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A NEW NUCLEOTIDASE OF RAT LIVER WITH ACTIVITY TOWARD 3'- AND 5'-NUCLEOTIDES*

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SUMMARY

1. A new nucleotidase (nucleotide phosphohydrolase) unique in its high activity toward deoxyribonucleotides, has been purified approximately 2000-fold from the 123 000 × *g* supernatant of rat liver homogenate.

2. The enzyme hydrolyzed deoxythymidine 3',5'-diphosphate more rapidly than the 5'-monophosphates of deoxythymidine, deoxyuridine, and deoxyguanosine, which were degraded at similar rates. Other 5'-nucleotides were only slightly or insignificantly degraded. The enzyme also possessed 3'-nucleotidase activity and hydrolyzed, in decreasing order of effectiveness, the 3'-monophosphate of deoxythymidine, uridine and guanosine. Other 3'-ribonucleotides were inactive as substrates. *p*-Nitrophenyl phosphate was dephosphorylated to a certain extent.

3. The pH optimum of the enzyme varied within the range of 5.6–6.4 depending on the substrate used. The enzyme was Mg^{2+} dependent, contained an essential SH group(s), and was moderately stable. It had a molecular weight of about 45 000. The apparent K_m values, which were higher for the 5'-nucleotides than for the 3'-isomers, ranged from 1.1 mM to 0.16 mM.

4. Identity of the 5'- and 3'-nucleotidase and the *p*-nitrophenyl phosphatase activities with a single protein was supported by the constant ratio of the activities during purification, parallell loss of activities upon storage, and elution of the activities in a single peak from both DEAE-Sephadex and Sephadex G-100 columns.

5. The enzyme activity with varied combinations of two different nucleotides indicated that the enzyme may possess either two catalytic sites hydrolyzing 5'- and 3'-nucleotides, respectively, or an allosteric site at which one of the nucleotides can bind and activate the dephosphorylation of the other.

INTRODUCTION

During the course of recent investigations in this laboratory on the dephos-

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phorylation of nucleotides in the 105 000 × *g* supernatant fluid of rat liver homogenates[1] two previously unknown nucleotidases with optimum activity around pH 6 were observed in the preparation[2]. One of these enzymes was purified[3] and was found to hydrolyze Ino-5′-*P*, Guo-5′-*P*, and dGuo-5′-*P* more rapidly than any of the other nucleotides tested. The other enzyme was detected by its high activity toward dThd-5′-*P* (refs. 2 and 3). The purification and properties of this enzyme are described in the present communication. The data show that the enzyme degrades 5′- as well as 3′-nucleotides with preference for those derived from deoxyribose. It is indicated that the enzyme possesses either two catalytic sites hydrolyzing 5′- and 3′-nucleotides, respectively, or an allosteric site at which one of the nucleotides can bind and activate the dephosphorylation of the other.

MATERIALS AND METHODS

DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia (Uppsala, Sweden). Cytochrome *c*, horse myoglobin, chymotrypsinogen A, egg albumin, bovine serum albumin, and blue dextran were obtained from Serva (Entwicklungslabor, Heidelberg, Germany). Dithiothreitol, 5-methyl-dCyd-5′-*P*, and dThd-3′,5′-P_2 were from CalBioChem (Los Angeles, Calif., U.S.A.), Urd-2′-*P* and Urd-3′-*P* from P-L Biochemicals, Inc. (Wisconsin, U.S.A.), dThd-*P*-dThd and Urd-3′:5′-*P* from Boehringer Mannheim GmbH (Mannheim, Germany). dThd-3′-*P* in the form of the barium salt was generously given by Dr. A. Holý, Institute of Organic Chemistry and Biochemistry, Praha, Czechoslovakia. It was converted to the sodium salt by digestion with Na_2SO_4. The other nucleotides, *p*-nitrophenyl phosphate, pyridoxal 5′-phosphate, ribose 5-phosphate, glycerol 2-phosphate, glucose 6-phosphate, and calcium phosphate gel were purchased from Sigma Chem. Corp. (St. Louis, U.S.A.).

Enzyme assays

Nucleotidase activity was assayed routinely with either dThd-5′-*P* or Urd-3′-*P* as substrate. The reaction mixture contained 50 mM Tris–maleate buffer (final pH 6.1 ± 0.1), 20 mM $MgCl_2$, 5 mM dThd-5′-*P* or 2.5 mM Urd-3′-*P*, 5 mM dithiothreitol, 0.1 mg of crystalline bovine serum albumin, and enzyme preparation in a total volume of 0.5 ml. Incubation was carried out at 37° for 20 min. The reaction was stopped by adding 0.25 ml of 25% (w/v) trichloroacetic acid. The phosphate liberated was determined by the very sensitive procedure described by CHEN *et al.*[4] with the exception that the color was developed at 45° in 30 min.

p-Nitrophenyl phosphatase activity was tested in the presence of 10 mM *p*-nitrophenyl phosphate and 50 mM $MgCl_2$ at a final pH of 5.8 ± 0.1. The composition of the reaction mixture and the incubation conditions were otherwise as described for the nucleotidase assay. The *p*-nitrophenol formed was determined as follows. The enzyme reaction was stopped by adding 2.0 ml of 0.07 M NaOH, the mixture was centrifuged, and the absorbance read at 410 nm. An experimentally determined absorbance of 0.343 corresponded to 0.05 μmole of *p*-nitrophenol formed.

