Two Low $K_m$ Hydrolytic Activities on Dinucleoside 5',5''-P',P'-tetraphosphates in Rat Liver

CHARACTERIZATION AS THE SPECIFIC DINUCLEOSIDE TETRAPHOSPHATASE AND A PHOSPHODIESTERASE I-LIKE ENZYME*

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Ninety per cent of total rat liver hydrolytic activity (1.4 units/g of fresh tissue) on diadenosine or diguanosine 5',5''-P',P'-tetraphosphate (Ap,A and Gp,G) present in isotonic homogenates sedimented at 37,000 x g. Supernatant activity corresponded to the earlier described, cytosolic and specific, bis(5'-guanosyl) tetraphosphatase or dinucleoside tetraphosphatase (EC 3.6.1.17; Lobatón, C. D., Vallejo, C. G., Sillero, A., and Sillero, M. A. G. (1975) Eur. J. Biochem. 50, 495-501). Particulate activity, as extracted with Triton X-100, is composed of two enzymes separable by gel filtration. One of them was a low $K_m$ (1.4 g/mM Gp,G, 6 g/mM Ap,A) 22,000-dalton enzyme, strongly inhibited by guanosine 5'-tetraphosphate ($K_i = 9$ mM), and likely identical to the cytosolic specific enzyme. The other Triton-extracted form was unspecific, with an estimated molecular weight of 150,000 (sucrose gradient) or 450,000 (gel filtration), both in the presence of detergent. Substrate specificity was broad, requiring a nucleoside 5'-phosphoryl residue with a free 3'-hydroxyl group, and acting on 5'-5' and 5'-3' compounds. $K_m$ values were $1.4$ g/mM (Gp,G) and $6$ g/mM (Ap,A). Guanosine 5'-tetraphosphate was a competitive inhibitor ($K_i = 2$ g/mM). It required bivalent cations since a residual activity after dialysis was abolished by EDTA and enhanced by Mg$^{2+}$, Mn$^{2+}$, or Ca$^{2+}$. In the absence of other added cations, the enzyme, inhibited by 1 mM EDTA, is fully reactivated by an equimolar amount of Zn$^{2+}$. The positive identity of this activity with phosphodiesterase 1 (EC 3.1.4.1; Razzell, W. E. (1963) Methods Enzymol. 6, 236-258) is discussed, and its potential role in the metabolism of dinucleoside tetraphosphates is indicated.

The presence of bis(5'-guanosyl) tetraphosphatase or diguanosine tetraphosphatase (EC 3.6.1.17) has been described in the cytosol of Artemia salina (Warner and Finamore, 1965; Vallejo et al., 1974), ascites tumor cells (Moreno et al., 1982), and several rat tissues (Lobatón et al., 1975; Cameselle et al., 1982). This enzyme cleaves dinucleoside tetraphosphates to the corresponding nucleoside mono- and triphosphates, has a molecular weight between 20,000 and 22,000 (rat), and is strongly inhibited (nanomolar $K_i$) by nucleoside 5'-tetraphosphates. The enzyme is equally active on Ap,A and Gp,G, two nucleotides present in biological sources (Zamecnik, 1969; Finamore and Warner, 1963; Sillero and Ochoa, 1971). This activity was originally named diguanosine tetraphosphatase by the Enzyme Commission on the basis of its earlier reported activity towards Gp,G (Warner and Finamore, 1965). Later studies on its substrate specificity made in our view more appropriate the name of dinucleoside tetraphosphatase (Vallejo et al., 1976). This denomination will be adopted through this work.

The increasing interest in the potential metabolic roles of both Gp,G and Ap,A (Renart et al., 1976; Sillero et al., 1977; Grummt et al., 1979; Rapaport et al., 1981; Yamakawa et al., 1982) made desirable the further investigation into the metabolism of these nucleotides. Previous results from our laboratory had shown that the hydrolytic activity on Gp,G present in the total homogenates from several rat tissues was higher than that recovered in the 27,000 x g supernatants (Cameselle et al., 1982). With rat liver, two thirds of the activity appearing in homogenates obtained in 50 mM Tris/HCl buffer, pH 7.5, 0.5 mM EDTA, sedimented at 27,000 x g, and one third remained in the supernatant. The last activity was characterized as the specific dinucleoside tetraphosphatase (see above and Lobatón et al., 1975b; Cameselle et al., 1982). The purpose of this article is to describe the two distinct activities on Gp,G detected in the particulate fraction. One of them could not be distinguished from the specific tetraphosphatase present in the cytosol, whereas the other one was of a higher molecular size and quantitatively predominant. The broad substrate specificity of the latter form makes likely that it corresponds to the previously described phosphodiesterase 1 (EC 3.1.4.1; Khorana, 1961). In order to facilitate the presentation of the results, this activity will be here named high molecular weight or unspecific dinucleoside tetraphosphatase in contrast to the specific one.

