Biochem. J. (1989) 259, 831–838 (Printed in Great Britain)

Characterization of the bis(5′-nucleosidyl) tetraphosphate pyrophosphohydrolase from encysted embryos of the brine shrimp *Artemia*

Mark PRESCOTT, Andrew D. MILNE and Alexander G. McLENNAN*
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

The P^1P^4-bis(5′-nucleosidyl) tetraphosphate *asymmetrical*-pyrophosphohydrolase from encysted embryos of the brine shrimp *Artemia* has been purified over 11000-fold to homogeneity. Anion-exchange chromatography resolves two major species with very similar properties. The enzyme is a single polypeptide of M_r 17600 and is maximally active at pH 8.4 and 2 mM-Mg^{2+}. It is inhibited by Ca^{2+} (IC_{50} = 0.9 mM with 2 mM-Mg^{2+}) but not by Zn^{2+} ions. It preferentially hydrolyses P^1P^4-bis(5′-nucleosidyl) tetraphosphates, e.g. P^1P^4-bis(5′-adenosyl) tetraphosphate (Ap^4A) ($k_{cat.}$ = 12.7 s^{-1}; K_m = 33 μM) and P^1P^4-bis(5′-guanosyl) tetraphosphate (Gp_4G) ($k_{cat.}$ = 6.2 s^{-1}; K_m = 5 μM). With adenosine 5′-P^1-tetraphospho-P^4-5‴-guanosine (Ap_4G) as substrate, there is a 4.5-fold preference for AMP and GTP as products and biphasic reaction kinetics are observed giving K_m values of 4.7 μM and 34 μM, and corresponding rate constants of 6.5 s^{-1} and 11.9 s^{-1}. The net rate constant for Ap_4G hydrolysis is 7.6 s^{-1}. The enzyme will also hydrolyse nucleotides with more than four phosphate groups, e.g. Ap_5G, Ap_6A and Gp_5G are hydrolysed at 25%, 18% and 10% of the rate of Ap_4A respectively. An NTP is always one of the products. Ap_2A and Gp_2G are not hydrolysed, while Ap_3A and Gp_3G are very poor substrates. When the enzyme is partially purified from embryos and larvae at different stages of development by sedimentation through a sucrose density gradient, its activity increases 3-fold during the first 12 h of pre-emergence development. This is followed by a slow decline during subsequent larval development. The similarity of this enzyme to other *asymmetrical*-pyrophosphohydrolases suggests that it did not evolve specifically to degrade the large yolk platelet store of Gp_4G which is found in *Artemia* embryos, but that it probably serves the same general function in bis(5′-nucleosidyl) oligophosphate metabolism as in other cells.

INTRODUCTION

Encysted embryos of the brine shrimp *Artemia* are virtually unique in possessing large quantities (2% of total dry weight) of the purine storage nucleotide P^1P^4-bis(5′-guanosyl) tetraphosphate (Gp_4G) [1]. Shortly after the discovery of this compound in 1963, an enzyme was isolated from *Artemia* cysts which had the properties expected which are necessary for the metabolism of this compound. This enzyme, which was originally named diguanosine tetraphosphate *asymmetrical*-pyrophosphohydrolase (EC 3.6.1.17), cleaved Gp_4G asymmetrically to yield equimolar amounts of GTP and GMP [2]. Studies on the partially purified enzyme showed it to be located primarily in the soluble fraction of the cell, to have a relative molecular mass of 17500 and to hydrolyse efficiently bis(5′-nucleosidyl) tetraphosphates such as Gp_4G, Ap_4A, Xp_4X and Up_4U [3,4]. It had little activity towards Gp_3G, which had also been found in significant quantities in *Artemia* embryos [2,5].

Two alternative schemes have been proposed for Gp_4G utilization which require the participation of this pyrophosphohydrolase activity. The first involves a complex shuttle mechanism which generates cytosolic Gp_4G from the yolk platelet store; this is then cleaved to give GTP and GMP with further conversions of the latter leading to the adenine nucleotides [6–8]. The second scheme involves a base substitution reaction between adenine and Gp_4G to give guanine and Ap_4G which is then converted to ATP and GMP by the pyrophosphohydrolase [8,9]. A third possibility which does not involve this enzyme requires the pyrophosphorolysis of Gp_4G to 2 mol of GTP by the enzyme which is also responsible for its synthesis, Gp_4G synthetase [6–8].

As yet there is no overwhelming evidence to favour any of these alternatives. However, the possible involvement of the *asymmetrical*-pyrophosphohydrolase in Gp_4G metabolism now requires re-examination in view of the more recent isolation of a similar enzyme from a wide range of cell types which do not contain Gp_4G, and the presence of an alternative substrate, Ap_4A, in all such cells. P^1P^4-bis(5′-nucleosidyl) tetraphosphate *asymmetrical*-pyrophosphohydrolases (*asymmetrical*-Np_4Nases) have now been isolated from rat liver and other rat tissues [4,10–12], mouse liver [13], mouse Ehrlich ascites cells [14], human leukaemic cells [15] and lupin seed meal [16], while a *symmetrical*-Np_4Nase, which yields 2 mol of NDP from Np_4N, has been purified from *E. coli* and other bacteria [17,18] and from *Physarum polycephalum* [19,20].

