Biochimica et Biophysica Acta, 438 (1976) 540—550

BBA 67854

NOVEL ACTIVITY OF POTATO NUCLEOTIDE PYROPHOSPHATASE

R. KOLE, HALINA SIERAKOWSKA and D. SHUGAR

Institute of Biochemistry and Biophysics, Academy of Sciences, 02-532 Warszawa (Poland)

(Received November 11th, 1975)

Summary

The classical Kornberg-Pricer procedure for purification of potato nucleotide pyrophosphatase (EC 3.6.1.9) has been modified to yield a preparation purified 2500-fold.

In addition to the known activity against pyrophosphate linkages in pyrophosphates located at the 5′-OH of nucleosides, and phosphodiester linkages in aryl esters of nucleoside-5′-phosphates, the enzyme has now been shown to catalyze the cleavage of: (a) aryl esters of nucleoside-3′-phosphates and orthophosphates, (b) nucleotide pyrophosphate linkages of the type (3′)-pp-(3′), and (c) pm^7G from m^7GpppG^m-terminated fragments of viral mRNA.

Activities against aryl esters of nucleoside-3′- and 5′-phosphates, and NAD, were shown to be due to the same protein by three criteria: (a) constant ratio of activities during purification and gel electrophoresis, (b) identical chromatographic properties in various systems, and (c) similarities in pH-dependence, heat inactivation, and the effects of cations and other substances.

Since potato nucleotide pyrophosphatase does not exhibit exonuclease or phosphatase activities against natural substrates for the latter enzymes, but does cleave synthetic aryl esters of nucleotide-3′- and 5′-phosphates and of orthophosphate, it follows that these substrates are not suitable for detection of such activities in higher plants.

Introduction

Nucleotide pyrophosphatase (EC 3.6.1.9), partially purified by Kornberg and Pricer [1] from potato tubers, has since been reported in a number of high-

Please adress correspondence to: Dr. R. Kole, Institute of Biochemistry and Biophysics, Academy of Sciences, 36 Rakowiecka St., 02-532 Warszawa, Poland.

Abbreviations: T*p*-nitrophenyl, thymidine-3′-(*p*-nitrophenylphosphate); nitrophenyl-*p*T, thymidine-5′-(*p*-nitrophenylphosphate); T*p*-naphthyl, thymidine-3′-(α-naphthylphosphate); naphthyl-*p*T, thymidine-5′-(α-naphthylphosphate).

er plants [2—4]. The enzyme is known to exhibit activity against the pyrophosphate linkage in nucleotide coenzymes and nucleoside di- and triphosphate [1] as well as the phosphodiester linkage in thymidine 5′-(*p*-nitrophenylphosphate) [2,5]. In the course of localization studies on a phosphodiesterase in wheat shoots active at a neutral pH, we observed an activity displaying the unique property of cleaving phosphodiester linkages in aryl esters of both thymidine 5′- and 3′-phosphates at comparable rates. Since other features of the wheat activity resembled those of potato nucleotide pyrophosphatase, it was considered of interest to purify the latter, and to study its specificity, principally with reference to its ability to cleave at comparable rates aryl esters of 3′- and 5′-nucleotides and orthophosphate, since such a property has not previously been noted with a single enzyme.

Materials

The various substrates employed were obtained from sources or by methods as indicated in the references and footnotes listed in Table II. Highly polymerized yeast RNA was obtained from Lodz Medical School (Lodz, Poland).

We are indebted to Dr. A.J. Shatkin (Roche Institute of Molecular Biology, Nutley, N.J., U.S.A.) for reovirus [*Me*-^{3}H]mRNA, reovirus [^{32}O]mRNA and [*Me*-^{3}H]m^{7}GpppG^m; and to Dr. M. Piechowska of this Institute for *Bacillus subtilus* [^{3}H]DNA, which was denatured by heating and rapid cooling.