MATERIALS AND METHODS

Proteins, Substrates, and Nucleotides

Auxiliary enzymes, molecular weight standards, NADP, NAD, NADH, AMP, glucose 6-phosphate, and fructose 6-phosphate were

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1 The abbreviations used are: Ap,A, diadenosine tetraphosphate or diadenosine 5',5''-P',P'-tetraphosphate; ApA, adenosine 5'-phosphate; Ap,A, adenosine 5'-phosphate; Gp,G, diguanosine tetraphosphate or diguanosine 5',5''-P',P'-tetraphosphate.
from Boehringer Mannheim. The rest of the substrates or nucleotides were obtained from Sigma, except for GpG which was purified from *A. salina* cysts as described by Vallejo et al. (1974).

**Detergents and Buffers**

The following detergents were used in this work: Triton X-100 (Sigma), sodium dodecyl sulfate (Merck), Zwittergent 3-14 (Calbiochem-Behring), and CHAPS (Serva). The two last products are zwitterionic surfactants which correspond to *N*-tetradecyl-NN,N-di-methyl-3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (Gonenne and Ernst, 1978) and 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (Hjelmeland, 1980), respectively. The adjustment of the pH values of Triton buffers was performed at the same temperature at which they were to be used.

**Enzyme Assays**

Unless otherwise stated, all the measurements were done in a volume of 1 ml and at 37 °C, with 50 mM Tris/ClCl buffer, pH 8.0, 0.5 mM MgCl₂.

**Direct Discontinuous Assay**—To measure the phosphohydrolatic activities on AMP and glucose 6-phosphate, the liberation of inorganic phosphate was determined. After incubation, the reaction (0.1-0.2 ml mixtures) was stopped with 3 ml of a solution prepared by mixing 0.3 M sodium dodecyl sulfate with 2 volumes of 10 mM ammonium heptamolybdate in 2.5 N H₂SO₄. After strong shaking, 0.1 ml of the filtrate and SubbaRow’s reducing reagent (prepared according to Leloir and Cardini, 1967) was added. The tubes were left for 1 h at room temperature before reading the absorbance at 660 nm. Sodium dodecyl sulfate removes Triton X-100 interference and makes deproteinization unnecessary (Dudley, 1975; Tashima, 1975). The reliability of the procedure was tested and found satisfactory up to at least 0.6 mg of protein and 12 mg of Triton/reaction mixture.

**Alkaline Phosphatase-coupled Method**—The procedure was the same as for the direct assay (see above) except that alkaline phosphatase was included in the reaction mixtures. A further modification was needed for the assay in crude extracts and pellets. In these cases, the reaction was finished with 1 ml of 0.5 M trichloracetic acid. Inorganic phosphate was determined in the supernatants of the supernatants as above described.

**Hyperchromicity Assay**—It was carried out as previously described in the presence of GpG, Ap₂A, or Ap₄A (Vallejo et al., 1974; Lobotáni et al., 1975b; Lobotáni et al., 1975a). A molar absorption coefficient of 4000 M⁻¹ cm⁻¹ was found for the hydrolysis of Ap₂A at pH 8.6.

**Other Enzyme Assays**—The hydrolysis of NAD by the hydrolysis of NAD, this assay was performed in two steps. The first one was an incubation with alkaline phosphatase and adenosine deaminase. When testing the sodium phosphate buffer, pH 7.0, and 0.05 mM EDTA.

