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THE ZINC REQUIREMENT OF DINUCLEOTIDE PYROPHOSPHATASE IN MAMMALIAN ORGANS

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SUMMARY

The enzyme in unfractionated rat kidney which cleaves UDPG to yield glucose 1-phosphate is slowly and completely inactivated by polyacetic-acid-chelating agents and may be fully reactivated only by Zn^{2+} out of a total of eighteen cations tested. Imidazole also inactivates, but not by removal of a metal, and the effect is reversible by dilution alone. A similar Zn^{2+} requirement was shown for the enzyme activity in eight other rat organs and the kidneys of eight other species. No difference in regard to inactivation and reactivation by Zn^{2+} was observed whether the substrate was UDPG, ADPG, CDPG, GDPG (rat kidney, liver, intestine and heart), DPN^+ or FAD (rat kidney). Kinetic evidence indicates that, at least in rat kidney and liver, a single enzyme is responsible for the major activity toward UDPG and ADPG.

INTRODUCTION

In the process of measuring UDPG pyrophosphorylase in rat kidney it was found that glucose 1-phosphate formation from UDPG was fully half as fast in the absence of PP_i as in its presence. This dinucleotide pyrophosphatase activity could be eliminated by incubation with chelating agents and be fully restored with Zn^{2+} but not with other cations tested. The enzyme is present in many organs at lower levels than in kidney and appears to be quite nonspecific.

MATERIALS AND METHODS

Most of the experiments were carried out with 235-g male Sprague–Dawley rats obtained from Holtzman Rat Company. Single members of the species were obtained locally. A fresh human kidney (from an adult male) was provided through the courtesy of the division of surgical pathology of this school. The human kidney contained renal stones, but the portion taken for enzyme measurements was judged to be normal; it contained both cortex and medulla.

Abbreviation: DTPA, diethylenetriaminepentaacetic acid.

Enzymes were purchased from Boehringer and Sons, except for heart lactic dehydrogenase (Worthington Biochem. Corp.) and UDPG dehydrogenase (Sigma Chem. Co.). Other chemicals were obtained from Calbiochem (GDPG), Aldrich Chem. Co. (diethylenetriaminepentaacetic acid (DTPA)), 2-amino-2-methyl-1,3-propanediol (Eastman) and the remainder from Sigma or Mallinckrodt Chemical.

Fresh, or sometimes frozen, tissues were homogenized at 0° in ground-glass grinders with 8–10 vol. of 0.05 M Tris–HCl (pH 8.2), unless otherwise indicated. These homogenates could be stored without loss of activity for several weeks at 4° or much longer at −10°. (Homogenates were less stable if prepared in imidazole buffer at pH 7.)

Pyrophosphatase activity with the sugar nucleotides was measured directly in a Farrand fluorometer in 1 ml of buffer at 25°. The system contained 0.5 mM nucleotide (UDPG, ADPG, CDPG, or GDPG), 5 mM $MgCl_2$, 0.01% bovine plasma albumin, 0.1 mM TPN^+, 4 μg/ml of yeast glucose-6-phosphate dehydrogenase, 16 μg/ml of muscle phosphoglucomutase, 0.1 M 2-amino-2-methyl-1,3-propanediol (pH 9.0), or 0.1 M Tris–HCl (pH 8.2), and two different levels of enzyme representing usually 20–100 μg of tissue. The half-time of the auxiliary enzyme system was 20–40 sec with glucose 1-phosphate standards. The enzymatic rate (increase in fluorescence) was linear with time after the first min. Pyrophosphatase activity toward DPN^+ was measured by incubating approx. 200 μg of tissue for 1 h in 1 ml of the same basic medium with 0.5 mM DPN^+ but with no auxiliary enzymes or TPN^+. The reaction was terminated by heating 5 min at 100°. An aliquot was taken for the measurement of liberated AMP by a fluorometric assay[1]. Pyrophosphatase activity toward FAD was measured by following the increase in fluorescence which occurs when the mononucleotide is liberated[2]. The reagent consisted of 0.1 M Tris–HCl buffer (pH 8.2), 54 nM FAD, 0.01% bovine plasma albumin and 5 mM $MgCl_2$, to which the equivalent of 30 μg of tissue was added.

RESULTS

Most of the studies were made with UDPG as substrate with unfractionated homogenates of rat kidney. With the sensitive fluorometric assay used, the permissible tissue dilution was so great (1:10 000–1:50 000) that other enzymes present did not interfere.

