0, and then the uridine diphosphatase was eluted with 4 ml. of 0.1M-KCl in 5 mM-tris-HCl buffer, pH 7.0. This preparation is referred to below as the 'partially purified enzyme'. A unit of uridine-diphosphatase activity is defined as the amount of enzyme that will hydrolyse 1 μ mole of substrate/min. at pH 8.1 and 30°.

From other plant tissues. For a survey of the distribution of uridine diphosphatase, 10 g. of each of the various tissues was ground in a mortar and the juice obtained by squeezing through fine muslin. Each extract was cooled to 0°, adjusted to pH 5.2 with 1N-HCl and centrifuged at 10 000 g for 10 min. After readjustment of the pH to 7.5 the supernatant was added to 0.2 g. of DEAE-cellulose (previously washed with 5 mM-tris-HCl buffer, pH 7.0) in a centrifuge tube. The DEAE-cellulose was washed twice with 5 ml. of 5 mM-tris-HCl buffer, pH 7.0, then with 5 ml. of 0.2M-KCl in 5 mM-tris-HCl buffer, pH 7.0. The protein fraction eluted with 0.2M-KCl was tested for uridine-diphosphatase activity.

Materials

The following compounds were obtained from commercial sources: uridine, UMP, UDP, UTP, adenosine, AMP, ADP, ATP, cytosine, CMP, CDP, CTP, guanosine, GMP, GDP, GTP, IDP, deoxyguanosine 5'-diphosphate, thymidine 5'-diphosphate and UDP-glucose (from Sigma Chemical Co., St Louis, Mo., U.S.A.); glucose 1-phosphate, fructose 6-phosphate, fructose 1-phosphate and fructose 1,6-diphosphate (from C. F. Boehringer und Soehne, Mannheim, Germany).

Analytical methods

Phosphatase activity. Except where otherwise indicated reaction mixtures contained: the partially purified enzyme (3.5 μ g. of protein), 0.5 μ mole of UDP (or of other nucleoside phosphates where specified), 0.4 μ mole of $MgCl_2$ and 5 μ moles of tris-HCl buffer, pH 8.1, in a total volume of 0.1 ml. Mixtures were incubated at 30° and the reactions were stopped by heating in a boiling-water bath for 2 min. After the addition of 0.01 ml. of 50 mM-EDTA, 0.02 ml. of the reaction mixture was placed on Whatman no. 54 chromatography paper and developed for 4 hr. with propyl acetate-90% (v/v) formic acid-water (11:5:3, by vol.) (Bielecki & Young, 1963). The solvent separated the nucleosides and their mono-, di- and tri-phosphate derivatives. The nucleotides were detected by viewing chromatograms under ultraviolet light: the area of the chromatogram that included the nucleotide was then cut out and placed in 2 ml. of 10 mM-potassium phosphate buffer, pH 7.0. After shaking for 2 hr. at room temperature to extract the nucleotide, the extinction of the solutions at the absorption maximum was determined. From the extinction values for the substrate and product the percentage hydrolysis of the nucleoside phosphates was calculated. Correction for trace contaminants in nucleotide samples was made by running zero-time controls. Paper blanks were determined on equal-size pieces of paper from the same chromatograms. Reaction products were identified by chromatography in propyl acetate-90% (v/v) formic acid-

water (11:5:3, by vol.) and propan-1-ol-aq. ammonia (sp.gr. 0.88)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949) and by their absorption spectra.

Determination of protein and of inorganic phosphate. The protein content of enzyme preparations was determined by the method of Warburg & Christian (1941); inorganic phosphate was determined as described by Allen (1940).

RESULTS

Purification of uridine diphosphatase. With unfractionated preparations from mature storage tissue of sugar cane UDP was hydrolysed five times as rapidly as UTP and 26 times as rapidly as UMP, at pH 8.1 (Table 1). Fractionation of the extract by the procedure described in the Experimental section resulted in a 43-fold purification of uridine-diphosphatase activity. The partially purified preparation attacked UDP at 53 times the rate for UTP whereas UMP was not hydrolysed. The results suggest that the residual activity with UTP was probably due to a contaminating phosphatase.