Pancreatic ribonuclease A, phosphocellulose and α-methyl-D-mannoside were purchased from Sigma (St. Louis, Mo., U.S.A.); ribonuclease T_1 from Calbiochem (San Diego, Calif., U.S.A.) and *Escherichia coli* alkaline phosphatase, BAPF, from Worthington (Freehold, N.J., U.S.A.). Concanavalin A-Sepharose, Sephadex G-200, and CM- and SE-Sephadex were obtained from Pharmacia (Uppsala, Sweden); DE-52 cellulose from Whatman (Maidstone, England); hydroxyapatite and materials for gel electrophoresis from Bio-Rad (Richmond, Calif., U.S.A.). Sepharose substituted with *O*-(4-aminophenyl)-*O*′-phenyl-thiophosphate [6] and ω-aminohexyl-Sepharose [7] were kindly donated by Dr. F. Eckstein (Max-Planck-Institut für Experimentalle Medizin, Gottingen, Germany) and Dr. H. Jakubowski (Institute of Biochemistry, College of Agriculture, Poznan, Poland), respectively. Calcium phosphate gel was prepared according to Keillin and Hartree [8]. All other reagents were of analytical grade.

Methods

Purification of enzyme.

All steps were carried out at 6° C, starting with 20 kg of a local variety of potato. Steps 1—4 (Table I) were essentially those of Kornberg and Pricer [1].

Step 5. 50 ml (342 mg protein) of the enzyme solution from step 4 was brought to 250 ml with 0.05 M acetate buffer pH 6.0 and applied to a 45 × 2.5 cm column of CM-Sephadex previously equilibrated with the same buffer. The column was washed with the same buffer to remove non-adsorbed protein, and elution then carried out with 1.2 l of a linear gradient formed between equal volumes of equilibrating buffer and the same buffer containing 0.6 M KCl, at a flow rate of 25 ml/h. Fractions eluted between 0.15 M and 0.35 M KCl were

TABLE I

PURIFICATION SCHEME FOR POTATO NUCLEOTIDE PYROPHOSPHATASE

Purification step	Volume (ml)	Total activity (units)	Protein (mg)	Specific activity (units/mg protein)	Yield (%)	Ratio of hydrolysis rates	
						NAD nitrophenyl-*p*T	NAD T*p*-nitrophenyl
Aqueous extract	—	—	—	0.02	—	—	—
(1) 40% saturated ammonium sulfate extract	30 000	3657	108 700	0.03	100	2.8	5.5
(2) Ammonium sulfate to 80% saturation	1 570	2560	39 300	0.07	70	3.0	5.6
(3) Calcium phosphate I	200	2283	7 740	0.83	62	3.0	5.5
(4) Calcium phosphate II	50	772	342	2.3	21	2.9	5.7
(5) CM-Sephadex	130	557	101	5.5	15	2.9	5.8
(6) DE-52 cellulose	20	472	29	16.3	13	2.9	5.6
(7) Concanavalin A-Sepharose	6	197	3.9	50.3	5	3.0	5.8

pooled, concentrated to 130 ml, and dialyzed against 0.02 M Tris · HCl buffer pH 7.2 in an Amicon instrument with a Diaflo UM-10 membrane.

Step 6. 130 ml of the foregoing dialyzate was loaded on a DE-52 cellulose column (27 × 1 cm) pre-equilibrated with 0.02 M Tris · HCl buffer pH 7.2, at a flow rate of 18 ml/h. Most of the activity was recovered in the breakthrough and subsequent washing with the same buffer, total eluate 140 ml. This was concentrated to 20 ml and dialyzed against 0.2 M acetate buffer pH 6.0 as above.

Step 7. 20 ml (29 mg protein) of the enzyme solution from step 6 was applied to a Concanavalin A-sepharose column (12 × 2 cm) pre-equilibrated with 0.2 M acetate buffer pH 6.0, and the column washed with this buffer to remove non-adsorbed protein. The enzyme was then eluted with 60 ml of 0.1 M α-methyl-D-mannoside in 0.2 M acetate buffer pH 6.0 at a flow rate of 1 ml/3 min, with collection of 2-ml fractions. The eluate was pooled, concentrated to 6 ml as above and stored as such at 4° C, with no loss of activity over a period of 2 months.

Assays of enzyme activity

The enzyme obtained from step 7 was employed throughout for specificity and rate studies, incubations being for 30 min at 37° C. The reaction rate was linear with time under these conditions. One unit of nucleotide pyrophosphatase activity is defined as the amount of enzyme which liberates 1 μmol *p*-nitrophenol from thymidine-5′-(*p*-nitrophenylphosphate) in 1 min at 37° C.