Abbreviations used: Ap_4A, P^1P^4-bis(5′-adenosyl) tetraphosphate; Gp_4G, P^1P^4-bis(5′-guanosyl) tetraphosphate; Np_nN, P^1P^n-bis(5′-nucleosidyl) oligophosphate; Ap_4G, adenosine 5′-P^1-tetraphospho-P^4-5‴-guanosine; other bis(5′-nucleosidyl) oligophosphates are abbreviated in a similar fashion; Ap_4, adenosine 5′-tetraphosphate; Np_4Nase, P^1P^4-bis(5′-nucleosidyl) tetraphosphate pyrophosphohydrolase (EC 3.6.1.17).

* To whom correspondence should be addressed.

It is now widely assumed that the Np_4Nase is involved in the regulation of intracellular levels of Ap_4A. The precise function of this nucleotide has yet to be elucidated although roles in the initiation of DNA replication and, at least in prokaryotes, in the response to oxidative stress, have been proposed [21–24]. Ap_4A is believed to have a high turnover rate: its concentration is inversely proportional to cellular proliferation rate [23,25] and in some cases fluctuates by two to three orders of magnitude during the cell cycle [23,26]. We have monitored the concentration of Ap_4A during pre-emergence development of *Artemia* embryos and have shown a 130-fold increase from 25 nM to 3.3 μM during the redevelopment of brine-stored, decapsulated cysts up to the point of hatching, followed by a more gradual decline during early larval development [27].

In contrast, the cytosolic concentration of Gp_4G has been estimated to be 1.2 mM [6,7]. In addition to its function as a purine ring store, Gp_4G has been assigned more controversial roles as a source of phosphate bond energy [8] and as a regulator of protein synthesis [6,7] and of certain enzyme activities including GMP reductase [28] and a trypsin-like protease [29]. At first sight it is difficult to imagine how the same enzyme could be responsible for the catabolism of two nucleotides of very different function and intracellular concentration. Since biochemical data on the *Artemia* Np_4Nase is limited, we have now purified it to homogeneity and made a detailed comparative study of this enzyme with the corresponding activities from other sources as a first step towards evaluating its relative importance to Ap_4A and Gp_4G metabolism in *Artemia*.

EXPERIMENTAL

Materials

Great Salt Lake *Artemia* cysts were from the Sanders Brine Shrimp Co., Ogden, UT, U.S.A., and were obtained in 1980. Nucleoside diphosphokinase, calf intestine alkaline phosphatase, and Ap_4A were from Boehringer. Ap_4A was also chemically synthesized in bulk for affinity chromatography [30]. Ap_4G was a gift from G. E. Taylor, Dept. of Chemistry, University of Sheffield. All other bis(5′-nucleosidyl) oligophosphates and protein molecular mass markers were from Sigma. Ap_4A-Sepharose was prepared by immobilizing Ap_4A to epoxy-activated Sepharose 6B (Pharmacia) according to the manufacturer's instructions. [^{3}H]Ap_4A (4.3 Ci/mmol) was from Amersham International.

Enzyme assays

Several different assay procedures were employed for the detection of Np_4Nase activity depending on whether speed, lack of auxiliary enzymes or precise quantification was most important.

1. Luminescence assays. With Ap_4A as substrate, these contained 20 mM-Hepes/KOH, pH 7.8, 5 mM-Mg acetate, 100 μM-Ap_4A and 25 μl of ATP-monitoring reagent (LKB) in 125 μl. The increase in luminescence was monitored over a 2 min period at 25 °C with an LKB 1250 Luminometer. With Gp_4G or Ap_4G as substrate, a two-step procedure was employed. First, Np_4Nase was incubated in 20 mM-bicine/KOH, pH 8.4, 5 mM-Mg acetate, 50 μM-Gp_4G (25 μl) for 10 min at 37 °C followed by 5 min at 90 °C to inactivate the Np_4Nase. Samples containing up to 10 pmol of GTP were then added to an assay containing 20 mM-Hepes/KOH, pH 7.8, 5 mM-Mg acetate, 0.5 μM-ADP, 1.5 U of nucleoside diphosphokinase and 25 μl of ATP-monitoring reagent in 125 μl, and the luminescence (in mV) was recorded at 25 °C.

2. Radiolabel assays (DEAE). These contained 20 mM-bicine/KOH, pH 8.4, 2 mM-Mg acetate, 5 units of alkaline phosphatase and 100 μM-[^{3}H]Ap_4A (40 mCi/mmol) in a total volume of 50 μl. After incubation for 10 min at 37 °C, assays were chilled on ice, and 300 μl of a 25% (v/v) suspension of DEAE–Sephacel in 10 mM-Tris/HCl, pH 7.5, were then added followed by centrifugation at 16000 *g* for 5 min. Aliquots (200 μl) of the supernatants were added to 4 ml of Optiphase-MP scintillant (LKB) and the radioactivity due to [^{3}H]adenosine released from the products by the alkaline phosphatase was determined.