**RESULTS**

**Washing Out of the Activity on Diguanylic Tetraphosphate from a Rat Liver Particulate Fraction**—As shown in the Introduction, about two thirds of the hydrolytic activity on dinucleoside tetraphosphates present in a total rat liver homogenate (Cameselle et al., 1982), obtained in 50 mM Tris/HCl buffer, pH 7.5, 0.5 mM EDTA, precipitated at 27,000 × g. In the following experiments and in order to preserve the integrity of subcellular structures, an isotonic extraction medium was used. A total liver homogenate was obtained and centrifuged at 37,000 × g, and the resulting precipitate was washed three times. The activities of two typically cytosolic enzymes (lactate dehydrogenase and glucose phosphate isomerase) and the hydrolytic activity on GpG were measured in the total homogenate, in the successive washes, and in the final precipitate (Table I). As expected, most of the activities of the cytosolic enzymes were found in the first wash, only 3% being retained in the last precipitate. The distribution of the total GpG hydrolytic activity was different since only 10% appeared in washes 1–4 (Table I) and the bulk of it remained in the precipitate. As shown later, the total activity is due to the presence of at least two different enzymes in the homogenate: the specific dinucleoside tetraphosphatase and another one, which unspecifically cleaves these compounds. The distribution of the specific dinucleoside tetraphosphatase among the fractions of Table I was calculated after its separation by Sephadex G-100 chromatography (results not shown). Two peaks of activity were apparent in this experiment, one of them being partly included and corresponding to the specific enzyme. The other peak, appearing in the void volume, was less prominent and amounted to 20 milliunits/g of fresh tissue (16% of the activity present in wash 1). Since the magnitude of the excluded peak varied with the conditions chosen for centrifugation (filling level of the tubes, rotor speed, or buffer density; results not shown), it can be tentatively assigned to the ultracentrifugal vesicles which, in our experimental procedure, may still contaminate the supernatants. Hence, we have not been concerned with this minor peak which, on the other hand, can be regarded as a modest loss of particulate activity.

From the results shown above, it seems that the specific and the unspecific tetraphosphatases behave as cytosolic and particulate enzymes, respectively. This point will be further discussed below.

**Dinucleoside Tetraphosphate Hydrolytic Activities from Liver Particulate Fraction: Solubilization with Triton X-100**—The precipitate obtained after successive washes with an isotonic medium was resuspended in 50 mM Tris/HCl buffer, pH 7.5, 10 mM MgCl₂, 0.5 mM EDTA (buffer B) supplemented with 4% Triton X-100. After an overnight incubation, the suspension was centrifuged at 37,000 × g for 2 h and the supernatant was taken (Table I, 4% Triton wash). Most of the activity present in the liver particulate fraction was obtained in that wash (Table I and Fig. 1).

**Gel Filtration of the Triton-solubilized Tetraphosphatase Activity from Liver Particulate Fraction**—A 4% Triton wash of the precipitate obtained as described above was applied to a Sepharose 4B column and eluted with buffer B (Fig. 2a). Fractions were collected and the activity on GpG was determined in them with the alkaline phosphatase-coupled assay. A rather broad profile of activity appeared between elution volumes 200 and 430 ml and a clear peak was apparent between 430 and 480 ml. The inclusion of 0.5% (w/v) Triton X-100 in the elution buffer changed the elution profile of the first peak (Fig. 2a), making it sharper, whereas the second peak was not affected by detergent. Similar results were obtained when other gel types (Sephadex G-100, Sephadex G-200, and Sephacryl S-300) were utilized. With 0.5% Triton in the elution buffer, two clear peaks were obtained; in the absence of Triton, the width of the first one also depended on the discriminating characteristics of the gel, i.e. it was sharp and broad after chromatography in Sephadex G-100 and Sepharose 4B columns, respectively. In every case, both peaks were fairly separated. An example of the elution profile on the Triton-solubilized precipitate in a Sephadex G-200 column and in the presence of 0.5% Triton in the elution buffer is shown in Fig. 2b. Altogether, these results show that the solubilized liver particulate fraction contains two different
**TABLE 1**

Washing out of the activity on Gp4G from a rat liver particulate fraction

<table>
<thead>
<tr>
<th>Lactate dehydrogenase</th>
<th>Glucose phosphate isomerase</th>
<th>Total Gp4G hydrolysis</th>
<th>Specific dinucleoside tetraphosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>units/g mg</td>
<td>units/g mg</td>
<td>millions/g</td>
<td>%</td>
</tr>
<tr>
<td>Homogenate</td>
<td>425 100</td>
<td>193 100</td>
<td>1414 100</td>
</tr>
<tr>
<td>Wash 1</td>
<td>352 83</td>
<td>160 80</td>
<td>125 9</td>
</tr>
<tr>
<td>Wash 2</td>
<td>22 5.2</td>
<td>11 5.7</td>
<td>16 1.1</td>
</tr>
<tr>
<td>Wash 3</td>
<td>7.4 1.7</td>
<td>2.9 1.5</td>
<td>6 0.4</td>
</tr>
<tr>
<td>Wash 4</td>
<td>4.6 1.1</td>
<td>1.9 1.0</td>
<td>5 0.4</td>
</tr>
<tr>
<td>Precipitate</td>
<td>12 2.8</td>
<td>5.2 2.7</td>
<td>1275 90</td>
</tr>
<tr>
<td>4% Triton wash</td>
<td>8.2 1.9</td>
<td>4.1 2.1</td>
<td>1600 113</td>
</tr>
</tbody>
</table>

*ND*, not determined.