Activity against unlabelled RNA was tested for as described by Bardon et al. [9].

Activity against labelled heat denatured DNA, reovirus mRNA and m^7GpppG^m was measured in an incubation volume of 25 μl 0.08 M Tris · HCl buffer 7.0 containing $3 \cdot 10^{-3}$ units of nucleotide pyrophosphatase, and one of the following substrates: (1) [*Me*-^{3}H]m^7GpppG^m, 2000 cpm; (2) 0.14 μg [*Me*-^{3}H]-mRNA, 2000 com; (3) 0.14 μg [*Me*-^{3}H]mRNA, 2000 cpm, predigested for 30 min with 25 μg RNAase T_1; (4) 0.14 μg [*Me*-^{3}H]mRNA, 2000 cpm, predisgested for 30 min with 12 μg RNAase A; (5) 0.07 μg [^{32}P]mRNA, 2000 cpm; (6) 0.04 μg [^{3}H]DNA, 8000 cpm. Controls without enzyme were run for each experiment. Following incubation, 20 μl was either (a) spotted on Whatman GF/C paper and the quantity of trichloroacetic acid-precipitable material determined by the method of Bollum [10], or (b) spotted on Whatman No. 1 paper and developed with isobutyric acid/0.5 M NH_4OH (10 : 6, v/v) with unlabeled pm^7G (R_f 0.52) added as internal standard. The chromatogram were dried, cut into 15-mm strips, and counted in a toluene-based scintillation fluid.

Pyrophosphatase activity against NAD was assayed according to the method of Kornberg and Pricer [1], except that 0.5 M Tris · HCl buffer pH 7.0 replaced the 0.5 M phosphate buffer and the volume of the incubation mixture was reduced to 0.5 ml.

For the other low-molecular-weight substrates the rates of hydrolysis were determined by incubation of 0.1 ml of 5 mM substrate in 0.1 M Tris · HCl buffer pH 7.0 with the appropriate amount of enzyme. For thymidine-(3′)-pp-(3′)-thymidine, following incubation with nucleotide pyrophosphatase, the sample was incubated for an additional 10 min with 1.5 unit alkaline phosphatase in 0.2 ml 0.2 M Tris · HCl buffer pH 9.0. The enzymatically liberated phosphate

was assayed, as in other cases, according to the method Lowry and Lopez [11]. The α-naphtol liberated from naphthyl esters was assayed as described in ref. 12 and the *p*-nitrophenol liberated from the *p*-nitrophenyl esters according to the method of Razzell and Khorana [13].

The kinetic parameters K_m, V and K_i were determined by the method of Lineweaver and Burk [14].

Heat inactivation

This was performed by immersion of the enzyme solution, in 0.2 M acetate buffer pH 6.0, in a water bath at 75° C. Aliquots were withdrawn after 5, 10 and 15 min, cooled in an ice-bath and assayed.

Polyacrylamide gel electrophoresis

This was performed at pH 4.5 as described by Gabriel [15], with 1.7 units (20 μg protein) of the enzyme preparation applied per gel. Gels were stained for protein with Coomassie brilliant blue [16]. Enzyme activity against α-naphthyl esters was localized in the gels by incubation of the gel columns for 1 h at 37° C in 3 ml medium containing 2 μmol substrate and 3 mg Fast Garnet GBC in 0.1 M acetate buffer pH 5.2.

Protein was determined according to the procedure of Lowry et al. [17].

Results

Initial attempts to purify potato nucleotide pyrophosphatase were based on the procedure originally described by Kornberg and Pricer [1]. It was only when we noted the broader substrate specificity of the enzyme, described below, that efforts were directed towards a more highly purified preparation for further studies.

The purification scheme, which includes the first four steps from Kornberg and Pricer [1], and illustrated in Table I, led to an increase in purification from the previous 750-fold to 2500-fold, with an overall yield of 5%. Additional attempts at simplification of the procedure have hitherto not given satisfactory results. In particular, affinity chromatography on Sepharose substituted with *O*-(4-aminophenyl)-*O'*-phenyl-thiophosphate was accompanied by irreversible adsorption of the enzyme; while the use of chromatography on Concanavalin A-Sepharose in the early stages of purification gave only a low increase in specific activity.