3. Modified radiolabel assays (DEAE). A modification of the above assay omitted the alkaline phosphatase from the primary incubation. After 10 min at 37 °C, the assays were heated at 90 °C for 10 min to inactivate the Np_4Nase and then incubated for a further 10 min at 37 °C with 5 units of alkaline phosphatase.

4. H.p.l.c. assays. Nucleotide substrates (400 μM) were incubated for 5 or 15 min at 37 °C in 20 mM-bicine/KOH, pH 8.4/2 mM-Mg acetate, with or without 5 units of alkaline phosphatase. Ammonium phosphate (70 μl; 50 mM; pH 5.2) was added, and 50 μl of the mixture were injected on to a 4.6 mm × 250 mm Partisil 10-SAX column (Whatman). The column was developed isocratically for 10 min with 5% buffer B followed by a 44 min gradient from 5–80% buffer B, where buffer A = 50 mM-ammonium phosphate, pH 5.2, and buffer B = 1 M-ammonium phosphate, pH 5.7 [20]. Areas of u.v.-absorbing peaks (254 nm) were integrated using a 1040 data capture unit and ChromMac software (Drew Scientific, London, U.K.) and were then manually adjusted to compensate for the hyperchromicity of the products.

Determination of protein

Protein concentrations were assayed by the bicinchoninic acid [31] (Pierce) and silver-binding [32] methods.

Purification of Np_4Nase from *Artemia* cysts

All steps were performed at 4 °C unless otherwise stated. Dry cysts (250 g) were hydrated overnight in 2.5 litres of sea water (final wet weight = 625 g) and then decapsulated [27]. The cysts were then filtered through nylon mesh, excess water was removed by squeezing and the wet cake was divided into three 250 g portions which were separately ground for 20 min each at 4 °C in a 7-inch (17.8 cm) unglazed mortar. The homogenate was then taken to a final volume of 1.25 litres with 50 mM-Tris/HCl, pH 7.5/0.25 M-sucrose/10 mM-$MgCl_2$/1 mM-β-mercaptoethanol/0.1 mM-EDTA, left for 15 min and then centrifuged at 1400 *g* for 15 min. The low-speed supernatant was then centrifuged at 152000 *g* for 90 min to yield 670 ml of crude extract from which the lipid pellicle was removed with cotton wool.

The crude extract was brought to 50% saturation by the addition of solid $(NH_4)_2SO_4$ and centrifuged at

20000 *g* for 30 min. The supernatant was then brought to 100% saturation and centrifuged as before. The pellet was redissolved in a final volume of 92 ml of Q-Sepharose loading buffer [20 mM-Tris/HCl, pH 8.0/10% (v/v) glycerol/1 mM-β-mercaptoethanol] and dialysed overnight against 2 x 10 vol. of loading buffer, yielding a final volume of 146 ml. This fraction was run on to a 5 cm x 16 cm column of Q-Sepharose at 193 ml/h, and after elution of the unbound protein, the column was developed with a 2.5 litre gradient of 0–0.6 M-NaCl in loading buffer. Active fractions were pooled and brought to 100% saturation with $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation at 20000 *g* for 30 min and redissolved in a final volume of 31.5 ml of Ultrogel AcA44 running buffer [50 mM potassium phosphate, pH 7.5/10% (v/v) glycerol/1 mM-EDTA/5 mM-β-mercaptoethanol].

This material was applied to a 5 cm x 95 cm column of Ultrogel AcA44 and eluted with running buffer at 80ml/h. Fractions containing Np_4Nase activity were pooled and dialysed overnight against 2 x 10 vol. of Mono-Q loading buffer (20 mM-Tris/acetate, pH 8.0/10% (v/v) glycerol/1 mM-β-mercaptoethanol) giving a final volume of 206 ml. This fraction was then applied at 4 ml/min to an 8 ml Mono-Q HR10/10 column at room temperature, and the column was eluted with a 160 ml gradient of 0–0.75 M-sodium acetate in loading buffer. The fractions comprising the major peak of Np_4Nase activity were combined and dialysed against 20 vol. of Ap_4A-Sepharose loading buffer [20 mM-Mes/KOH, pH 6.0/10% (v/v) glycerol/1 mM-β-mercaptoethanol/0.1 mM-EDTA]. This fraction (7.6 ml) was applied to a 1 ml column of Ap_4A–Sepharose, and the column was washed successively under gravity with 2 ml of loading buffer, 2 ml of loading buffer containing 50 mM-NaCl, and 8 ml of loading buffer containing 100 μM-Ap_4A. The activity which eluted with Ap_4A was dialysed overnight against 2 x 1 litre of HPHT equilibration buffer (10 mM-potassium phosphate, pH 6.8/10 μM-$CaCl_2$) and 5.8 ml was applied at room temperature to a 100 mm x 7.8 mm Bio-Gel HPHT column (Bio-Rad) at 0.7 ml/min. After elution of unbound protein, the column was eluted with a 31.5 ml gradient of 10–500 mM-potassium phosphate, pH 6.8/10 μM-$CaCl_2$. Fractions containing Np_4Nase activity were re-run on the same HPHT column under identical conditions, and finally concentrated by chromatography on Mono-Q as described above, except that Tris/HCl and NaCl replaced the Tris/acetate and sodium acetate in the buffers. Active fractions were brought to 50% (v/v) glycerol/100 μg of bovine serum albumin/ml and stored in aliquots at −70 °C. Homogeneous enzyme was obtained on three occasions using this procedure.