The livers from two female rats (14 g of fresh weight) were homogenized in a motor-driven Potter apparatus with a glass pestle in the presence of 29 ml of 35 mM Tris/HCl buffer, pH 7.7, 70 mM KCl, 9 mM MgCl₂, 0.25 M sucrose (buffer A). A 3:2 ml sample of the homogenate was taken, and the remaining 40 ml were distributed equally among eight 30-ml tubes and centrifuged at 18,000 rpm (37,000 × g average) and 2°C for 1 h. The supernatants were decanted, pooled, and kept at 3°C. Each pellet was resuspended in 3.5 ml of buffer A with the help of a glass rod and centrifuged as described above. This step was repeated two more times, and four supernatants were obtained (washes 1–4). After the last centrifugation, the pellets were resuspended either in buffer A (precipitate in the table) or in buffer B containing 4% (w/v) Triton X-100. Aliquots of every step were dialyzed overnight against 200 volumes of buffer A, except for the Triton-resuspended fraction which was dialyzed against buffer B supplemented with 4% Triton X-100. The latter preparation, after dialysis, was centrifuged as described above for 2 h, and the supernatant was taken (4% Triton wash in the table). Total hydrolytic activity on Gp4G (0.6 mM) with the alkaline phosphatase-coupled assay and the activities of lactate dehydrogenase and glucose phosphate isomerase were measured in those samples. The specific dinucleoside tetraphosphatase was evaluated (Gp4G same assay as described above) after chromatography of the extracts in a Sephadex G-100 column, except for the value assigned to the homogenate, which is the sum of the activities found in washes 1–3.

**Fig. 1. Solubilization of particulate hydrolytic activity on Gp4G by Triton X-100.** A liver precipitate was obtained as described in the legend to Table 1. That pellet was resuspended in buffer B. Six 3-ml aliquots of that suspension received 0.75 ml of buffer B supplemented with various amounts of Triton X-100 sufficient to bring the final detergent concentrations to 0, 0.1, 0.5, 1, 2, and 4% (w/v), respectively. The six samples were dialyzed overnight against 100 volumes of buffer B alone or supplemented with Triton X-100 up to the same concentration to which each sample had been brought. After centrifugation in 30-ml SS-34 Sorvall rotor tubes at 18,000 rpm and 2°C for 2 h, the hydrolytic activity on Gp4G (Gp4G) was assayed in the supernatants with the alkaline phosphatase-coupled method and 0.6 mM Gp4G as substrate. The results are expressed as percentage of the activity present in the untreated precipitate. Protein content (●) was determined by a procedure which removes Triton X-100 with the help of a glass rod and centrifuged as described above. The high molecular weight form of dinucleoside tetraphosphatase activities with dissimilar apparent molecular weights.

**Characterization of the Low Molecular Weight Form of the Triton-solubilized Dinucleoside Tetraphosphatase Activity from Liver Particulate Fraction—**This enzyme form was obtained as described above; the washed precipitate (Table I) was treated with 4% Triton, and the supernatant was applied to a Sephadex G-100 column and eluted without detergent. Two fully resolved peaks of hydrolytic activity on Gp4G appeared (results not shown). The three fractions with the maximal activity from the peak corresponding to the low molecular weight form were pooled, and the enzymatic activity was characterized as described by Lobatón et al. (1975b). The more relevant results were as follows. The enzyme was equally active on Gp4G and Ap4A, with $K_m$ values of 1 and 5 μM, respectively. The products of the reaction, with Gp4G as substrate and characterized by spectrophotometric coupled methods or by thin layer chromatography, were GTP and GMP. The enzyme required Mg²⁺, was maximally active at pH 7.5, and was inhibited by guanosine 5'-tetraphosphate ($K_i$ = 9 nM) and by Ca²⁺; the apparent molecular weight was 22,000 as determined by gel filtration in Sephadex G-100. In our view, these results are sufficient to characterize this enzyme as the specific dinucleoside tetraphosphatase previously described in the rat liver supernatant (Lobatón et al., 1975b; Cameselle et al., 1982).