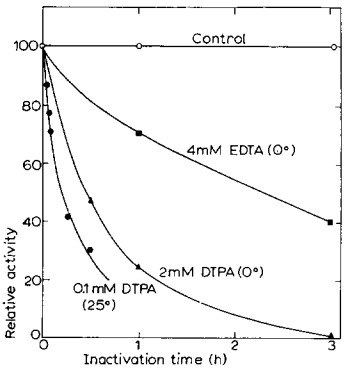
The Michaelis constant (measured with UDPG) is markedly affected by pH. In the case of kidney, it was found to be 0.04 mM at pH 8.2 and 0.67 mM at pH 9.0. In the case of liver, it is 0.23 mM at pH 9.0.

With 0.5 mM UDPG, a sharp pH optimum was observed at pH 9. A similar pH optimum has been reported for UDPG-pyrophosphatase activity in seminal plasma[3] and in liver[4]. At a higher level of UDPG (5 mM), the pH optimum was broader, ranging from pH 9.2 to 9.7.

The enzyme activity can be diminished by chelating agents but in two distinctly different fashions. Imidazole produces an immediate inhibition, reversible by dilution. In contrast, EDTA and DTPA slowly inactivate and their effects are only reversed if Zn^{2+} is added in excess.

Inactivation by EDTA and DTPA

At 0°, with 4 mM EDTA, activity was lost with a half-time of about 2 h (Fig. 1).



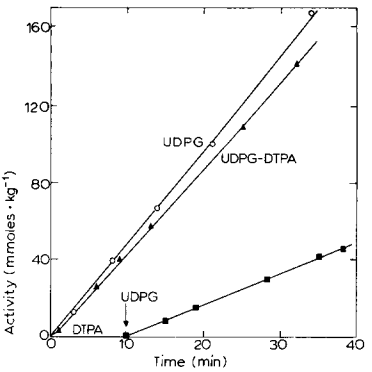


Fig. 1. Time-dependent inactivation of rat kidney UDPG pyrophosphatase. For the inactivation at 0°, homogenate (1:50 in 0.1 M imidazole–HCl, pH 7.0) was incubated with DTPA or EDTA and aliquots were assayed in Tris–HCl buffer .The inactivation at 25° was carried out directly in the Tris–HCl buffer (1:30 000 tissue dilution), and activity was determined by introducing the assay components at the time indicated.

Fig. 2. UDPG protection from DTPA inactivation of rat kidney UDPG pyrophosphatase. Homogenate (1:30 000 tissue dilution) was incubated in Tris–HCl buffer with all of the assay components, except 0.5 mM UDPG. UDPG, DTPA or a UDPG–DTPA mixture was introduced at time zero. UDPG was added to the DTPA sample at the time shown. The DTPA level was 1 mM. Activity is calculated per kg original tissue.

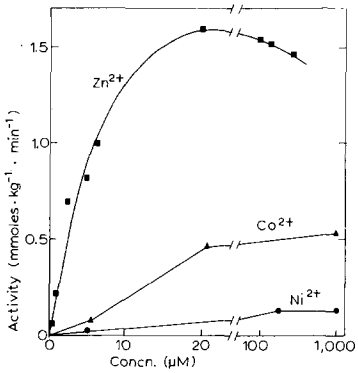
DTPA is more effective, 2 mM producing half inactivation in 30 min under the same conditions. At 25° the rate is much faster. With lower DTPA (0.1 mM), only 12 min were required for 50% inactivation (Fig. 1). Inactivation by DTPA is blocked by the substrate (Fig. 2).

Reactivation by Zn^{2+} and lack of Mg^{2+} requirements

After inactivation, activity can be fully restored by diluting out the DTPA and adding Zn^{2+}. This finding did not fully establish a Zn^{2+} requirement, since it left the possibility that the metal was merely acting to complex the remaining DTPA. A more conclusive experiment consisted of prolonged dialysis of the enzyme against a DTPA solution followed by removal of the DTPA by further dialysis. Although this resulted in some permanent loss of activity, the effects of Zn^{2+} were unambiguous. Only Zn^{2+}, Co^{2+} and Ni^{2+} of eighteen cations tested had substantial reactivating effects, and Zn^{2+} was by far the most effective cation (Fig. 3). (The Ni^{2+} effect is so small that it might have been the consequence of Zn^{2+} contamination.) Under the conditions tested, half-maximal activation was obtained with 4 μM Zn^{2+}. No activity was obtained with Mg^{2+}, Sn^{2+}, V^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, Pb^{2+}, Cd^{2+}, Ba^{2+}, Be^{2+}, Cu^{2+}, Ag^{+} or Sr^{2+}. Mn^{2+} and Ca^{2+} gave a trace of activity.