RESULTS

Purification of Np_4Nase from *Artemia* cysts

Throughout the purification, fractions were assayed immediately with the luminescence assay. Pooled fractions were subsequently quantified with the radiolabel assay (DEAE, method 2). Np_4Nase activity was eluted from Q-Sepharose as two or three overlapping peaks between 0.25 M- and 0.35 M-NaCl (results not shown). Such a charge heterogeneity has been noted before during chromatography of the *Artemia* enzyme

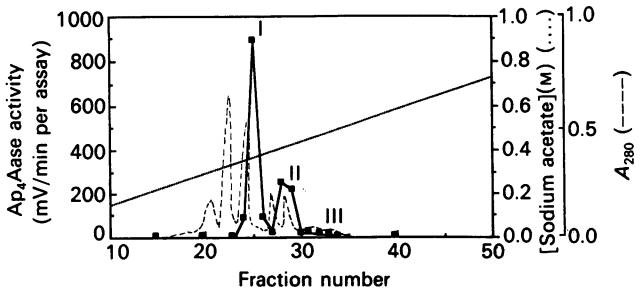

Fig. 1. Chromatography of Np_4Nase on Mono-Q

Conditions for chromatography were as described in the Experimental section. Fractions (3 ml) were collected and 2 μl portions were assayed for Ap_4Aase activity with the luminescence assay, method 1 (■). Fractions 25 and 26 (peak I, 60% of total) were combined for further purification and fractions 27–29 (peak II, 30% of total) were pooled separately and stored at −70 °C. Absorbance at 280 nm (——) and the sodium acetate gradient (.....), are shown.

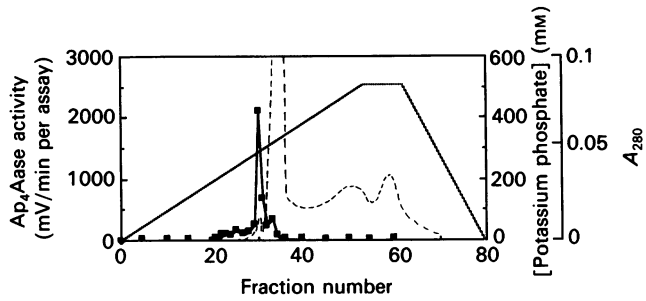

Fig. 2. Chromatography of Np_4Nase on Bio-Gel HPHT

Conditions for chromatography were as described in the Experimental section. Fractions (0.5 ml) were collected and 5 μl portions were assayed for Ap_4Aase activity with the luminescence assay, method 1 (■). 70% of the eluted activity was contained in fractions 28–31, which were retained. Absorbance at 280 nm (——) and the potassium phosphate gradient (.....), are shown.

on CM-cellulose [2] but has not been reported for similar enzymes from other sources. All these forms eluted together as a single peak from Ultrogel AcA44. This column efficiently separated the Np_4Nase from a specific Np_3Nase which eluted with an M_r of 110000–120000 (M. Prescott, A. D. Milne & A. G. McLennan, unpublished work).

The high resolving power of FPLC on Mono-Q clearly shows the heterogeneity of the Np_4Nase with two principal peaks (peaks I and II) eluting in the ratio 2:1 followed by a third minor activity (peak III) (Fig. 1). This does not appear to be the result of proteolysis since the pattern is reproducible and, although proteinase inhibitors were not employed in the bulk preparation because neutral proteinase activity in the cysts is extremely low, inclusion of phenylmethanesulphonyl fluoride (1 mM), leupeptin (0.1 mM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (10 μM), EDTA (1 mM), pepstatin (1 μM), bestatin (10 μM) and soybean trypsin inhibitor (100 μg/ml) during small-scale extractions affected neither the yield nor the distribution of enzyme activity between peaks I and II (results

not shown). Peak I was further purified by stepwise affinity elution from Ap_4A–Sepharose. This is the first time that such an affinity medium has been reported in the purification of an Ap_4A-binding protein. Final purification to homogeneity was achieved by chromatography on Bio-Gel HPHT with the activity eluting near the start of the gradient (Fig. 2). The large u.v.-absorbing peak eluting just after the Np_4Nase is residual Ap_4A from the affinity elution step. After rechromatography on Bio-Gel HPHT (to remove final traces of contaminants) and concentration, the final fraction was judged to be homogeneous by SDS/polyacrylamide-gel electrophoresis and silver staining [33,34] (Fig. 3). The overall yield was 2.7%, with a purification of 11000-fold over the crude extract (Table 1). This table shows that the BioGel HPHT gave a particularly high degree of purification. Using figures of 4000 cells/embryo and 9×10^4 embryos/g wet wt. of cysts [35], we estimate that there are approx. 10^5 molecules of the Np_4Nase per cell.