The presence of this enzyme in the liver supernatant and in the successive washes of the precipitate was investigated by chromatography of the samples in a Sephadex G-100 column to allow for the separation of the low molecular weight tetraphosphatase. As shown in Table I, the percentage of this activity extracted with the successive washes was very similar to those of lactate dehydrogenase and glucose phosphate isomerase, pointing to a cytosolic localization of the specific tetraphosphatase. However, an appreciable amount (20%) of the specific enzyme remains in the precipitate and is liberated after detergent treatment. This result could favor the possibility that a certain amount of this enzyme is also located in the rat liver particulate fraction.

**Characterization of the High Molecular Weight Form of the Triton-solubilized Dinucleoside Tetraphosphatase Activity from Liver Particulate Fraction—**The apparent molecular weight of this activity was studied by gel filtration in a Sephadex G-200 column. The enzyme sample was a 4% Triton wash of the last liver precipitate (Table I). The elution was accomplished with buffer B containing 0.5% (w/v) Triton X-100 (Fig. 3). The calibration of the column was performed with markers of known molecular weight (ferritin, 450,000; catalase, 240,000; lactate dehydrogenase, 140,000; cytochrome c, 12,500) which were chromatographed under the same experimental conditions as the sample. The high molecular weight form of dinucleoside tetraphosphatase activity eluted...
Molecular weights were calculated, with catalase as a marker, determined with dextran blue.

...column (1.6 cm) preparation to the above one were applied to a Sephadex G-200 column (Fig. 2) were used. The flow rate was 3.2 ml/h and 1.7-ml fractions were collected. The following samples were run successively: 1) 4 ml of the 4% Triton wash of a liver precipitate (Table I) and 2) 4 ml of a solution containing 7.5 mg of ferritin, 4.5 mg of cytochrome c, 0.7 mg of catalase, and 25 units of lactate dehydrogenase in elution buffer. The elution profiles are represented in arbitrary units. One arbitrary unit equals 6.7 milliunits/ml (Gp4G hydrolysis; alkaline phosphatase-coupled assay with 0.6 mM Gp4G), 1 absorbance unit (ferritin; measured at 400 nm; O), 160 units/ml (catalase; x), 0.22 unit/ml (lactate dehydrogenase; A), or 0.4 absorbance units (cytochrome c; measured at 400 nm; △). The arrow marks the elution volume of the tetraphosphatase activity.

The molecular size of this activity was also studied by means of sucrose gradient centrifugation. The source of enzyme was the same as in the preceding experiment. A 0.4-ml portion of that preparation was layered on the top of a continuous sucrose gradient (10-30%) in buffer B. Fractions were collected and analyzed for Gp4G hydrolysis by the alkaline phosphatase-coupled method with 0.6 mM Gp4G. The recovery of activity in the two above experiments was 62 and 71%, respectively. In separate experiments, a 0.4-ml portion of catalase and the hydrolytic activity on Gp4G were measured. The latter activity sedimented in two peaks, whose apparent molecular weights were assigned to it. What follows is the characterization of the latter activity with the same in the five gradients (Fig. 4). Referring to the control and to the KCl-, Zwittergent-, CHAPS-, and Triton-supplemented gradients, the calculated molecular masses for the small form were 30, 27, 23, 32, and 21 kilodaltons, respectively. The corresponding values for the high molecular weight form were 224, 213, 152, 225, and 150 kilodaltons. In agreement with the results presented above using gel filtration, the first activity corresponds to the specific dinucleoside tetraphosphatase. The molecular mass calculated for the second form was a third of that obtained by gel filtration in Sephadex G-200 (see above), both cases in the presence of Triton X-100.

For purposes of kinetic characterization, the large form of dinucleoside tetraphosphatase activity, which had been isolated by gel filtration, was subjected to an additional purification step. Fractions corresponding to elution volume 70-83 ml of a Sephadex G-200 column (Fig. 2b) were pooled and applied to a DEAE-cellulose column (1.6 x 13 cm) equilibrated with 0.5% (w/v) Triton X-100 in buffer B. The column was then washed with the same buffer until the protein detected in the eluate was negligible. Further elution was accomplished with 200 ml of a linear gradient (0-0.35 M) of KCl in the starting buffer. The hydrolytic activities on several substrates were determined in the collected fractions (Fig. 5). The activity on Gp4G eluted as a single peak coinciding with another one on ApA and ApA. Two peaks of activity were apparent when AMP was used as the substrate. This result points to the existence in those fractions of at least two enzymatic activities, one acting on AMP and the other one with capacity to hydrolyze molecular structures with inner phosphates. What follows is the characterization of the latter activity with a pool of fractions 47-60 in Fig. 5.