The enzyme exhibited activity against NAD, the *p*-nitrophenyl esters of 5′ and 3′-thymidylic acids, bis-*p*-nitrophenylphosphate and *p*-nitrophenylphosphate. As can be seen from the two last columns in Table I, the rates of hydrolysis of nitrophenyl-*p*T and T*p*-nitrophenyl, each measured with reference to NAD as standard, were found to be reasonably constant throughout all purification steps, pointing to the activity against these substrates being a property of one protein. To test this further, the enzyme obtained after step 4 was subjected to column chromatography on Sephadex G-200, SE-Sephadex, phosphocellulose, hydroxyapatite, ω-aminohexyl-Sepharose [7] and Sepharose substituted with *O*-(4-aminophenyl)-*O'*-phenyl-thiophosphate [6]; none of these led to separation of activities against the aforegoing three substrates.

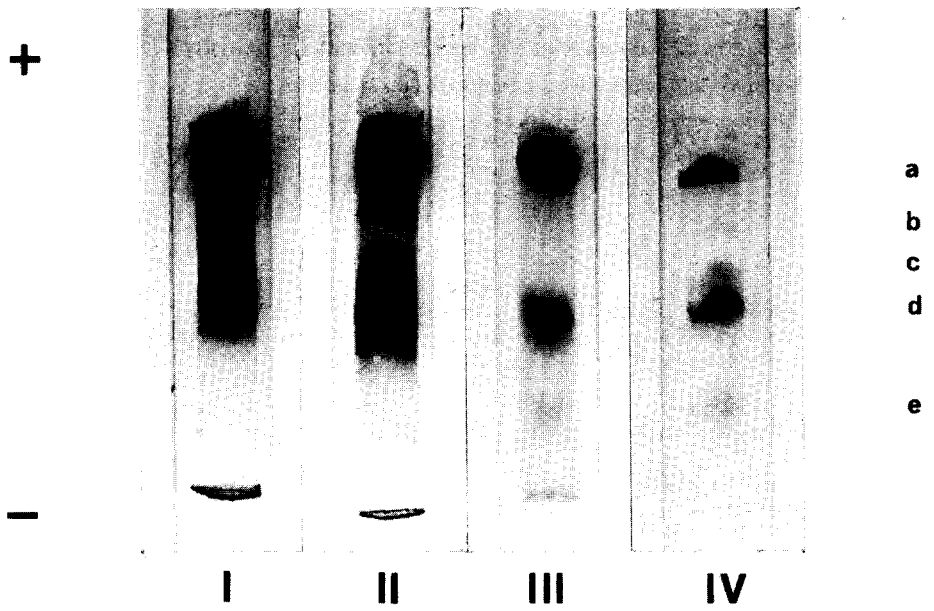

Fig. 1. Polyacrylamide gel electrophoresis of purified potato nucleotide pyrophosphatase, showing activities against: (I) naphthyl-*p*T, (II) T*p*-naphthyl, (III) α-naphthylphosphate, and (IV), staining for protein.

Polyacrylamide gel electrophoresis similarly failed to separate these activities. Gel electrophoresis of the final enzyme preparation at pH 4.5 gave 2 major protein band (Fig. 1, panel IV, a and b) and three minor ones (Fig. 1, panel IV, b, c and e). Bands a, b and d exhibited activities against the α-naphthyl esters of 3′- and 5′-thymidylic acid (Fig. 1, panels I and II). Band c, which partially merges with d, can occasionally be resolved upon incubation with these substrates. Bands and and d exhibited activity against α-naphthylphosphate (Fig. 1 panels I and II). On prolonged incubations with α-naphthylphosphate somewhat diffuse bands corresponding to b and c become detectable. Combined extracts from bands a plus b, and c plus d, cleaved NAD, the *p*-nitrophenyl and α-naphthyl esters of 5′- and 3′-thymidylic acid and of orthophosphate, the ratio of these activities being equal to that in the enzyme preparation prior to electrophoresis. Moreover, repeated electrophoresis of extracts from band c plus band d upon incubation with α-naphthyl esters of thymidine 3′ and 5′-phosphates again gave the same picture as in Fig. 1, panels I and II, suggestive of enzyme aggregation. Aggregation has also been observed on concentration of the enzyme with Aquacide I, the aggregate failing to penetrate the gel in electrophoresis.