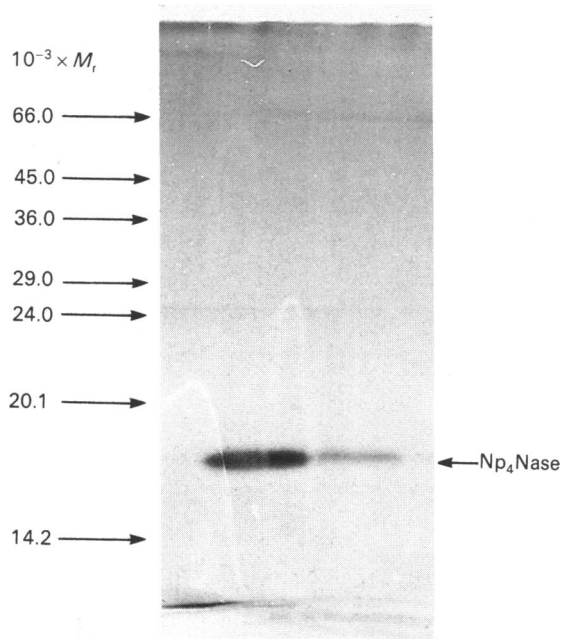

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified Np_4Nase

Portions (0.1 μg) of each of the two peak fractions from the final Bio-Gel HPHT purification step were run on a 15% polyacrylamide gel in the presence of SDS and stained with silver [34]. M_r markers (Sigma) were lactalbumin (14200), soybean trypsin inhibitior (20100), trypsinogen (24000), carbonic anhydrase (29000), glyceraldehyde 3-phosphate dehydrogenase (36000), ovalbumin (45000) and bovine serum albumin (66000).

Molecular mass

Calibration of the Ultrogel AcA44 column with standard proteins gave a native M_r of 17000 for the Np_4Nase (not shown). The latter values agrees well with previous estimates for the *Artemia* enzyme [4]. *Asymmetrical*-Np_4Nases from other sources have relative molecular masses of 17500 (human) [15], 18500 (lupin) [16], 20000–22000 (rat) [10,12], 19800 (Ehrlich ascites mouse cells) [14] and 64000 (mouse liver) [13]. The polypeptide M_r of 17600 shows the native enzyme to be a monomer (Fig. 3) and therefore similar in this respect to the other enzymes.

Effect of pH and monovalent cations on Np_4Nase activity

Maximum activity in the absence of auxiliary assay enzymes (assay methods 3 and 4) was displayed at pH 8.4 in bicine/KOH. Activity was 20% of maximum in Mes/KOH at pH 6.5, and 27% of maximum in glycine/KOH at pH 10.0. All buffers were used at a constant ionic strength of 0.1. No significant buffer effect within the pH range 7.5–9.5 was observed with a variety of buffers except for Tris (65–70% of the maximum). This may reflect an irreversible loss of activity in Tris which has been noted during storage of the enzyme in Tris buffers and which probably accounts for the losses of activity during purification when Tris buffers are used.

The activity was neither inhibited nor stimulated by Na^+, NH_4^+ or K^+ ions up to 0.1 M. At 0.2 M these ions inhibited activity by 55%, 18% and 5% respectively.

Effect of divalent cations on Np_4Nase activity

Artemia Np_4Nase is similar to all other asymmetrically-cleaving enzymes in its absolute requirement for a divalent cation. Mg^{2+} was the most effective in the range 1–5 mM, with Mn^{2+} 35% as effective at 0.1 mM (Fig. 4*a*). Mn^{2+} also promotes partial activity of the other Np_4Nases. In combination, these ions stimulated activity

Table 1. Purification of bis(5′-nucleosidyl) tetraphosphate pyrophosphohydrolase from *Artemia* embryos

Details of the purification are given in the Experimental section. Activities of each pooled fraction were determined by assay method 2 after dialysis of a sample to remove salts. One unit of Np_4Nase activity is defined as the amount which degrades 1 nmol of Ap_4A per min at 37 °C.

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Crude supernatant	670	7640	20712	2.7	100
50–100% ammonium sulphate cut	146	4220	22075	5.2	107
Q-Sepharose	31.5	246	9323	38	45
Ultrogel AcA44	206	95	6773	71	33
Mono-Q	7.6	9.4	2468	262	12
Ap_4A–Sepharose	5.8	1.6	1276	798	6.2
Bio-Gel HPHT	2.0	0.019	569	29947	2.7