The compounds tested as substrates were: (a) dinucleoside polyphosphates (GpG, ApA, ApA, ApA, NAD, 3-acetylpyrididine adenosine dinucleotide, and FAD), (b) other molecules with inner phosphates (ApA, ADP-ribose, ADP-Glc, UDP-
Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Relative rate</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GpG</td>
<td>0.6</td>
<td>0.62</td>
<td>8.0</td>
</tr>
<tr>
<td>ApA</td>
<td>0.6</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>ApA</td>
<td>0.6</td>
<td>2.9</td>
<td>0.3</td>
</tr>
<tr>
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<td>0.6</td>
</tr>
<tr>
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<td>0.3</td>
</tr>
<tr>
<td>AcPyNAD</td>
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</tr>
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<td>0.3</td>
</tr>
<tr>
<td>NAD</td>
<td>0.6</td>
<td>2.4</td>
<td>0.3</td>
</tr>
<tr>
<td>FAD</td>
<td>0.6</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>ApA</td>
<td>0.6</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>0.6</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>ADP-Glc</td>
<td>0.6</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>0.6</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>3'-Deoxy-CoA</td>
<td>0.6</td>
<td>2.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Bis(p-nitrophenyl)</td>
<td>0.6</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>dT-5'-(4-nitrophenyl)</td>
<td>0.6</td>
<td>3.8</td>
<td>0.3</td>
</tr>
<tr>
<td>dT-3'-(4-nitrophenyl)</td>
<td>0.6</td>
<td>3.8</td>
<td>0.3</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.6</td>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>0.6</td>
<td>2.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Fig. 4. Sucrose gradient centrifugation of the Triton-solubilized dinucleoside tetraphosphatase activities. Five 10-ml continuous gradients (sucre, 10-30%) were accomplished in buffer B supplemented with one of the following reagents: nothing (a), 1 M KCl (b), 0.5% (w/v) CHAPS (c), 0.3% (w/v) Zwittergent 3-14 (d), 0.5% (w/v) Triton X-100 (e) (see under “Materials and Methods” for detergent data). A 0.4-ml sample of a 4% Triton wash of the liver precipitate (Table I), having 0.25 mg of catalase added, was layered onto each gradient. After 14.5 h at 58,000 rpm in a Beckman SW 41 rotor, the gradients were fractionated and catalase (G) and the Gp,G hydrolytic activity (D) were assayed. For the latter activity, the alkaline phosphatase-coupled method with 0.6 mM GpG was used. Enzyme activities are expressed in arbitrary units equaling 100 milliunits/ml (catalase) or 10 milliunits/ml (Gp,G hydrolysis).

Gluc, 3'-dephospho-CoA, bis(p-nitrophenyl) phosphate, dT-5'-(4-nitrophenyl phosphate), dT-3'-(4-nitrophenyl phosphate), and cAMP, and (c) two molecules with terminal phosphates (AMP and glucose 6-phosphate). The enzymatic activities were followed in conditions of linearity with both time and amount of extract. For each substrate, the concentration at which it was tested, the relative velocity of hydrolysis, and, when pertinent, the K<sub>m</sub> value are included in Table II. All the substrates were efficiently hydrolyzed with the exception of bis(p-nitrophenyl) phosphate, dT-5'-(4-nitrophenyl phosphate), glucose 6-phosphate, and cAMP. It is relevant that, contrary to the 5'-derivative, dT-3'-(4-nitrophenyl phosphate) was not a substrate of the reaction. Similarly, the molecules which did not have a free 3'-OH end were poor substrates of the enzyme. This activity most probably corresponds to phosphodiesterase I (EC 3.1.4.1; Khorana, 1961). The activity towards AMP (Table II) is likely due to an enzyme different from that acting on Gp,G (and the rest of the substrates; Table II) for two reasons: the DEAE-cellulose elution profile (Fig. 5) and different inactivation curves. The DEAE-cellulose preparation lost 50% of activity on Gp,G (and also on ApA, APbA, NAD, and dT-5'-(4-
nitrophenyl phosphate) after 5 days at 4 °C, whereas the activity on ATP remained unchanged through that period.