Michaelis constant. For studies with UDP as substrate the amount of enzyme and incubation time were adjusted to give between 40 and 70% hydrolysis of the nucleotide. A time-course study of a reaction mixture containing UDP (5 mM) showed that the reaction was linear with time until at least 90% of the UDP had been hydrolysed. The assay method was not sufficiently sensitive to determine the Michaelis constant (K_m) for UDP. However, the enzyme was saturated with respect to UDP at a concentration of 0.2 mM so that the K_m would be less than 0.1 mM.

Product. The product of the reaction on UDP co-chromatographed with authentic uridine 5'-phosphate in two solvents and had an identical

Table 1. *Purification of uridine diphosphatase*

Reaction mixtures contained either the 0-70% $(NH_4)_2SO_4$ fraction (103 μ g. of protein), the frozen-and-thawed 0-70% $(NH_4)_2SO_4$ fraction (75 μ g. of protein), the pH 4.8 supernatant (16 μ g. of protein), or the DEAE-cellulose eluate (3.5 μ g. of protein). Otherwise their composition was as described in the Experimental section. With UDP as substrate the incubation time was 60 min., and with UTP or UMP it was 120 min. The unit of phosphatase activity is defined in the Experimental section.

Fraction	Total units	Concn. of protein (mg./ml.)	Substrate and specific activity (units/mg. of protein)	Ratio of activity	
				UDP UTP	UDP UMP
0-70% $(NH_4)_2SO_4$, dialysed	11.9	20.5	0.038 (UDP)	—	—
0-70% $(NH_4)_2SO_4$, frozen and thawed	11.9	15.0	0.010 (UTP) 0.053 (UDP) 0.002 (UMP)	5.3	26
pH 4.8 supernatant	6.7	1.6	0.025 (UTP) 0.385 (UDP) 0.005 (UMP)	15.5	77
DEAE-cellulose eluate (0.1M-KCl)	3.1	0.41	0.031 (UTP) 1.75 (UDP) 0.0 (UMP)	53	∞

absorption spectrum. Inorganic phosphate and UMP were formed in equimolar amounts.

Effect of pH. With UDP as substrate the enzyme had optimum activity between pH 7.6 and 8.2, and showed 50 % of its maximum activity at pH 5.8 and 8.9 (Fig. 1).

Activators and inhibitors. The activity of the partially purified enzyme was increased by approx. 40 % when magnesium chloride was added to give a final concentration of 4 mM (Table 2). Higher con-

centrations of magnesium chloride were inhibitory. Calcium chloride (4 mM) had no effect on activity, and 4 mM- and 10 mM-manganese chloride inhibited activity by 35 and 55 % respectively. Even at a concentration of 70 mM, fluoride inhibited by only 45 %. EDTA (6 mM) inhibited activity by 92 % in the presence of 4 mM-magnesium chloride and by 96 % in the absence of magnesium chloride.

Inorganic phosphate inhibited uridine-diphosphatase activity by 40 % at a concentration of 50 mM. When added at the same concentration as the substrate, UTP and UMP inhibited activity by only 10 and 7 % respectively; ADP had no effect.

Substrate specificity. The partially purified uridine-diphosphatase preparation attacked UTP at less than 2 % of the rate for UDP and was inactive towards UMP (Table 1). The same enzyme preparation was inactive towards ATP, ADP, AMP, GTP, GMP, CTP, CMP, UDP-glucose, glucose 1-phosphate, fructose 1-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate. Of the nucleoside diphosphates tested most were hydrolysed at a significant rate but none were attacked at more than 45 % of the rate for UDP (Table 3). The relative rates at which the nucleoside diphosphates were hydrolysed remained constant during the purification of uridine diphosphatase.

Enzyme stability. The partially purified enzyme preparation, which contained 0.41 mg. of protein/ml., lost about 10 % of its original activity during storage for 3 months at -10° . Activity was completely destroyed by heating at 100° for 2 min. The enzyme was relatively stable under acid conditions, being unaffected by exposure to pH 4.5 for 10 min. at 5° .

Fig. 1. Effect of pH on uridine-diphosphatase activity. Reaction mixtures were as described in the Experimental section except that a tris-maleate buffer was used. A solution containing 0.2M-tris and 0.2M-maleic acid was adjusted to different pH values with KOH. The final concentration of both tris and maleate in the reaction mixture was 20 mM. The incubation time was 60 min.

Table 2. *Activators and inhibitors of uridine diphosphatase*

The composition of standard reaction mixture (complete system) is given in the Experimental section. The concentration of compounds added to the standard reaction mixture is shown in the Table. The incubation time was 60 min.