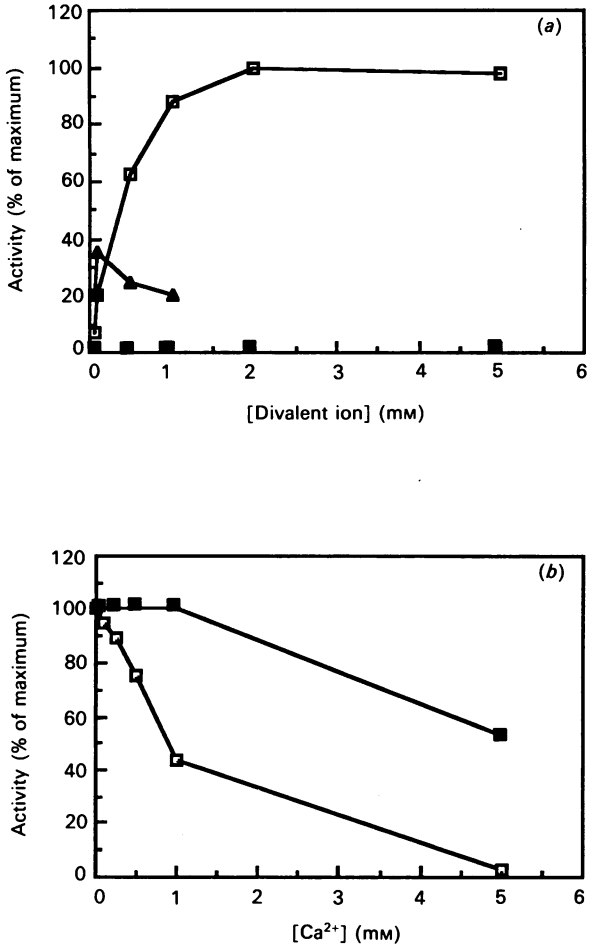

Fig. 4. (*a*) Stimulation and (*b*) inhibition of Np_4Nase activity by divalent cations

Np_4Nase (2.7 ng) was incubated under the conditions of assay method 2 in the presence of (*a*) different concentrations of Mg^{2+} (□), Mn^{2+} (▲) or Ca^{2+} (■); or (*b*) 2 mM-Mg^{2+} (□) or 20 mM-Mg^{2+} (■) and different concentrations of Ca^{2+}. Control experiments showed that the excess alkaline phosphatase was still sufficiently active under all these conditions not to affect the outcome of the assay. Points represent the averages of triplicate determinations.

of the *Artemia* enzyme by a further 20% at 0.2 mM-Mn^{2+} and 2 mM-Mg^{2+}. Ca^{2+} did not support activity at any concentration, as has also been reported for the rodent and lupin enzymes [11,12,14,16]. In both these cases, as here, Ca^{2+} actually inhibited the Mg^{2+}-dependent reaction (Fig. 4*b*). The inhibition would appear to be competitive with Mg^{2+} as it was relieved by increasing the Mg^{2+} concentration to 20 mM.

The role of Zn^{2+} in the modulation of cellular Ap_4A levels has been stressed as a result of their stimulation of the synthesis of Ap_4A by aminoacyl-tRNA synthetases and their inhibition of the activity of certain degradative Np_4Nases [23,26]. However, although rat liver Np_4Nase [37] and Ap_4A-hydrolysing activity in crude extracts of BHK cells and sea urchins [36,38] are inhibited by low concentrations of Zn^{2+} (e.g. IC_{50} for rat liver enzyme of 2 μM), the *Artemia* enzyme is unaffected by Zn^{2+} up to a concentration of 100 μM even when pre-incubated with Zn^{2+} in the absence of Mg^{2+}. Zn^{2+} can in fact promote

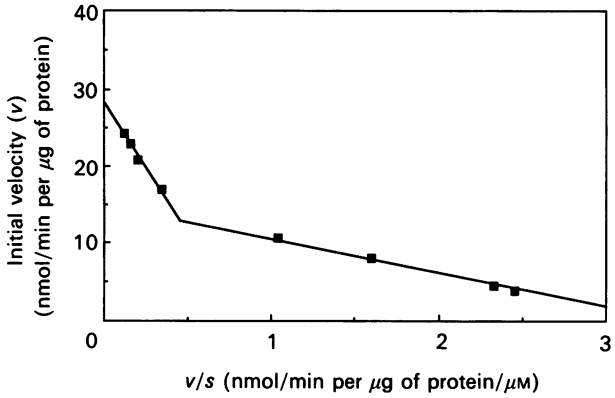

Fig. 5. Eadie–Hofstee plot of the hydrolysis of Ap_4G by *Artemia* Np_4Nase

Np_4Nase (1.1 ng) was incubated for 2, 5 or 10 min with Ap_4G (0.2–150 μM) under the conditions described for the luminescence assay (method 1). Initial rates of ATP + GTP production were then determined from the results. Points are averages of triplicate determinations and were fitted by linear least-squares analysis.

activity (7% of maximum at 2 mM in the absence of Mg^{2+}).