Glycerol-2-phosphatase activity was assayed in the presence of 0.1 M glycerol-2-phosphate, 75 mM $MgCl_2$, and 0.1 M acetate buffer (final pH 6.2 ± 0.1)[1]. The

composition of the reaction mixture and the incubation conditions were otherwise as described for the nucleotidase assay.

One unit of enzyme is defined as that amount which hydrolyzes 1 nmole of phosphate ester per min under the specified incubation conditions. Specific activity is expressed in units per mg of protein.

Assay of protein

Prior to determination of protein by the method of LOWRY *et al.*[5], substances such as Tris buffer, $(NH_4)_2SO_4$, and dithiothreitol in the protein samples, which interfere with the assay, were removed by dialysis for 18 h against 2 l of 5 mM phosphate buffer (pH 8). The recovery of protein in model experiments with crystalline bovine serum albumin was almost quantitative.

RESULTS

Purification of enzyme

All operations were carried out at 0–4°. Centrifugation of precipitated protein was carried out at 10 000 × *g* for 10 min. The saturated $(NH_4)_2SO_4$ solution contained 2 mM EDTA and was adjusted to pH 6.3 with NH_4OH. The steps of a typical purification experiment are summarized in Table I.

TABLE I

PURIFICATION OF NUCLEOTIDASE

Fractions were assayed for activity with dThd-5′-*P* or Urd-3′-*P* as substrate, as described in MATERIALS AND METHODS. The amount of enzyme protein used was approx. 120 μg of Fractions E1, E2 and E3, 5 μg of E4, 0.5 μg of E5, and 0.2 μg of E6.

Fraction	*Volume (ml)*	*Total protein (mg)*	*Total activity with dThd-5′-P (units)*	*Specific activity with dThd-5′-P (units/mg)*	*Specific activity with Urd-3′-P (units/mg)*
E1 (123 000 × *g* supernatant)	182	1929	17 400	9	3
E2 (39–49% $(NH_4)_2SO_4$ precipitate)	11	289	6 550	23	11
E3 (Supernatant after dialysis at pH 6.0)	16	191	5 800	30	16
E4 (Supernatant after calcium phosphate gel adsorption)	18	8.3	3 350	405	204
E5 (DEAE-Sephadex eluate)*	13	0.29	1 520	5 200	2650
E6 (Sephadex G-100 eluate)*	17	0.086	1 080	12 600	6500

* The data refer to the peak tubes. Only the peak tubes of E5, after concentration to about 5 ml by vacuum dialysis, was applied to the Sephadex G-100 column.

Step 1. Preparation of the 123 000 × g supernatant fluid. The livers from 6 rats were homogenized for 0.5 min in 0.25 M sucrose, and the particle-free supernatant fluid (soluble fraction) prepared as described previously[1] except that centrifugation was carried out for 1 h with the Spinco rotor type 50.1 at an average *g*-value of 123 000. The supernatant fluid was diluted with an equal volume of 0.04 M Tris–

maleate buffer (pH 6.3) giving a final concentration of about 10 mg of protein per ml (Fraction E1).

Step 2. Fractionation with $(NH_4)_2SO_4$. In accordance with data described earlier[3] Fraction E1 was brought to 39% saturation with $(NH_4)_2SO_4$ by dropwise addition of saturated $(NH_4)_2SO_4$ solution with stirring over a 20-min period followed by a further 10-min period before the precipitate was removed by centrifugation. The supernatant fluid was brought to 49% saturation by the addition of $(NH_4)_2SO_4$ in the same manner. The precipitate was collected by centrifugation and dissolved in 5 mM Tris–maleate buffer (pH 6.0) containing 1 mM $MgCl_2$ and 1 mM dithiothreitol (Fraction E2).

Step 3. Dialysis at pH 6.0. In accordance with previous findings[3] Fraction E2 was dialyzed at pH 6.0 for 18 h against 2 l of the buffer in Step 2, and the precipitate formed was centrifuged and discarded. The supernatant fluid (Fraction E3) contained the bulk of the enzyme.

Step 4. Calcium phosphate gel treatment. To Fraction E3 was added calcium phosphate gel (about 20 mg of 22% gel per mg of protein), and after stirring for 15 min the gel was centrifuged and discarded. The supernatant fluid, which contained the bulk of the enzyme, was dialyzed for 18 h against 2 l of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM $MgCl_2$ and 1 mM dithiothreitol to give Fraction E4.