Expt. no.	Reaction mixture composition	Uridine-diphosphatase activity (μmoles/mg. of protein/min.)
1	Complete system*	1.51
	Complete system but with $MgCl_2$ (1 mM)	1.18
	Complete system but with $MgCl_2$ (10 mM)	1.35
	Complete system, $MgCl_2$ omitted	1.09
	Complete system + $CaCl_2$ (4 mM), $MgCl_2$ omitted	1.11
	Complete system + $MnCl_2$ (4 mM), $MgCl_2$ omitted	0.69
	Complete system + $MnCl_2$ (10 mM), $MgCl_2$ omitted	0.48
	Complete system + KF (70 mM)	0.60
	Complete system + EDTA (6 mM)	0.11
	Complete system + EDTA (6 mM), $MgCl_2$ omitted	0.04
2	Complete system*	1.43
	Complete system + inorganic phosphate (50 mM)	0.99
	Complete system + UTP (5 mM)	1.26
	Complete system + UMP (5 mM)	1.32
	Complete system + ADP (5 mM)	1.45

* Including $MgCl_2$ (4 mM).

Table 3. *Substrate specificity with nucleoside diphosphates*

Reaction mixtures contained either the 0–70% $(\text{NH}_4)_2\text{SO}_4$ fraction (centrifuged after freezing and thawing) (150 μg . of protein) or the partially purified enzyme (3–5 μg . of protein), and were otherwise of the composition described in the Experimental section. Incubation times varied between 30 and 90 min. depending on the substrate being examined. The phosphatase activities given are those of the partially purified enzyme. The values for the 0–70% $(\text{NH}_4)_2\text{SO}_4$ fraction are not shown but the purification for each substrate is given.

Substrate	Phosphatase activity ($\mu\text{moles/mg. of protein/min.}$)	Purification compared with 0–70% $(\text{NH}_4)_2\text{SO}_4$ fraction
UDP	1.56	29-fold
IDP	0.71	24-fold
GDP	0.60	26-fold
Deoxyguanosine 5'-diphosphate	0.18	29-fold
CDP	0.13	24-fold
Thymidine 5'-diphosphate	0.11	28-fold
ADP	0.0	—

Occurrence of uridine diphosphatase in different tissues from sugar cane and other plants. For the studies described above the tissue used to prepare the enzyme consisted of storage parenchyma and vascular tissue. These two types of tissue were separated and extracts were prepared from each. Uridine-diphosphatase activity was found only in the preparation from storage parenchyma.

Because of the prevalence of non-specific phosphatases in tissue extracts, juice from the various tissues examined was subjected to a purification procedure based on that used for uridine diphosphatase of sugar-cane storage tissue. As a check on specificity the rates of hydrolysis of UTP, UDP and UMP with each preparation were compared. Preparations that hydrolysed UDP at least five times as rapidly as UTP or UMP were obtained from immature storage tissue and leaves of sugar cane, potato tuber, green-pea seeds and shoots of 7-day-old oat seedlings. Activity was not detected in preparations from sugar-cane roots, mature leaves and stem of spinach, oat roots and wheat germ. Before fractionation, extracts from immature storage tissue and leaves of sugar cane hydrolysed UMP to uridine more rapidly than UDP was converted into UMP. Failure to detect activity in some preparations may have been due to loss of enzyme during fractionation.

DISCUSSION

The sugar-cane uridine diphosphatase described above apparently requires a bivalent metal ion for activity. The partially purified enzyme did not

show an absolute requirement for added Mg^{2+} ions, but EDTA (6 mM) inhibited activity when added either with or without magnesium chloride (4 mM). A similar partial requirement for added Mg^{2+} ions has been observed with other purified alkaline phosphatases (Schmidt & Thannhauser, 1943; Heppel & Hilmo, 1951). It is concluded that most of the metal ion essential for uridine-diphosphatase activity remained bound to the enzyme during precipitation with ammonium sulphate, dialysis for 18 hr., and adsorption on and then elution from DEAE-cellulose.