Michaelian kinetics was obtained when Gp,G, Ap,A, Ap,a, Ap,A, NAD, or bis(p-nitrophenyl) phosphate was used as substrate. The $K_m$ values calculated were 12 $\mu$M, 8 $\mu$M, 11 $\mu$M, 22 $\mu$M, 10 $\mu$M, and 5 mM, respectively. It seemed to us of interest to test the inhibition of the enzyme by guanosine 5'-tetraphosphate. This nucleotide is a very strong competitive inhibitor ($K_i \sim 10$ nM) of the specific dinucleoside tetraphosphatase from rat liver (Lobatón et al., 1975b; and this work). The effect of this nucleotide was tested on the high molecular weight form obtained by chromatography on a Sepharose 4B column in the presence of 0.5% Triton X-100 (Fig. 2a).

Guanosine 5'-tetraphosphate was also a competitive inhibitor of Ap,A and Ap,A hydrolysis by the unspecific tetraphosphatase with $K_i$ values of 2 $\mu$M in both cases. A fixed concentration (22 $\mu$M) of guanosine 5'-tetraphosphate was also tested as inhibitor of the hydrolysis of several substrates of the enzyme, Ap,A, Ap,A, NAD, and dT-5'- (4-nitrophenyl phosphate), all of them at a 35 $\mu$M concentration. In those experimental conditions, the inhibitions obtained were 66, 59, 57, and 66%, respectively, in relation to control without guanosine 5'-tetraphosphate. The cation requirements were studied with the same preparation. After extensive dialysis, the enzyme exhibited a residual activity in the absence of added bivalent cations (Fig. 6). This activity was completely abolished by 1 mM EDTA. Full reactivation was achieved with an equimolar amount of Zn$^{2+}$, but not of Mg$^{2+}$, showing that probably the enzyme has a strict requirement for Zn$^{2+}$. Other cations behaved as activators, such as Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$. Maximal velocity obtained with Ca$^{2+}$ was twice as much as that with Mg$^{2+}$. The concentration giving rise to half-maximal activation was about 0.5 mM for both Ca$^{2+}$ and Mg$^{2+}$. Quite different was the behavior of the enzyme in the presence of Mn$^{2+}$. A sharp peak of activity was reached at around 50 $\mu$M, decreasing also sharply at a concentration of 0.1–0.5 mM. After a slight increase in activity, the enzyme was inhibited by higher, up to 4 mM, Mn$^{2+}$ concentrations (Fig. 6). Maximal activity was found at pH 8.5, with both Ap,A or Ap,A as substrates. At pH 7.0, the activity was less than 10% of that obtained at pH 8.5. In the presence of cysteine, reduced glutathione, or $\beta$-mercaptoethanol, each one at a concentration of 8 mM, the activity was, respectively, 10, 45, and 85% of that obtained in the absence of thiols.

**FIG. 6. Cation effect on the high molecular weight, unspecific dinucleoside tetraphosphatase activity.** Enzyme from the Sepharose 4B step was used (elution with Triton X-100; Fig. 2a). That preparation was thoroughly dialyzed for 24 h against 125 volumes of 50 mM Tris/HCl buffer, pH 7.5, 0.5 mM EDTA, which was renewed 3-fold during that period. Enzyme samples of 40 $\mu$l were incubated at 23 °C with 50 mM Tris/HCl buffer, pH 7.5, 28 $\mu$m Ap,A as substrate and various concentrations of MgCl$_2$ (O), MnCl$_2$ (C), or CaCl$_2$ (X). The increase in absorbance at 259 nm was recorded (hyperchromicity assay).

**DISCUSSION**

Two different hydrolytic activities on dinucleoside tetraphosphates have been solubilized by Triton X-100 from the isotonic rat liver precipitate. Both activities differ markedly in abundance, molecular weight, kinetic properties, and, possibly, in subcellular localization. We have obtained strong evidence that the smaller one is the specific dinucleoside tetraphosphatase (EC 3.6.1.17) which was previously reported in the cytosol of several rat tissues (Lobatón et al., 1975b; Cameselle et al., 1982). The soluble and the detergent-extracted specific enzymes have the same size as evaluated by gel filtration, the same cation requirements, the same pattern and extent of inhibition by guanosine 5'-tetraphosphate and Ca$^{2+}$, and very similar $K_i$ values for Ap,A and Gp,G. Furthermore, the same enhydride bond is split by both enzymes, yielding 1 mol of nucleoside 5'-triphosphate and 1 mol of nucleoside 5'-monophosphate/mol of dinucleoside tetraphosphate hydrolyzed. Hence, it appears that the specific tetraphosphatase could be particulate to some extent. Under our experimental conditions, the detergent-extracted specific enzyme represents about 20% of the activity found in the cytosol and can be compared with the corresponding percentage (3%) obtained for lactate dehydrogenase and glucose phosphate isomerase. Relevant to the discussion is the fact that the last two enzymes have been reported to be present in the nucleoplasm (Price and Stevens, 1982). If lactate dehydrogenase and glucose phosphate isomerase activities which can be extracted with Triton X-100 are from nuclear origin, it follows that the specific tetraphosphatase that appears in the same preparation should be at least considered as a component of the rat liver precipitate. Obviously, both the cytosolic and the particulate specific dinucleoside tetraphosphatases could correspond to the same protein and, in fact, we think likely that it is so. The assumption that this enzyme is present in the nucleoplasm is tempting, since Ap,A has been related to DNA synthesis (Grunmmt, 1978). However, this picture must remain speculative for the moment.