Step 5. Chromatography on DEAE-Sephadex A-50. The column (1.5 cm × 20 cm) was equilibrated with the buffer mixture (pH 7.5) used for dialysis in Step 4. Fraction E4 was applied, and the column was eluted with about 85 ml of the same buffer. A linear $(NH_4)_2SO_4$ gradient was then begun. The mixing vessel contained 300 ml of the buffer used for equilibration, and the reservoir contained 300 ml of the same

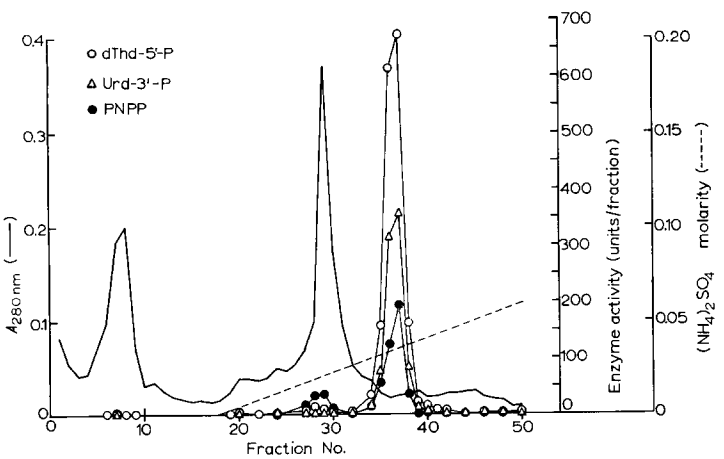

Fig. 1. Chromatography of enzyme Fraction E4 on DEAE-Sephadex A-50. The column (1.5 cm × 20 cm) was equilibrated with a buffer mixture consisting of 50 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, and 1 mM dithiothreitol. The enzyme preparation was applied, and the column was eluted with 85 ml of the buffer mixture. A linear $(NH_4)_2SO_4$ gradient was begun as indicated. The mixing vessel contained 300 ml of the buffer mixture. The reservoir contained 300 ml of the buffer mixture in which 0.15 M $(NH_4)_2SO_4$ was included. The fraction size was 6.5 ml, flow rate 13 ml/h. The $(NH_4)_2SO_4$ concentration in the eluted fractions was determined by conductivity measurements. Enzyme activities were measured as described in MATERIALS AND METHODS. Protein was determined by its absorbance at 280 nm. PNPP, *p*-nitrophenyl phosphate.

buffer in which 0.15 M $(NH_4)_2SO_4$ was included. The fraction size was 6.5 ml, flow rate 13 ml/h. The enzyme was eluted as a sharp peak (Fig. 1) when the $(NH_4)_2SO_4$ concentration reached 35 mM. The eluted enzyme was called Fraction E5. Total recovery of applied enzyme activity was about 90%.

Step 6. Gel filtration on Sephadex G-100. The column (2.5 cm × 38 cm) was equilibrated with a buffer mixture containing 50 mM Tris–HCl (pH 7.5), 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 1 mM dithiothreitol. The most active fractions from

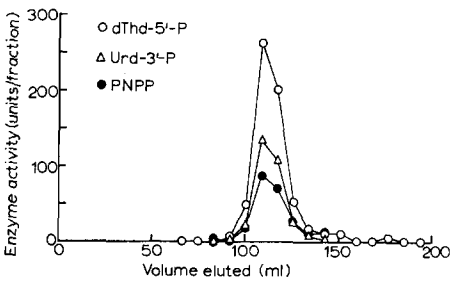

Fig. 2. Gel filtration of enzyme fraction E5 on Sephadex G-100. The column (2.5 cm × 38 cm) was equilibrated with a buffer mixture consisting of 50 mM Tris–HCl (pH 7.5), 10 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, and 1 mM dithiothreitol. Enzyme preparation (5 ml) was applied, and the column eluted with the buffer mixture. The fraction size was 8 ml, flow rate 16 ml/h. Enzyme activities were measured as specified in MATERIALS AND METHODS. PNPP, *p*-nitrophenyl phosphate.

Step 5, concentrated to about 5 ml by vacuum dialysis in collodion bags, were applied to the column and eluted with the buffer mixture used for equilibration. Fraction size was 8 ml, flow rate 16 ml/h. The enzyme was eluted as a sharp peak (Fig. 2). After an aliquot had been reserved for protein assay, crystalline bovine serum albumin (0.1 mg/ml) was added to stabilize the enzyme. The enzyme solution was called Fraction E6. Total recovery of applied enzyme activity was about 90%.

The purification procedure has been repeated a number of times and showed very good reproducibility.

TABLE II

REACTIVATION OF ENZYME BY DITHIOTHREITOL AFTER STORAGE

Undialyzed enzyme from Step 4 was used as enzyme source. The enzyme solution was adjusted to pH 7.5 with Tris buffer, final concentration 10 mM. The preparation was stored at 0°. Enzyme activity (approx. 5 μg of enzyme protein) was assayed with dThd-5′-*P* as substrate as described in MATERIALS AND METHODS except that serum albumin was not present, and dithiothreitol was varied as indicated.

Period of storage (days)	*Dithiothreitol in incubation mixture (mM)*	*Specific activity (units/mg protein)*
0	0	542
20	0	15
20	1	225
20	4	265

Effect of dithiothreitol and serum albumin on enzyme activity

Table II shows that undialyzed enzyme* from step 4 lost 97% of its activity when stored for 20 days at 0°. A partial restoration of the original activity was obtained when the enzyme was incubated in the presence of dithiothreitol. The observation indicates that the enzyme contains an essential sulfhydryl group(s). In accordance with this finding all incubation mixtures contain 5 mM dithiothreitol, and 1 mM dithiothreitol has been included in all enzyme preparations. Other thiols were less efficient for restoration and conservation of enzyme activity.