The relative activities of the phosphatase with different nucleoside phosphates lead to the following conclusions about the structural requirements of the substrate for activity: (a) Only nucleoside 5'-diphosphates were hydrolysed. (b) Irrespective of whether the base was a purine or a pyrimidine, nucleotides with a 6-hydroxyl substituent in the pyrimidine ring were active whereas those with a 6'-amino substituent were either inactive (ADP) or very weakly active (CDP). (c) The substituent in position 2 of the pyrimidine ring did not have a profound influence. Though the greater activity of UDP compared with IDP and GDP may have been due to the different substituents at position 2, the lower activity of the purine nucleotides was probably due to the imidazole ring. (d) The relative rates of hydrolysis of GDP and deoxyguanosine 5'-diphosphate showed that activity was decreased by approx. 70% when the ribose moiety was replaced by deoxyribose. The fact that thymidine 5'-diphosphate was only 6% as active as UDP may have been due to the combined effect of the deoxyribose and the 5'-methyl substituent of thymidine 5'-diphosphate.

Sucrose (Cardini, Leloir & Chiriboga, 1955), sucrose phosphate (Leloir & Cardini, 1955), glycogen (Leloir & Cardini, 1957) and starch (Leloir, de Fekete & Cardini, 1961) are formed enzymically by reactions that may be described by the following equation in which 'X' is an acceptor sugar or polysaccharide:

UDP-glucose or other nucleoside diphosphate sugar derivatives may also be involved in the synthesis of cell-wall polysaccharides (Setterfield & Bailey, 1961). The equilibrium for the reactions that form sucrose phosphate (Mendicino, 1960) and glycogen (Kornfeld & Brown, 1962) strongly favour synthesis, but the reaction for sucrose formation has an equilibrium constant of about 6 (Cardini *et al.* 1955). A physiological function for specific nucleoside diphosphatases could be to prevent the reversal of reactions of the type described in the above equation. In the sugar-cane stalk, uridine diphosphate glucose-fructose trans-

glucosylase is confined to the vascular tissue (Hatch *et al.* 1963), whereas uridine diphosphatase is only present in the storage parenchyma.

SUMMARY

1. A phosphatase that attacks certain nucleoside 5'-diphosphates, but not the corresponding mono- and tri-phosphates, was purified 43-fold from sugar-cane storage tissue.

2. The relative activities for the 5'-diphosphates of uridine, of inosine, of guanosine, of deoxyguanosine, of cytidine, of thymidine and of adenosine were 1.0, 0.45, 0.38, 0.11, 0.08, 0.06 and 0.0 respectively. The relationship between activity and the structure of substrates is discussed.

3. Added Mg^{2+} ions increased activity by only 40% but the enzyme was completely inhibited by EDTA. Activity was depressed to some extent by fluoride, inorganic phosphate and Mn^{2+} ions.

4. The enzyme operated at more than 50% of its maximum rate between pH 5.8 and 8.9. The Michaelis constant for UDP was less than 0.1 mM.

5. Phosphatase preparations that attacked UDP much more rapidly than they did either UMP or UTP were obtained from a number of other plant tissues.

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Biochem. J. (1963) **88**, 427

Glucose Utilization in Pregnant Sheep

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(Received 22 January 1963)

There has been much speculation on the role of glucose in ruminant metabolism. This has, in part, arisen because the concentration of blood glucose is lower than in non-ruminants, and because the evidence from many experiments in ruminants is that little glucose is absorbed from the alimentary canal. For example, Allcroft & Strand (1933) and Bell & Jones (1945) found that no obvious hyperglycaemia developed in the sheep and cow after a test meal of glucose; Schambye (1951) and Annison, Hill & Lewis (1957), working with sheep, were unable to demonstrate significant portal-carotid differences in glucose concentration. Also, most of the dietary energy of the ruminant is absorbed in the form of short-chain volatile fatty acids that are produced by fermentation of carbohydrates in the rumen. It is therefore likely that metabolism in the ruminant is adapted towards acetate as the main

source of energy and the main substrate for lipogenesis. Lindsay (1959) reviewed the available evidence on carbohydrate metabolism in the ruminant and concluded that most of the glucose presented to the tissues must arise by gluconeogenesis from propionate and protein. During pregnancy, however, the foetal demand for fructose (Huggett, Warren & Warren, 1951) is believed to cause a relatively great increase in overall hexose requirements (Kronfeld, 1958).

It was therefore decided to measure turnover rates of glucose in pregnant sheep and compare these rates with those obtained by other investigators in non-pregnant sheep.

The constant-infusion isotope-dilution method has been used by Steele, Wall, de Bodo & Altszuler (1956) for measuring glucose-utilization rates in dogs and by Annison & White (1961) and Kronfeld