The high molecular weight form of the splitting activity on Gp,G and other nucleotides (Table II) is clearly different from the specific tetraphosphatase. Its apparent molecular weight, as estimated by Sephadex G-200 gel filtration in the presence of 0.5% Triton X-100, is very near to 450,000 since ferritin and the unspecific tetraphosphatase activity eluted practically at the same volume. Nevertheless, when detergent was not included in the elution buffer, the latter enzyme seemed to aggregate, itself spreading towards the void volume of a Sepharose 4B column (exclusion limit 2-150 dalton). On the other hand, its apparent molecular weight was smaller when estimated in sucrose gradient centrifugation. In this case and depending on the detergent used, the values varied from 150,000 to 240,000. Altogether, these results could indicate that the high molecular weight form of the Gp,G-lytic activity is an integral membrane protein that can be extracted with Triton and aggregates when the detergent is eliminated. According to this and to the special features of the protein-detergent complexes, the molecular weights quoted above for the unspecific tetraphosphatase activity are to be considered as an index of its behavior during gel filtration or gradient centrifugation, but not as a measure of actual protein size. In this regard, the amount of lipid and/or detergent bound to protein is not known, and the protein-Triton X-100 complexes are featured by a low sedimentation coefficient and a high Stokes radius (Heleneius and Simons, 1975), mainly due to the high partial specific volume of Triton X-100. Hence, factors such as nature and concentration of detergent can affect size estimations (e.g. see EY and Ferber, 1977). It seems reasonable
to assume that the true molecular weight of the unspecific dinucleoside tetraphosphatase complex is between the values estimated by gel filtration and sedimentation analysis. These results, together with the kinetic data presented above, indicate that this activity may correspond to the phosphodiesterase I (EC 3.1.4.1), earlier characterized by others in the rat liver particulate fraction (Touster et al., 1970; Prospero et al., 1973). It is known that 50% of the total phosphodiesterase I is located in the microsomal fraction (Touster et al., 1970). Under our experimental conditions, almost all the enzyme sedimented in the 37,000 \( g \) precipitate (see the first heading under “Results”). Phosphodiesterase I can be isolated from several sources, requires a nucleoside 5’-phosphoryl residue with a 3’-hydroxyl group, and is equally active on 5’-5’ and 5’-3’ phosphodiester linkages (Khorana, 1961; Razzell, 1963). The enzyme presents maximal activity at pH 8.5 (Schissel et al., 1965), is inhibited by EDTA, and requires bivalent cations (Prospero et al., 1973), with a strict requirement for Zn\(^{2+}\) (Lau and Carlson, 1981). Thiols are also inhibitors of the enzyme (Razzell, 1963). The properties summarized above are in good accord with those obtained here for the unspecific high molecular weight dinucleoside tetraphosphatase. From Table I it could be also inferred that the activity is inversely related to the length of the inner phosphate chain, considering the decreasing activities on Ap2A, ApA, and ApA. The hydrolysis of ApA and ApA by the kidney phosphodiesterase I had been incidentally reported (Razzell, 1963). As shown under “Results,” the activity on AMP is due to a different enzyme, probably a nucleotidase (EC 3.1.3.5), in our preparation. In the presence of this activity, it is difficult to assess the products of the reaction catalyzed by the phosphodiesterase I on the different substrates which have been tested. It is also pertinent to recall that confusion exists in the bibliography on this enzyme. Three entries of the Enzyme bibliography on this enzyme. Three entries of the Enzyme...

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Two low Km hydrolytic activities on dinucleoside 5',5'''-P1,P4-tetraphosphates in rat liver. Characterization as the specific dinucleoside tetraphosphatase and a phosphodiesterase I-like enzyme.

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