Enzyme eluted from the DEAE-Sephadex column (enzyme Fraction E5) showed low and variable activity in parallell assay experiments in spite of the fact

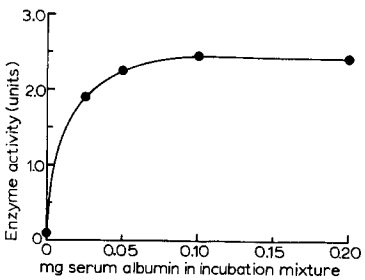

Fig. 3. Effect of serum albumin in incubation mixture. Enzyme activity (Fraction E5, 1.5 μg of enzyme protein) was assayed with dThd-5′-*P* as substrate under the conditions described in MATERIALS AND METHODS except that the amount of serum albumin in the incubation mixture was varied as indicated.

that adequate amounts of dithiothreitol were present. However, when serum albumin was added to the incubation mixture a considerably increased activity level (Fig. 3) and excellent reproducibility were obtained. The optimal level of serum albumin was 0.1 mg/0.5 ml of incubation mixture. The observed effect is probably due to protection of enzyme against denaturation during incubation.

TABLE III

STABILITY OF ENZYME TO STORAGE

The enzyme fractions were stored at 0° for the time indicated. Enzyme activity was assayed with dThd-5′-*P* as substrate, as described under MATERIALS AND METHODS. The amount of enzyme protein used was approx. 5 μg of Fraction E4, 1.5 μg of E5, and 0.6 μg of E6.

Enzyme fraction	*Period of storage (days)*	*Addition to enzyme preparation at zero time*	*Remaining activity (%)*
E4	5	None	78
E5	2	None	78
E5	12	None	26
E5	13	0.1 mg of serum albumin/ml	85
E6	2	0.1 mg of serum albumin/ml	87
E6	10	0.1 mg of serum albumin/ml	42

* In the standardized purification procedure the enzyme is dialyzed against a buffer containing 1 mM dithiothreitol.

With newly prepared enzyme (Fraction E5 stored for 2 days) no significant effect of dithiothreitol (0 to 10 mM) in the incubation mixture was observed. However, with enzyme stored for 12 days dithiothreitol (5 mM) was necessary to obtain optimal and reproducible enzyme activity.

Stability of enzyme

The enzyme was moderately stable when stored in ice at 0°, but was inactivated with freezing. Table III shows that enzyme Fraction E4 lost 22% of its activity in 5 days at 0°. By further purification (Fraction E5) the activity decreased still more rapidly. The activity could be stabilized to a certain extent by addition of serum albumin (0.1 mg/ml) to the enzyme preparation. Higher levels of serum albumin had no further stabilizing effect. The activity of enzyme fraction E6 decreased almost linearly with time at a rate of 6% per day during a 10-day period. Dilution of the enzyme preparations led to a more rapid inactivation.

Substrate specificities

The relative activities of the nucleotidase toward a number of nucleotides and other phosphate esters are shown in Table IV. It can be seen that the enzyme exerts high activity against the 5'-phosphates of dThd, dUrd, and dGuo. The other deoxynucleoside 5'-phosphates and the 5'-ribonucleotides were only slightly or insigni-

TABLE IV

RELATIVE ENZYME ACTIVITY WITH VARIOUS SUBSTRATES

Fraction E6 (approx. 0.6 μg of enzyme protein) was used as enzyme source. The substrates were tested at 5 mM concentration under the conditions for assay of nucleotidase activity, except for *p*-nitrophenyl phosphate and glycerol-2-phosphate which were assayed at higher concentrations under slightly different conditions. The incubation conditions are specified in MATERIALS AND METHODS. The rate of dephosphorylation of the substrates is given relative to that of dThd-5'-*P*, which is assigned a value of 100.

Substrate	*Relative activity*	*Substrate*	*Relative activity*
dThd-5'-*P*	100	dThd-3',5'-P_2	159
dUrd-5'-*P*	108	dThd-3':5'-*P*	2
dGuo-5'-*P*	88	Urd-3':5'-*P*	2
dAdo-5'-*P*	18	Urd-2':3'-*P*	1
dCyd-5'-*P*	1	dThd-5'-*P*-*p*-nitrophenyl	0
5-Methyl-dCyd-5'-*P*	2	dThd-*P*-dThd	6
		Urd-*P*-Urd	13
Ino-5'-*P*	13	dThd-5'-*P*-*P*	0
Guo-5'-*P*	12	dThd-5'-*P*-*P*-*P*	4
Urd-5'-*P*	10		
Ado-5'-*P*	1	*p*-Nitrophenyl phosphate	30
Cyd-5'-*P*	2	Pyridoxal-5'-*P*	4
		Ribose-5-*P*	0
dThd-3'-*P*	79	Glycerol-2-*P*	0
Urd-3'-*P*	49	Glucose-6-*P*	0
Urd-2'-*P*	32		
Guo-3'-*P*	44		
Guo-2'-*P*	1		
Cyd-3'-*P*	4		
Cyd-2'-*P*	1		
Ado-3'-*P*	0		
Ado-2'-*P*	2		

ficantly degraded. Surprisingly, the enzyme showed relatively high activity toward certain 3′-nucleotides, *viz.* the 3′-phosphates of dThd, Urd, and Guo. Guo-2′-*P* was not significantly degraded in spite of the fact that Urd-2′-*P* was dephosphorylated relatively efficiently. Urd-2′-*P* should be free of contaminating isomers according to specifications given by P-L Biochemicals, Inc. No significant dephosphorylation of the 2′- and 3′-phosphates of Cyd and Ado was observed. dThd-5′-*P* and Urd-3′-*P* have been used as routine substrates for assay of the 5′- and 3′-phosphohydrolase activities, respectively.