Reactivation was not instantaneous. With 20 μM Zn^{2+}, activation was 75% maximal in 10 min. Although, as shown in Fig. 2, the substrate blocks inactivation by DTPA, it does not block reactivation with Zn^{2+}.

There appears to be no requirement for Mg^{2+}. The standard assay system requires Mg^{2+} for the sake of the phosphoglucomutase step. When Mg^{2+} was omitted from the assay system for 21 min, the rapid increase in fluorescence upon subsequent Mg^{2+} addition demonstrated that UDPG had been hydrolyzed as fast in the absence of Mg^{2+} as in its presence (Fig. 4).



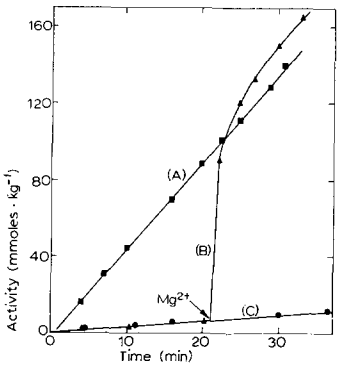


Fig. 3. Reactivation of DTPA-inhibited rat kidney UDPG pyrophosphatase by heavy metals. Homogenate (1 ml of 1:10 tissue dilution in 0.1 M Tris–HCl, pH 8.2) was dialyzed 18 h at 4° against 2 l of 5 mM DTPA in 0.05 M Tris–HCl (pH 8.2) then for two 3-h periods against 1 l of deionized water. An aliquot (45–240 μg tissue) was incubated 10 min at 25° in 1 ml of 0.1 M Tris–HCl (pH 8.2) containing one of the metals at the levels indicated. Activity was determined by adding 0.5 mM UDPG, and after 25 min the amount of glucose 1-phosphate that had been formed was determined by introducing the remaining assay components. Activity is calculated per kg original tissue.

Fig. 4. Activity of rat kidney UDPG pyrophosphatase in the absence of Mg^{2+}. Rat kidney homogenate was inactivated with DTPA followed by reactivation with Zn^{2+} (Table II). Enzyme activity was determined in 0.1 M Tris–HCl with 0.2 mM EDTA included. 5 mM Mg^{2+} was present from the beginning (A) or was added at 21 min (B). The inactivated enzyme unexposed to Zn^{2+} was assayed with Mg^{2+} present from the beginning (C). Activity is calculated per kg of original tissues.

Inactivation by imidazole

Imidazole is a potent inhibitor of the pyrophosphatase with a K_i of 12 mM (Fig. 5). Unlike the inactivation by DTPA, the inhibition is immediate, is immediately reversible by dilution and is not influenced by the presence of substrate (Fig. 6). It is also not affected by the level of substrate (not shown). Addition of Zn^{2+} at levels up to 0.5 mM does not diminish the inhibition by imidazole. Conversely, the presence of imidazole neither accelerates nor delays the inhibition by DPTA (not shown).

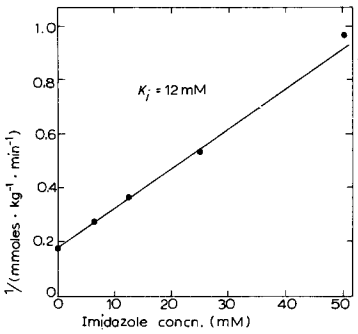
The rapid reversal of imidazole inhibition by dilution and the lack of a Zn^{2+} effect suggests that this inhibitor does not act by removal of a metal. This was more conclusively shown by an experiment in which the enzyme was dialyzed first against 100 mM imidazole buffer and then against water. After this treatment the enzyme had 105% of the original activity and was not influenced whatsoever by low levels of Zn^{2+}.

Effect of other chelating agents

o-Phenanthroline inhibited in a manner similar to that of DTPA but at a much slower rate. Tested at 25° and pH 8 with 1:100 rat kidney homogenate, 0.6 and 5 mM *o*-phenanthroline reduced activity 20 and 50%, respectively, in 90 min. In the second case, activity was restored to 86% of the original level with 50 μM Zn^{2+}. α,α′-Dipyridyl also inactivated but extremely slowly. At 5 mM concentration under the above conditions, activity was reduced only 15% in 2.4 h.