Kinetic parameters for Np_4N substrates

These were calculated with the 'HYPER' hyperbolic curve-fitting computer program and from Eadie–Hofstee plots. When the Np_4Nase was assayed by method 2 (alkaline phosphatase present), a K_m of 33 μM and a rate constant of 12.7 s^{-1} were determined for Ap_4A. This K_m value is much higher than previous estimates of 2 μM for the partially purified enzyme [4] and of 4.2 μM from our own previous work using the luminometer assay (without alkaline phosphatase) [39]. Values for other asymmetrical Np_4Nases are all reported within the range 0.5–5 μM. When K_m and $k_{cat.}$ were redetermined by assay method 3 (alkaline phosphatase omitted), values of 4.4 μM and 1.5 s^{-1} respectively were obtained, which represent 7.5- and 8.5-fold reductions. The latter value for the rate constant is in close agreement with that calculated for the homogeneous lupin enzyme [16] of 1.2 s^{-1}. The reason for this discrepancy is not clear. However, pretreatment of the Ap_4A with alkaline phosphatase results in the higher values of K_m and $k_{cat.}$, suggesting the possible presence of an alkaline phosphatase-sensitive uncompetitive inhibitor in the commercial Ap_4A. Some adenine nucleotide preparations have previously been shown to contain adenosine 5′-tetraphosphate (Ap_4) [37,40], a powerful competitive inhibitor of Np_4Nase [3,10,14]. The nature of the material causing the inhibition in our experiments remains to be determined; nevertheless we report this finding here to indicate that some previous determinations of the kinetic parameters of Np_4Nases may not have allowed for such factors, particularly when commercial preparations of Ap_4A have been used without repurification. Assuming that the inhibitor may be related to Ap_4A (cf. Ap_4), the co-incubation with alkaline phosphatase employed here ensures compensation for any spontaneous generation of inhibitor which might occur upon storage.

The K_m for Gp_4G (pretreated with alkaline phosphatase and assayed by method 1) was 5 μM as previously

Table 2. Substrate specificity of the *Artemia* Np_4Nase

Rates of substrate hydrolysis relative to Ap_4A (100%) were determined in duplicate by h.p.l.c. (assay method 4, with alkaline phosphatase) at a fixed concentration of each nucleotide (400 μM), and therefore do not necessarily reflect true relative maximum velocities, as kinetic parameters were not determined for all substrates. The identity of the products was determined in the absence of alkaline phosphatase.

Substrate	Relative hydrolysis (%)
Ap_2A	0
Ap_3A	0.5
Ap_4A	100
Ap_5A	25
Ap_6A	18
Gp_2G	0
Gp_3G	2
Gp_4G	48
Gp_5G	10
Ap_4G	60

shown [4], with $k_{cat.} = 6.2\,s^{-1}$. Specificity constants ($k_{cat.}/K_m$) for Ap_4A and Gp_4G are therefore $3.84 \times 10^5\ M^{-1} \cdot s^{-1}$ and $1.22 \times 10^6\ M^{-1} \cdot s^{-1}$ respectively. These values show that the enzyme has an approx. 3-fold preference for Gp_4G over Ap_4A as a substrate.

As Ap_4G has been proposed as a substrate for the *Artemia* Np_4Nase *in vivo* [9], we have also investigated its behaviour as a substrate for this enzyme *in vitro*. With this nucelotide, the Np_4Nase exhibited biphasic reaction kinetics as illustrated by an Eadie–Hofstee plot (Fig. 5). From this plot, apparent K_m values of 4.7 μM and 34 μM were calculated and the corresponding rate constants were $6.5\,s^{-1}$ and $11.9\,s^{-1}$. The net rate of Ap_4G hydrolysis observed by h.p.l.c. (assay method 4) at an Ap_4G concentration of 400 μM was $7.6\,s^{-1}$.

Substrate specificity and products

The Np_4Nase from lupin seeds has been shown to required a minimum of four bridging phosphates for activity and always produces an NTP as one of the products [16]. The *Artemia* enzyme shows the same specificity, giving appreciable hydrolysis of Ap_5A, Ap_6A and Gp_5G in addition to the preferred substrates Ap_4A, Gp_4G and Ap_4G (Table 2). An NTP was always one of the products. Low but detectable activity was also found with Ap_3A and Gp_3G (0.5% and 2% of maximum respectively); in this case the products were the respective NMP and NDP. Ap_2A and Gp_2G were not substrates. When the products of Ap_4G cleavage were investigated by h.p.l.c. after digestion to completion, a marked asymmetry in the cleavage pattern was found: AMP and GTP were present in a 4.5-fold excess over GMP and ATP.

Comparison of peak I and peak II Np_4Nase activities

The clear resolution of two major forms of the *Artemia* Np_4Nase with different net charges but the same molecular mass contrasts with what has been observed with other Np_4Nases. This may simply reflect the high resolving power of the Mono-Q column which we have used in our studies or it may be an *Artemia*-specific phenomenon. Since the existence of two Np_4Nases, one relatively specific for Ap_4A and the other for Gp_4G, would offer a neat solution to the problem of degradation specificity, the assay requirements and kinetic parameters of the peak II fraction from Mono-Q were examined. However, no significant differences were found between the two activities. The significance of these two forms of the enzyme therefore remains unclear.