The aforegoing results indicate that bands a, b, c and d are due to one protein. Band e (see Fig. 1, IV) can be ascribed to contaminating protein.

Specificity

Table II gives the rates of hydrolysis of various compounds by potato nucleotide pyrophosphatase. Nucleotide coenzymes such as FAD, NAD, NADP etc. have already been studied and found to be cleaved by the enzyme [1]; hence only NAD and ATP were included in this study for purpose of reference. As

TABLE II

POTATO NUCLEOTIDE PYROPHOSPHATASE ACTIVITY TOWARDS VARIOUS SUBSTRATES

Figures in brackets are references to preparative procedures for the substrates

Substrate	Rate μmol substrate hydrolyzed/mg protein/30 min at 37°C	V	K_m (mM)
NAD *	4300		
Thymidine-(3′)-pp-(3′)-thymidine [18]	90		
Thymidine-5′-(p-nitrophenylphosphate) **	1500	2400	0.8
Thymidine-3′-(p-nitrophenylphosphate) ***	800	1300	1.3
Bis-p-nitrophenylphosphate *	3200	6800	1.3
Thymidine-5′-(α-naphthylphosphate) **	14	22	2.5
Thymidine-3′-(α-naphthylphosphate) [12]	19	30	2.9
Bis-α-naphthylphosphate [19]	13		
Uridine-3′-(α-naphthylphosphate) [20]	5		
Uridine-3′-(phenylphosphate) [21]	8		
Adenosine-3′-(α-naphthylphosphate) [22]	3		
5′-ATP *	400		
5′-ADP *	300		
5′-AMP *	11		
3′-AMP *	11		
2′-AMP *	3		
5′-UMP *	7		
3′-UMP *	7		
p-Nitrophenylphosphate *	700	1200	1.2
α-Naphthylphosphate †	26		
Glucose 6-phosphate †	2		
β-Glycerophosphate *	2		
RNA	≪2		

* Sigma (St. Louis, Mo., U.S.A.).
** Merck (Darmstadt, West Germany).
*** Raylo (Edmonton, Alta., Canada).
† Schuchardt (Munchen, West Germany).

can be seen from Table II, the enzyme cleaves not only the pyrophosphate linkages at nucleoside 5′-phosphates but also those at nucleoside 3′-phosphates (T(3′)T). It also liberates aryl substituents from esters of nucleoside 3′- and 5′-phosphates and of orthophosphate.

The rate of cleavage of NAD, a (5′)pp(5′)-type pyrophosphate, appreciably exceeds that of all other substrates, including T(3′)pp(3′)T. The rate of hydrolysis of diesterified phosphate depends mainly on the leaving tendency of the substituent, the *p*-nitrophenyl esters are hydrolyzed from 40 to 250-fold more rapidly than the corresponding α-naphthyl esters (See also V values in Table II).

The rate of cleavage of monoesterified phosphate likewise depends on the nature of the substituent, *p*-nitrophenylphosphate being hydrolyzed 28 times more rapidly than α-naphthylphosphate. Nucleoside 2′-, 3′- and 5′-phosphates, β-glycerophosphate and glucose 6-phosphate are hydrolyzed slowly (see first column of Table II) and it is not certain whether this is due to nucleotide pyrophosphatase activity, or to contamination of the preparation with traces of phosphomonoesterase activity (see Fig. 1, III—IV, band e).

The data in Table II show that the K_m values for the various esters of nucleotides and orthophosphate fall within a range of one order of magnitude, and are comparable to those reported by Kornberg and Pricer [1] for NADO, ATP and thiamine pyrophosphate. Only NAD exhibits appreciably higher affinity for the enzyme, with a K_m = 0.15 mM.