Enzyme distribution and specificity

UDPG-pyrophosphatase activity was found in nuclear, mitochondrial and



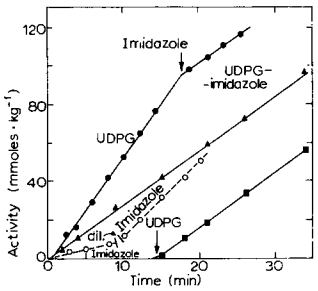


Fig. 5. Imidazole inhibition of rat kidney UDPG pyrophosphatase. Tissue homogenate was added to the assay media (Tris–HCl) containing varying concentrations of imidazole (final tissue dilution, 1:30 000). Activity is calculated per kg original tissue.

Fig. 6. Reversibility of imidazole inhibition and failure of substrate to block the inhibition. Rat kidney homogenate was added to Tris–HCl medium with all assay components except 0.5 mM UDPG. At zero time, UDPG, imidazole, or a UDPG–imidazole mixture were added (solid lines). At the times indicated imidazole or UDPG were added. In each case the final imidazole concentrations were the same (12 mM). The dotted lines represent a two-step experiment. For the first 8 min homogenate was incubated in the standard assay medium with 46 mM imidazole. At the arrow the sample was diluted 1:8.5 with fresh, complete, assay medium. The rates are all calculated per kg of original tissue.

microsomal fractions of rat kidney, but the activity was negligible in the 105 000 $\times g$ supernatant fraction (Table I). Of the total amount of activity in the original homogenate, approx. 64% was recovered in the various fractions, 57% of the recovered activity was located in the mitochondrial fraction, and 30% in the microsomal fraction.

Dinucleotide-pyrophosphatase activity was measured in nine rat tissues (Table II). Kidney, liver and intestine were the most active. The enzyme was fully active in the native state (no enhancement by Zn^{2+}). DTPA treatment resulted in complete inactivation in every case, except that there appeared to be some residual activity in the case of spleen. Subsequent treatment with Zn^{2+} restored activities almost to the

TABLE I

SUBCELLULAR DISTRIBUTION OF RAT KIDNEY UDPG-PYROPHOSPHATASE

Rat kidney was homogenized in 10 vol. of 0.25 M sucrose and fractionated by differential centrifugation at 0°. The pellets were resuspended in 0.1 M imidazole–HCl buffer (pH 7.0) containing 2 mM DTPA and stored at 4°. After 15 h, an aliquot was incubated 30 min at 0° with 3 mM zinc acetate in the same buffer and assayed in 0.1 M Tris–HCl buffer (pH 8.2). Activity is expressed on a wet-weight basis.

Fraction	*Activity (mmoles · $kg^{-1} \cdot h^{-1}$)*	*Recovery (%)*
Whole homogenate	308	(100)
Nuclei and debris (800 × *g*, 10 min)	27	8.8
Mitochondria (18 000 × *g*, 10 min)	111	36.0
Microsomes (105 000 × *g*, 60 min)	59	19.2
Supernatant fluid	< 1	0

TABLE II

UDPG-PYROPHOSPHATASE DISTRIBUTION IN RAT ORGANS

Homogenates were made in 0.05 M Tris–HCl (pH 8.2) at dilutions of about 1:10. Portions of each sample were treated three different ways at a tissue dilution of 1:100. (1) Samples labeled "Zn^{2+}" were incubated for 30 min at 0° with 3 mM zinc acetate in 0.1 M imidazole–HCl buffer (pH 7.0). (2) Samples labeled "DTPA" were incubated 3–5 h at 0° with 5 mM DTPA in the same buffer. (3) Samples labeled "DTPA–Zn^{2+}" were first treated with DTPA, as in (2), and then with 3 mM zinc acetate, as in (1). Assays were made in each case with two levels of tissue at pH 9. Depending on the expected activity, homogenate equivalent to 20–200 μg of tissue was used in each assay (1 ml). Activities are calculated on a wet-weight basis. Numbers in parentheses are the range for three animals.