The high rate of dephosphorylation of deoxythymidine 3′,5′-diphosphate might suggest that the enzyme shows a preference for the cleavage of the 3′-phosphate group in the presence of a 5′-phosphate substitution or *vice versa*. Should this be the case, the enzyme might be able to split off terminal 3′- or 5′-phosphate groups from polynucleotides. This possibility has not yet been investigated. The enzyme showed no significant activity toward cyclic 2′:3′- and 3′:5′-phosphates, dThd-5′-*P*-paranitrophenyl ester, dinucleoside phosphates of dThd and Urd, and di- and triphosphates of dThd. Sugar phosphates were inactive as substrates, but *p*-nitrophenyl phosphate was appreciably dephosphorylated.

Consideration of the structure of the nucleotides which are actively dephosphorylated by the enzyme indicates that an oxygroup in the 4-position of the pyrimidine and in the 6-position of the purine component is essential for significant enzyme activity. Moreover, deoxyribonucleotides are dephosphorylated more rapidly than ribonucleotides, particularly when the phosphate is attached at the 5′-position.

Purity of the enzyme

The enzyme was purified about 2000-fold from the 123 000 × *g* supernatant fluid, and during the course of this purification the ratios between the rate of dThd-5′-*P* dephosphorylation and the dephosphorylation of Urd-3′-*P* remained essentially constant (Table V). The ratio between dephosphorylation of dThd-5′-*P* and *p*-nitrophenyl phosphate seemed to reach a constant value after purification step 5 had been carried out. Moreover, the activity ratios remained constant in Fraction E6 which decayed to 20% of the initial activity during storage. The fact that glycerol-2-phosphate, sugar phosphates, and a number of nucleotides were not dephosphoryl-

TABLE V

RATIOS OF SPECIFIC ACTIVITIES

The enzyme fractions from two different purification experiments (1 and 2) were assayed for respective activities as described under MATERIALS AND METHODS. The amounts of enzyme protein used were as in Table I. PNPP, *p*-nitrophenyl phosphate.

Enzyme fraction	*dThd-5′-P: Urd-3′-P*		*dThd-5′-P: Glycerol-2-P*		*dThd-5′-P: PNPP*	
	1	2	1	2	1	2
E1	2.98	2.66	1.16	2.88	0.186	0.322
E2	2.08	2.16	1.35	2.86	0.532	0.904
E3	1.95	2.10	1.16	2.56	0.561	0.942
E4	1.98	2.02	6.61	2.47	2.23	1.21
E5	1.96	1.99	34.0	27.6	2.88	3.13
E6	1.95	1.95	∞	∞	3.02	2.97

ated by Fraction E6 (Table IV) shows that nonspecific phosphatase was absent and could not be responsible for dephosphorylation of *p*-nitrophenyl phosphate. Chromatography of the enzyme on DEAE-Sephadex and subsequent gel filtration on Sephadex G-100 (Steps 5 and 6 in the purification procedure) showed that the eluted enzyme activity for dThd-5'-*P*, Urd-3'-*P*, and *p*-nitrophenyl phosphate exactly coincided in a sharp peak in both experiments (Figs. 1 and 2). All in all, the evidence seems to suggest strongly that the dephosphorylating activities of Fractions E5 and E6 are associated with the same protein.

Molecular weight

A 2-ml aliquot of enzyme Fraction E5 containing 0.1 mg of serum albumin/ml was subjected to gel filtration on a 2.5 cm × 38 cm column of Sephadex G-100. The column was calibrated with the following marker proteins: Cytochrome *c*, mol. wt. 12 400; horse myoglobin, mol. wt. 17 800; chymotrypsinogen A, mol. wt. 25 000; egg albumin, mol. wt. 45 000; bovine serum albumin, mol. wt. 67 000. The proteins showed close adherence to linear relationship between partition coefficient and log molecular weight[6]. From the standard curve obtained the partition coefficient of the nucleotidase indicated a mol. wt. of 45 000.

Kinetics

The amount of phosphate released from dThd-5'-*P* and Urd-3'-*P* was proportional with incubation time and with amount of enzyme in the ranges investigated (up to 30 min; up to 4 enzyme units measured with dThd-5'-*P* as substrate).