Hydrolysis of pyrophosphate bond in mRNA

In view of the finding that the 5′-termini of viral and eukaryotic mRNA (ref. 23 and papers quoted therein), as well as of some types of small nuclear RNA [24], include a 5′-5′-pyrophosphate linkage to methylated guanosine, it became of interest to establish whether such a linkage is susceptible to potato nucleotide pyrophosphatase.

Using paper chromatography to follow the course of the reaction (see Methods), the enzyme was found to release [*Me*-^{3}H]pm^{7}G (R_f = 0.52) from m^{7}GpppG^m (R_f = 0.33), from m^{7}GpppG^m-terminated reovirus mRNA (R_f = 0), from m^{7}GpppG^m-terminated reovirus mRNA predigested with excess RNAase A (R_f = 0.16), and from m^{7}GpppG^m-terminated reovorus mRNA predigested with excess RNAase T_1 (R_f = 0.16), at relative rates of 1.0, 0.2, 0.5 and 0.8, respectively. From these results it appears that even relatively large oligonucleotide fragments attached to the m^{7}GpppG^m terminus do not abolish the activity of the enzyme towards the pyrophosphate linkage.

However, closer examination demonstrated that, although our purified enzyme preparation was inactive against unlabelled RNA, it exhibited detectable endonucleolytic activity against reovorus [^{32}P]mRNA and denatured *B. subtilis* [^{3}H]DNA. It consequently proved impossible to establish unequivocally whether to potato enzyme is able to cleave the pyrophosphate linkage in intact viral mRNA. Since (a) up to 12% of total label from [*Me*-^{3}H]mRNA was released as pm^{7}G without concomitant appearance of fragments smaller than m^{7}GpppG^m-terminated tetranucleotides, and (b) release of as much as 43% of total label as pm^{7}G preceded the disappearance of labelled material from the start on the chromatogram, it is clear that the enzyme acts on fragments at least appreciably larger than m^{7}GpppG^m-terminated tetranucleotides.

Other properties

The pH-dependence of the nucleotide pyrophosphatase activity towards nitrophenyl-*p*T, T*p*-nitrophenyl and *p*-nitrophenylphosphate was similar for all three substrates, the optimal pH covering the range 5—7. The kinetics of heat inactivation of the enzyme were also similar for the different substrates, e.g. 5 min heating at 75° C led to a decrease in activity of 55% towards NAD, nitrophenyl-*p*T, T*p*-nitrophenyl and *p*-nitrophenylphosphate; while 15 min heating at the same temperature completely liquidated activity towards all four substrates.

The effects, on nucleotide pyrophosphatase activity, of $MgCl_2$, $CuCl_2$, $HgCl_2$, EDTA and 2-mercaptoeethanol at concentrations of 10 mM, and of *p*-chloromercuribenzoate and iodoacetate at levels at 1 mM, were identical in assays against nitrophenyl-*p*T, T*p*-nitrophenyl and *p*-nitrophenylphosphate. Only Cu^{2+} and Hg^{2+} were found to be inhibitory, the extent of inhibition being 45% and 25%, respectively, against all three substrates. The remaining compounds were

without effect on activity, irrespective of the substrate. The absence of inhibition by EDTA and Mg^{2+} is in agreement with analagous data of Razzell [2] for activity against nitrophenyl-*p*T.

Attention was then directed to the effect of 5′-AMP, 3′-AMP and P_i on enzyme activity against the foregoing three substrates. 5′-AMP proved to be a much more effective inhibitor (K_i = 0.5 mM) of activity against nitrophenyl-*p*T than 3′-AMP (K_i = 1 mM) and P_i (K_i = 4 mM). This is in agreement with a previous report (1) on inhibition of nucleotide pyrophosphatase activity against NAD by 5′-AMP, but not by 3′-AMP or other phosphate esters. Furthermore, 3′-AMP and P_i were more effective inhibitors of activity against T*p*-nitrophenyl (K_i = 1.4 and 0.3 mM, respectively) and *p*-nitrophenylphosphate (K_i = 0.7 and 0.4 mM, respectively) than against nitrophenyl-*p*T. This points to some differences in the mechanism of cleavage of these substrates but does not warrant postulating the existence of distinct active centers.