Organ	*Activity (mmoles · kg^{-1} · h^{-1})*			
Pretreatment:	*Control*	*Zn^{2+}*	*DTPA*	*DTPA–Zn^{2+}*
Kidney	394 (85)	398 (66)	2 (4)	388 (32)
Liver	185 (53)	204 (23)	<1	194 (23)
Intestine	118 (45)	104 (38)	<1	98 (46)
Heart	48 (0)	48 (6)	<1	53 (6)
Lung	37 (5)	36 (6)	<1	40 (5)
Muscle	16 (5)	13 (5)	<1	10 (3)
Fat pad	14 (9)	10 (5)	<1	14 (6)
Spleen	20 (7)	14 (13)	5 (5)	19 (4)
Brain	4 (1)	3 (2)	1	3 (4)

original levels (Table II). The pyrophosphatase was found in kidney from nine species examined but the activity varied 10-fold from mouse (highest) to dog; human kidney was among the lowest four tested (Table III). The activity in most cases was reduced to negligible values by DTPA and restored with Zn^{2+}; however, restoration was not always complete.

Measurements with other organic pyrophosphates suggest the presence in rat tissues of a single predominant enzyme with broad specificity but do not rule out the possibility of a family of similar enzymes (but see below). In four rat tissues, and in human kidney, UDPG, ADPG, CDPG and GDPG were all attacked at comparable rates and the activities were similarly inhibited by DTPA and restored by Zn^{2+} (Table III). The same is true for rabbit kidney except that restoration with Zn^{2+} was only about 50%. Modest but real differences in the relative rates among the substrates were observed for the three species examined.

DPN^+ cleavage in the kidneys of the three species and in rat liver was also completely inactivated by DTPA and in rat was largely restored with Zn^{2+} (Table III). In human kidney restoration was only 60% complete and in rabbit kidney only 30%, which might suggest the possibility that more than one enzyme is involved.

FAD cleavage in rat kidney was also found to be inhibited by DTPA and restored in activity by Zn^{2+} (not shown). The reaction may therefore be catalyzed by the same enzyme. The rate, measured with a very low level of substrate (54 nM), was 0.28 mmole · kg^{-1} · h^{-1}. If the rate is calculated for UDPG at a similar level, the activity would be 0.51 mmole · kg^{-1} · h^{-1}. Thus it seems that the level of activity with FAD may be of the same order as with the other dinucleotides examined.

Evidence that in rat kidney a single enzyme cleaves both UDPG and ADPG is provided by experiments with mixtures of the two substrates. If there is a specific enzyme for each substrate, the rates with both substrates present would be expected

TABLE III

PYROPHOSPHATASE SPECIFICITY AND SPECIES VARIATION

Assay conditions and pretreatment of the samples are as described in Table II, except for the use of different substrates, which were 0.5 mM in each case. 5′-AMP release was measured when DPN^+ was the substrate (MATERIALS AND METHODS). Data are for a single animal in each case. Results are expressed as mmoles·kg^{-1} wet wt.·h^{-1}.

	UDPG			*ADPG*			*CDPG*			*GDPG*			*DPN+*		
	Control	*DTPA*	*DTPA–Zn^{2+}*	*Control*	*DTPA*	*DTPA–Zn^{2+}*	*Control*	*DTPA*	*DTPA–Zn^{2+}*	*Control*	*DTPA*	*DTPA–Zn^{2+}*	*Control*	*DTPA*	*DTPA–Zn^{2+}*
Rat kidney	392	<1	374	370	<1	382	346	<1	367	244	<1	239	176	<1	146
liver	237	<1	219	167	<1	151	219	<1	214	154	<1	156	171	<1	120
intestine	157	10	96	107	<1	90	114	<1	89	67	<1	53			
heart	64	<1	46	54	<1	38	61	<1	38	39	<1	30			
Rabbit kidney	130	8	73	205	4	101	132	1	79	135	4	77	129	<1	41
liver	28	7	16	17	8	14	23	8	15	15	9	14	<1	<1	<1
Mouse kidney	482	13	370												
Beef kidney	278	38	201												
Pig kidney	194	6	161												
Sheep kidney	98	7	61												
Guinea pig kidney	50	10	39												
Dog kidney	39	4	22												
Human kidney	109	8	102	69	10	69	104	5	84	84	8	76	32	<1	19