Effect of pH on enzyme activity

The nucleotidase exhibited a sharp activity optimum between pH 6.0 and 6.1 when dThd-5'-*P* was used as substrate (Fig. 4). The same pH optimum, although

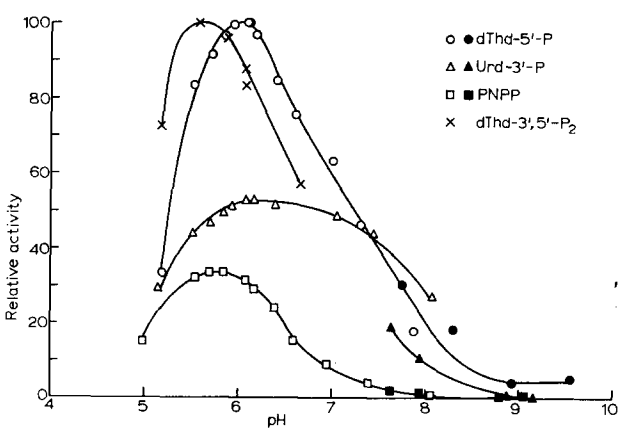

Fig. 4. Effect of pH on enzyme activity. Fraction E5 or E6 (1.1 or 0.5 μg of enzyme protein, respectively) was used as enzyme source and was measured with the substrates shown in the figure under assay conditions specified in MATERIALS AND METHODS, except that pH was varied as indicated. Open symbols, Tris–maleate buffer; closed symbols, glycine buffer. pH was adjusted to the desired value with NaOH and was measured at 20° in the complete incubation mixture. PNPP, *p*-nitrophenyl phosphate.

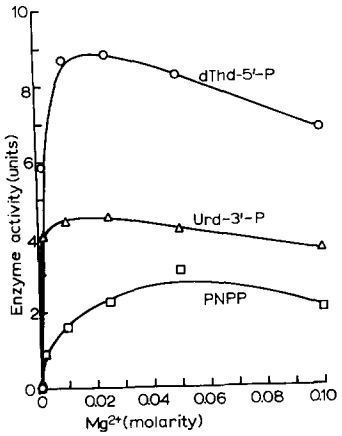

Fig. 5. Effect of Mg^{2+} on enzyme activity. The enzyme (Fraction E5) was dialyzed for 18 h against 2 l of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol. Activity (1.1 μg of enzyme protein) was measured with the substrates shown in the figure under the assay conditions specified in MATERIALS AND METHODS, except that Mg^{2+} was varied as indicated. PNPP, *p*-nitrophenyl phosphate.

slightly less sharp, was found with dUrd-5′-*P*, dGuo-5′-*P*, Guo-3′-*P* or Urd-2′-*P*. With Urd-3′-*P* a somewhat broader pH optimum around 6.2 occurred. The same pH dependence was observed with dThd-3′-*P* as substrate. With dThd-3′,5′-diphosphate optimal enzyme activity was at pH 5.6, and with *p*-nitrophenyl phosphate at pH 5.8.

Effect of Mg^{2+} on enzyme activity

The enzyme was completely inactive in the absence of Mg^{2+} with either dThd-5′-*P*, Urd-3′-*P* or *p*-nitrophenyl phosphate as substrate (Fig. 5). Optimal Mg^{2+} concentration was around 0.02 M with dThd-5′-*P* and Urd-3′-*P*, and was 0.05 M with *p*-nitrophenyl phosphate as substrate.

Effect of substrate concentration

Fig. 6 shows the relationship of substrate concentration to enzyme activity for four types of substrates, *viz.* dThd-5′-*P*, Urd-3′-*P*, dThd-3′,5′-P_2, and *p*-nitrophenyl

TABLE VI

APPARENT K_m VALUES FOR VARIOUS SUBSTRATES

The K_m values were estimated from Lineweaver-Burk plots based on activity measurements described in the legend to Fig. 6.

Substrate	K_m *(mM)*
dThd-5′-*P*	0.89
dUrd-5′-*P*	0.72
dGuo-5′-*P*	1.1
dThd-3′-*P*	0.16
Urd-3′-*P*	0.16
Guo-3′-*P*	0.24
dThd-3′,5′-P_2	0.36
p-Nitrophenyl phosphate	2.1