Discussion

The aforegoing results show that purified potato nucleotide pyrophosphate exhibits activity towards pyrophosphate linkages in pyrophosphate groups at the 3′- and 5′-OH of nucleosides and towards phosphodiester linkages in synthetic aryl esters of nucleoside 5′- and 3′-phosphates and of orthophosphate.

Using selected examples of each group, activities against these substrates were shown to be due to one protein by: (a) a constant ratio of activities throughout purification and following gel electrophoresis; (b) co-chromatography of the activities in various systems; and (c) similarities in dependence on pH, heat inactivation, and effects of cations and other substances.

In connection with these results, it should be noted that an apparently homogenous preparation of yeast nucleotide pyrophosphates has also been reported to exhibit activity against *p*-nitrophenyl esters of both thymidine 5′- and 3′-phosphates, and bis-*p*-nitrophenylphosphate [25]. The ratio of these activities, however, was 1 : 0.01 : 0.1, respectively, as compared to 1 : 0.5 : 2.1 for potato nucleotide pyrophosphatase, pointing to appreciable differences in the degree of specificity of the two enzymes.

Potato nucleotide pyrophosphatase activity against synthetic substrates is seen to depend primarily on the acidity of the phosphate substituent. Such activity, resulting from a mixed phosphoanhydride type linkage between the phosphoryl residue and its acidic substituent [26], is also known for several other enzymes [5,25—27] and is probably of little metabolic significance.

It is worth noting that esters of nucleoside 3′- and 5′-phosphates, in particular their *p*-nitrophenyl derivatives, have been considered specific substrates for various types of exonuclease [28]. Their specificity has been questioned for bacterial systems [26] and, to some extent, for mammalian ones [27]. Such reservations also appear to be valid with regard to higher plants [5,25,29]. The present results show that esters of both nucleoside 3′- and 5′-phosphates are cleaved by potato nucleotide pyrophosphatase, an enzyme probably devoid of activity against 3′ → 5′ internucleotide linkages (Table II and ref. 5), and hence not a true phosphodiesterase.

Similar reservations may be entertained with regard to the specificity of *p*-

nitrophenylphosphate as a substrate for plant phosphomonoesterases. This substrate is readily cleaved by potato nucleotide pyrophosphatase although the enzyme exhibits negligible, if any, activity against a number of other phosphate esters. Doubts concerning the inherent nature of phosphatase activity in nucleotide pyrophosphatase preparations have already been expressed by Kornberg and Pricer [1]. Regardless of whether nucleotide pyrophosphatase actually exhibits slight phosphatase activity or is contaminated with it, it is evident that in higher plants cleavage of *p*-nitrophenylphosphate cannot be considered as proof of presence of phosphatase activity.

The activity of nucleotide pyrophosphatase against such synthetic substrates as nitrophenyl-*p*T, bis-*p*-nitrophenylphosphate and *p*-nitrophenylphosphate raises doubts as to the validity of previous reports of acid phosphodiesterase activity in oat leaves [30] and phosphodiesterase-phosphatase activity in barley seedlings [31]. Both these activities may have been due, at least in part, to nucleotide pyrophosphatase.

Because of the traces of endonucleolytic activity in our preparation, we have been unable to establish whether nucleotide pyrophosphatase is able to cleave the pyrophosphate linkage in intact mRNA. We may, nonetheless, conclude that the enzyme, which does readily hydrolyze m^7GpppG^m itself to completion, exhibits pyrophosphatase activity towards fragments larger than m^7GpppG^m-terminated tetranucleotides. It therefore resembles the enzyme from HeLa cells which cleaves specifically pm^7G from m^7GpppN^m-terminated oligonucleotide up to 10 nucleotides in length [32]. If it were found feasible to remove the residual endonuclease activity from our purified preparation, one could then examine the activity against intact viral mRNA in the hope that it might prove a useful tool for studying the role of the 5'-terminus of viral and eukaryotic mRNA.