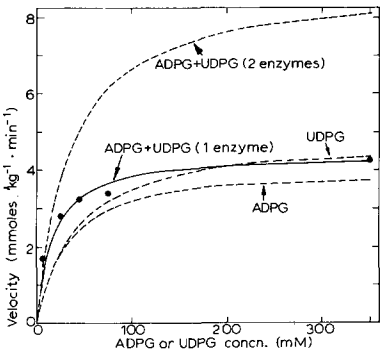


Fig. 7. Evidence that one enzyme in rat kidney cleaves both ADPG and UDPG. The two substrates were added together to the reagent to give equal concentrations (total concentration twice that shown on the abscissa). The five points are observed. The four curves are theoretical for ADPG alone (A), UDPG alone (U) and ADPG *plus* UDPG (A + U) with either a single non-specific enzyme or two enzymes, one specific for ADPG and one specific for UDPG. The curves are based on the maximum velocities and Michaelis constants observed when each substrate was tested alone. The curve for two enzymes is simply the sum of Curve A and Curve U. The curve for A + U with one enzyme is calculated from the formula

$$v = \frac{v_A\,[A]K_U + v_U[U]K_A}{[A]K_U + [U]K_A + K_AK_U}$$

where v_A and v_U are respectively 4.07 and 4.85 mmoles·kg^{-1}·min^{-1} and K_A and K_U are respectively 29 and 39 μM. Reactions were carried out in 0.1 M Tris–HCl (pH 8.2).

(in the absence of complications) to be additive. This was found not to be the case. Instead the rates were those expected for mutual competition for a single enzyme (Fig. 7). Similar results were obtained for rat liver (not shown). To obtain these results with two different specific enzymes, it would be necessary that each substrate be an inhibitor for the other enzyme. Moreover, to explain quantitatively in this way the results of Fig. 7 would demand the rather remarkable coincidence that the Michaelis constant for each substrate be the same as its inhibitor constant for the other enzyme.

DISCUSSION

Pyrophosphatases capable of splitting a variety of dinucleotides (UDPG, ADPG, DPN$^+$, FAD, *etc.*) have been identified in a number of mammalian tissues[3–11]. This is apparently the first report of a Zn^{2+} requirement, but previous findings are not inconsistent with this. SCHLISELFELD *et al.*[4] found that nucleotide pyrophosphatase in rat liver is inactivated by preincubation with EDTA. Activity was stimulated slightly by Co^{2+}; Zn^{2+} was not tested; Mg^{2+} was not effective. Similar results were obtained by PATTABIRAMAN *et al.*[9] for sheep brain nucleotide pyrophosphatase. A discrepancy may exist in regard to bovine seminal pyrophosphatase[3]. When this enzyme was inactivated by treatment with Dowex 50, it could be partially reactivated by 10 mM Mg^{2+} but not by 10 mM Zn^{2+}. However, it is possible that the high level of Mg^{2+} may have contained a sufficient trace of Zn^{2+} to reactivate. Moreover, the level of Zn^{2+} tested is so high that it would completely inhibit dinucleotide pyrophosphatase activity of the type present in kidney.

It is concluded from the present results that dinucleotide pyrophosphatase of

mammalian tissues can be inhibited with chelating agents either by removal of the metal ligand or by combining with the metal without removal. Both of these phenomena have been observed before. Examples of the first are well known. Inhibition by cyanide of alkaline phosphatase[12] and inhibition of alcohol dehydrogenase by *o*-phenanthroline[13], as well as by imidazole[14] may be cited as examples of the second phenomena. In each of these instances reactivation occurs by simple dilution, as shown here for imidazole inhibition.

The presence of substrate prevents inactivation (presumably by Zn^{2+} removal). On the other hand, substrate fails to prevent reactivation by Zn^{2+}. This suggests that UDPG is more tightly bound when Zn^{2+} is present than when it is absent. The prevention by substrate of removal of activating metal is reminiscent of the prevention by substrate of activation of phosphoglucomutase through removal of inhibitory metal (also presumably Zn^{2+}) with chelating agents[15,16].

Some of the previous studies have indicated the existence of a number of specific dinucleotide pyrophosphatases. A specific ADPG pyrophosphatase from *Escherichia coli* has been isolated[17]. RODRIGUEZ *et al.*[11] have reported an ADPG pyrophosphatase from rabbit and calf liver that is totally inactive toward UDPG. On the other hand, the presence of less specific enzymes in mammalian tissues has been demonstrated by a number of laboratories[3,4,8–10]. The present study suggests that if there are specific enzymes for UDPG, ADPG, CDPG, GDPG or DPN^+, they are either present in relatively small amount in kidney, liver, intestine and heart or they all have a Zn^{2+} requirement. In the organs tested most thoroughly (rat kidney and liver), the kinetic evidence presented argues strongly for a single major enzyme splitting both UDPG and ADPG.

ACKNOWLEDGMENTS

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