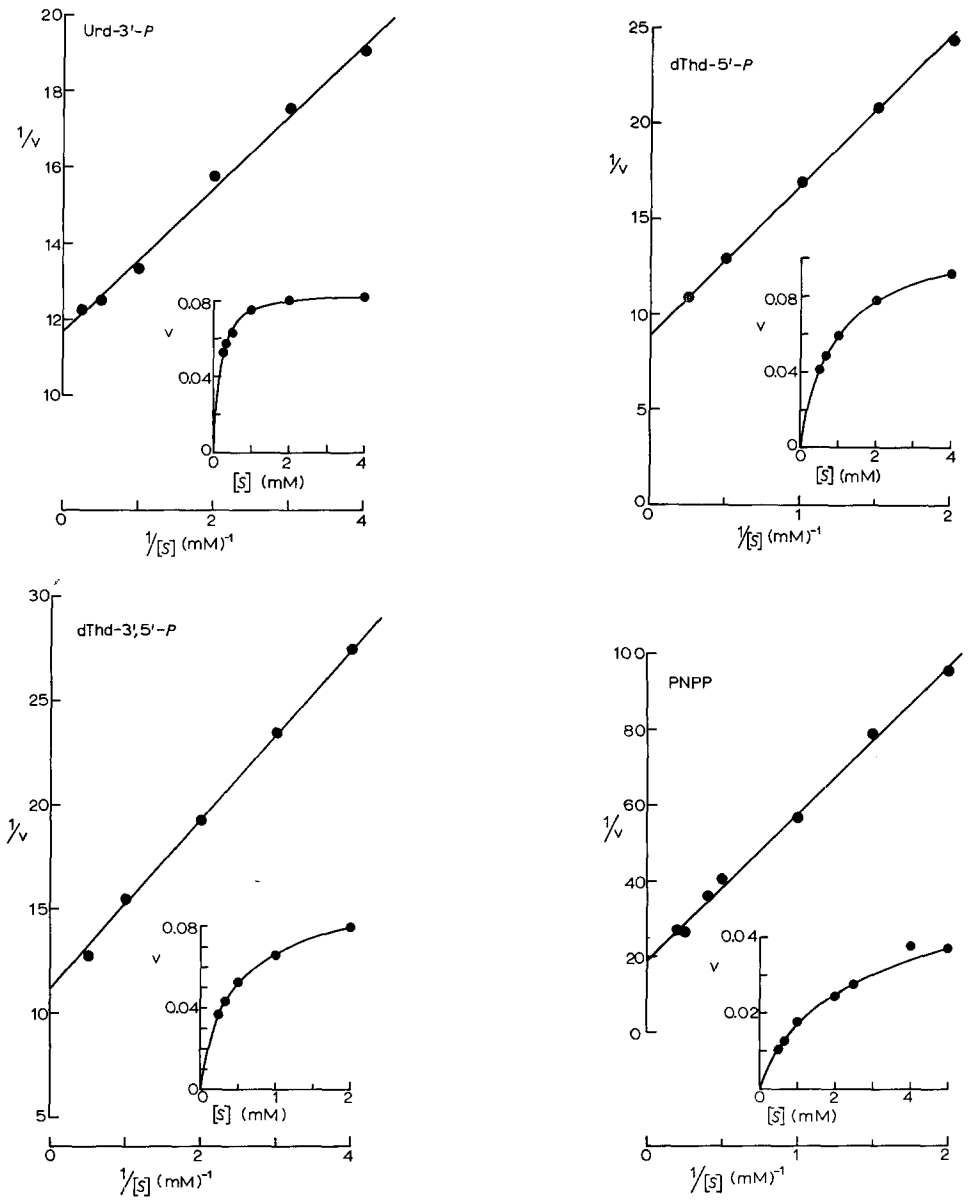

Fig. 6. Effect of substrate concentration on enzyme activity. The rate of dephosphorylation of the nucleotides and of *p*-nitrophenyl phosphate was measured under the assay conditions for nucleotidase and *p*-nitrophenyl phosphatase, respectively, as specified in MATERIALS AND METHODS, except that the substrate concentration [*S*] was varied as indicated. At each substrate concentration, incubations were carried out for 7.5 and 15 min in duplicate. The amount of enzyme (approx. 1.5 or 0.2 μg of enzyme protein from fraction E5 or E6, respectively) was adjusted so that less than 14% of the substrate was dephosphorylated. In all experiments P_i released was proportional with time over the assay period. The absorbance of the colorimetric assay of P_i (or *p*-nitrophenol) released in the 7.5-min enzyme reaction is used to express reaction velocity *(v)*. An absorbance of 0.01 corresponds to 1.65 nmoles or 1.46 nmoles of P_i released from nucleotides or *p*-nitrophenyl phosphate, respectively. PNPP, *p*-nitrophenyl phosphate.

phosphate. It can be seen that almost optimal enzyme activity was obtained with Urd-3'-*P* at concentrations above 2 mM, whereas higher concentrations were required for the other substrates to reach saturating levels. The apparent K_m values for different substrates were estimated from the double reciprocal plots according to LINEWEAVER AND BURK[7] and are quoted in Table VI. The 5'-nucleotides have consistently higher K_m values than the 3'-nucleotides. dThd-3',5'-diphosphate has K_m 0.36 mM and may kinetically be more closely related to the 3'-nucleotides than to the 5'-derivatives. It seems reasonable that the artificial substrate *p*-nitrophenyl phosphate, which happens to be degraded by the enzyme, has a higher K_m value than any of the other substrates.

Enzyme activity with combinations of two different substrates

Table VII shows that if two different 5'-nucleotides were present in the incubation mixture (Expts. 1 and 2), the enzyme activity was almost equal to that obtained with a single substrate. The small difference may be explained as a result of increased substrate level. Essentially the same was found when a mixture of two

TABLE VII

ENZYME ACTIVITY WITH COMBINATIONS OF TWO DIFFERENT SUBSTRATES

Fraction E6 (approx. 0.6 μg of enzyme protein) was used as enzyme source. The substrates were tested under the conditions for assay of nucleotidase activity, as specified in MATERIALS AND METHODS. The concentration of the 5'-nucleotides was 5 mM and that of the 3'-nucleotides 2.5 mM. The rate of dephosphorylation of single substrates and substrate combinations is given relative to that of dThd-5'-*P*, which is assigned a value of 100.