Acknowledgments

We should like to express our appreciation to Dr. A.J. Shatkin for gifts of labelled reovirus mRNA and m^7GpppG^m, as well as useful discussions. We are also indebted to Dr. F. Eckstein for Sepharose substituted with *O*-(4-aminophenyl)-*O'*-(phenylthiophosphate), to Dr. H. Jakubowski for ω-aminohexyl-Sepharose, to Dr. M. Zan-Kowalczewska for uridine-3'-(phenylphosphate), to K. Lang for preparation of thymidine-(3')-pp-(3')-thymidine, and to Mrs. Halina Szemplinska for competent technical assistance. This investigation was carried out as Project 09.3.1 of the Polish Academy of Sciences, and profited also from the partial support of the Agricultural Research Service, U.S. Department of Agriculture (FG-Po-307).

References

1 Kornberg, A. and Pricer, W.E. (1950) J. Biol. Chem. 182, 763—778
2 Razzell, W.E. (1966) Biochem. Biophys. Res. Commun. 22, 243—247
3 Clayton, R.A. and Hanselman, L.M. (1960) Arch. Biochem. Biophys. 87, 161—166
4 Roberts, D.W.A. (1959) J. Biol. Chem. 234, 655—657
5 Razzell, W.E. (1968) Can. J. Biochem. 46, 1—7
6 Frischauf, A.M. and Eckstein, F. (1973) Eur. J. Biochem. 32, 479—485
7 Jakubowski, H. and Pawelkiewicz, J. (1973) FEBS Lett. 34, 150—154

8 Keilin, D. and Hartree, E.K. (1938) Proc. R. Soc. Lond. B 124, 397—403
9 Bardon, A., Sierakowska, H. and Shugar, D. (1976) Clin. Chim. Acta 67, 231—244
10 Bollum, F.J. (1966) in Procedures in Nucleic Acid Research (Cantoni, G.L. and Davies, D.R., eds.), pp. 296—300, Harper and Row, New York
11 Lowry, O.H. and Lopez, J.A. (1946) J. Biol. Chem. 162, 421—426
12 Sierakowska, H. and Sjugar, D. (1971) Acta Biochim. Polon. 18, 143—152
13 Razzell, W.E. and Khorana, H.G. (1961) J. Biol. Chem. 236, 1144—1149
14 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658
15 Gabriel, O. (1971) in Methods in Enzymology (Jakoby, W.B., ed.), Vol. 22, pp 565—578, Academic Press, New York
16 Hoffstetter, H., Monstein, H.J. and Weissman, C. (1974) Biochim. Biophys. Acta 374, 238—251
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Turner, A.F. and Khorana, H.G. (1959) J. Am. Chem. Soc. 81, 4651—4656
19 Friedman, O.M. and Seligman, A.M. (1950) J. Am. Chem. Soc. 72, 624—632
20 Kole, R. and Sierakowska, H. (1971) Acta Biochim. Polon. 18, 187—197
21 Zan-Kowalczewska, M., Bardon, A., Sierakowska, H. and Shugar, D. (1974) Biochim. Biophys. Acta 341, 138—156
22 Kole, R., Sierakowska, H. and Shugar, D. (1972) Biochim. Biophys. Acta 289, 323—330
23 Muthukrishnan, S., Both, G.W., Furuichi, Y. and Shatkin, A.J. (1975) Nature 255, 33—37
24 Ro-Choi, T.S., Reddy, R., Choi, Y.C., Raj, N.B. and Henning, D. (1974) Fed. Proc. 33, 1548
25 Haroz, R.K., Twu, J.S. and Bretthauer, R.K. (1972) J. Biol. Chem. 247, 1452—1457
26 Spahr, P.F. and Gasteland, R.F. (1970) Eur. J. Biochem. 12, 270—284
27 Bernardi, A. and Bernardi, G. (1971) in The Enzymes (Boyer, P.D., ed.), Vol. 4, pp. 329—336, Academic Press, New York
28 Razzell, W.E. (1961) J. Biol. Chem. 236, 3028—3030
29 Kole, R., Sierakowska, H., Szemplinska, H. and Shugar, D. (1974) Nucleic Acids Res., 1, 699—706
30 Udvardy, J., Marre, E. and Farkas, G.L. (1970) Biochim. Biophys. Acta 206, 392—403
31 Holbrook, J., Ortanderl, F. and Pfleiderer, G. (1966) Biochem. Z. 345, 427—439
32 Nuss, D.L., Furuichi, Y., Koch, G. and Shatkin, A.J. (1975) Cell 6, 21-32