Expt. No.	*Substrate*	*% of rate of dThd-5'-P dephosphorylation*
1	dThd-5'-*P*	100
	dUrd-5'-*P*	108
	dThd-5'-*P* + dUrd-5'-*P*	125
2	dGuo-5'-*P*	88
	dThd-5'-*P* + dGuo-5'-*P*	101
3	Urd-3'-*P*	48
	dThd-3'-*P*	76
	Urd-3'-*P* + dThd-3'-*P*	76
4	Urd-3'-*P* + dThd-5'-*P*	180
5	dThd-3'-*P* + dThd-5'-*P*	243

different 3'-nucleotides was incubated (Expt. 3). However, if a combination of a 3'- and a 5'-nucleotide was present in the incubation mixture (Expts. 4 and 5), the enzyme activity exceeded the sum of the activities with the individual substrates. The excess of activity was 22% for Urd-3'-*P* *plus* dThd-5'-*P*, and was 38% for dThd-3'-*P* *plus* dThd-5'-*P*.

Nucleoside triphosphates such as Ado-5'-*P*-*P*-*P* and dThd-5'-*P*-*P*-*P* (not shown) did not exert any effect on the rate of dephosphorylation of dThd-5'-*P* or Urd-3'-*P*.

DISCUSSION

The nucleotidase reported in the present communication has a unique substrate specificity in that it degrades 5′-deoxyribonucleotides far more rapidly than 5′-ribonucleotides, in contrast to the 5′-nucleotidases studied previously in rat liver[1–3,8–11], other animal tissues[12–17], *Escherichia coli*[18], and plants[19]. A nucleotidase present in *Bacillus subtilis* after phage infection[20] showed significant activity only toward dThd-5′-*P*. The relative activities of the enzyme toward certain 3′-nucleotides further show that it is distinctly different from previously described enzymes with 3′-nucleotidase activity[21–30]. These facts clearly distinguish the present nucleotidase from other nucleotidases previously detected, and indicate that the nucleotidase is a new enzyme of rat liver.

The experimental evidence for identification of the 3′- and 5′-nucleotidase activities with the same protein is discussed under RESULTS, *Purity of the enzyme*. Additional evidence is provided by the finding that the P_i released in the presence of combinations of 3′- and 5′-nucleotides exceeded significantly the sum of the activities with single substrates (Table VII, Expts. 4 and 5). It would be difficult to explain this excess of activity if two enzymes were responsible for the dephosphorylations. If one accepts that the two activities are intrinsic properties of the same enzyme molecule, one of two possible consequences must follow: (1) The enzyme possesses two catalytic sites which degrade 3′- and 5′-nucleotides, respectively. Binding of substrate at one site induces a conformational change of the enzyme molecule, resulting in increased activity of the other site. The phenomenon of two catalytic sites in enzymes related to nucleotide degradation has been reported for bacterial cyclic phosphodiesterases having 3′-nucleotidase activity[24,31,32]. (2) The enzyme possesses an allosteric site at which a 5′- or 3′-nucleotide can bind and activate the dephosphorylation of a 3′- or 5′-nucleotide, respectively. The validity of the hypotheses will be investigated by means of isotopically labeled nucleotides.

The activity of the nucleotidase toward *p*-nitrophenyl phosphate is surprising in view of the difference in structure between this compound and the nucleotides. It is possible that the activity may be related in some way to the 3′-nucleotidase activity of the enzyme. Thus, it has been reported[27,33] that phosphodiesterases with 3′-nucleotidase activity also exerted activity toward *p*-nitrophenyl phosphate. It may be significant that this enzyme also possessed two substrate-binding sites[24,31,32]. The observations may point out the uncertainty of using dephosphorylation of *p*-nitrophenyl phosphate as a measure of nonspecific phosphatase activity.

The physiological significance of the enzyme is not known, although its specificity for a limited number of 3′- and 5′-nucleotides and the possible existence of allosteric regulation of its activity suggest that it may have some function in the control of nucleotide metabolism. The enzyme resembles in some respects a bacterial 3′-nucleotidase described by BECKER AND HURWITZ[25], which catalyzed the selective removal of terminal 3′-phosphoryl groups from DNA. This enzyme also hydrolyzed deoxythymidine 3′,5′-diphosphate and deoxythymidine 3′-phosphate. Although its substrate specificity was otherwise somewhat different from that of the present enzyme, the possibility appears that our nucleotidase exerts activity toward phosphate termini of polynucleotides. The relatively high K_m values of the substrates tested so far could suggest that the true substrate has not yet been found. On the

other hand, the K_m values recorded in the present paper were determined with only one substrate present in the incubation mixture. Our data indicate that the simultaneous presence of 3'- and 5'-nucleotides will influence the kinetic characteristics of the enzyme. However, should the enzyme turn out to hydrolyze phosphate termini from polynucleotides, it might be expected to have a direct influence on the DNA template activity. It is well known that a 3'-phosphate end in DNA is a potent inhibitor of DNA polymerase, and that hydrolytic removal of this 3'-phosphoryl group is essential for the priming function of the DNA chain[34].

Previous observations indicate that the enzyme undergoes activity variations in growing tissues, at least when the growth stimulus is a carcinogen. Thus, a temporary and striking increase in dephosphorylation of dThd-5'-*P* at pH 6 occurred in the 105 000 × *g* supernatant fluid of rat liver homogenates during induction of carcinogenesis by 2-acetylaminofluorene[35,36]. A slight increase in activity level also seemed to occur during liver regeneration[1]. In fact, these observations prompted the search for the present enzyme[2]. The importance of further investigations on the biological role of the enzyme seems to be indicated.

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