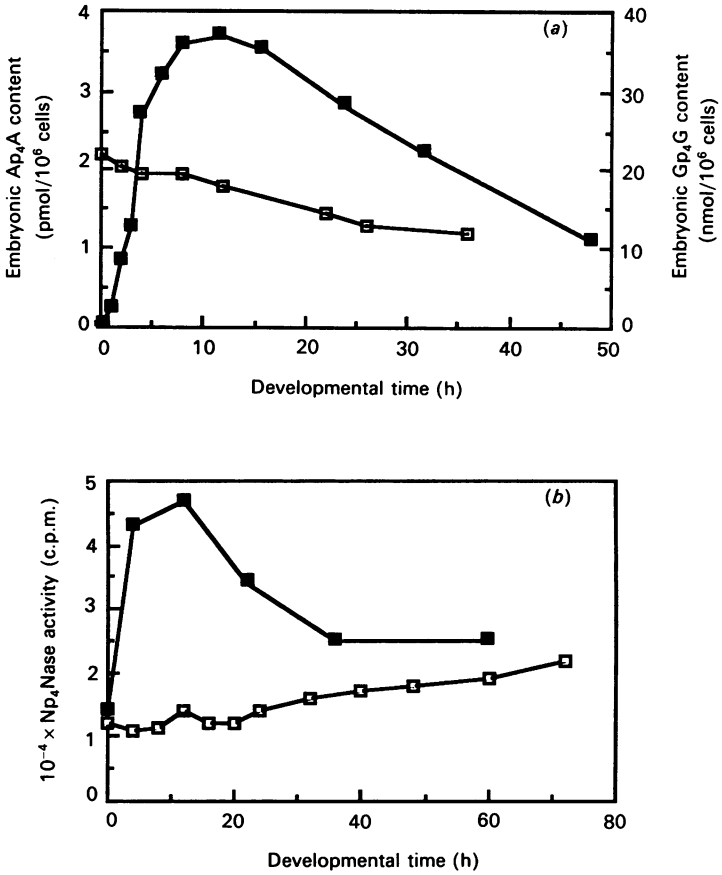

Fig. 6. Changes in (*a*) bis(5′-nucleosidyl) tetraphosphate concentrations and (*b*) Np_4N pyrophosphohydrolase activity during embryonic and early larval development of *Artemia*

(*a*) Ap_4A (■) and Gp_4G (□) pool sizes were measured as previously described [27,41]. (*b*) Crude extracts were prepared by homogenizing 1 g of decapsulated cysts or larvae per ml of 10 mM-potassium phosphate, pH 7.5/10% (v/v) glycerol/5 mM-2-mercaptoethanol/1 mM-EDTA/1 mM-phenylmethylsulphonyl fluoride, 100 μg of soybean trypsin inhibitor/ml with a Potter–Elvehjem homogenizer. After high-speed centrifugation and dialysis, 200 μl samples were layered on 5–20% (w/v) sucrose gradients in homogenizing buffer and centrifuged at 182000 *g* for 21 h. Crude extract (0.5 μl) (□) and 0.5 μl of gradient fractions were assayed for Np_4Nase activity by method 2. The Np_4Nase activity of active fractions was summed for each gradient (■). Gradients were run in duplicate. Similar results were obtained from two preparations of cysts.

Changes in Np_4Nase activity during embryonic and early larval development

One possible way of determining the relative importance of the *Artemia* Np_4Nase to the metabolism of

adenine- and guanine-containing bis-(5′-nucleosidyl) oligophosphates is to correlate its activity with the pool sizes of these nucleotides in cysts and embryos. Gp_4G pools show a slow decline from the onset of redevelopment at a rate of 0.2 pmol/h per embryo, increasing to 1.5 pmol/h per embryo after hatching [1,6,7,41]. In contrast, Ap_4A accumulates rapidly up to the point of hatching to reach a maximum level of 15 fmol/embryo (3.7 pmol/10^6 cells). Thereafter it declines at an average rate of about 0.35 fmol/h per embryo [27] (Fig. 6*a*).

When Np_4Nase activity was determined in crude extracts of decapsulated cysts and larvae at various times after the re-initiation of development, little change was observed until hatching; however, a gradual 1.8-fold increase in activity occurred between hatching and 75 h (Fig. 6*b*). This is in good agreement with original observations on this enzyme [42]. However, a very different picture emerged when the extracts were subjected to sucrose-gradient sedimentation analysis and the activities of the fractions containing Np_4Nase summed. In this case, activity was seen to increase by 3.2-fold up to hatching and thereafter fall again to the level characteristic of 72 h larvae (Fig. 6*b*). This pattern more closely reflects changes in the Ap_4A pool over the same period rather than Gp_4G. The reason why this change in activity is masked in crude extracts is not clear but could reflect the separation of the Np_4Nase from an inhibitor or regulatory factor. Further work will be required to clarify this point.

DISCUSSION

The relatively mild extraction conditions employed in this study appear to have avoided the extraction of non-specific phosphodiesterases which would interfere with the quantification of the Np_4Nase at early stages of the purification. It is the only enzyme capable of hydrolysing Ap_4A and Gp_4G in our extracts.

Except in the case of Zn^{2+}, the behaviour of the *Artemia* Np_4Nase towards divalent cations is similar to the other enzymes of this class. As the lupin Np_4Nase is also different in being only slightly inhibited by Zn^{2+}, the proposed role of Zn^{2+} as a general regulator of Ap_4A metabolism and therefore cellular proliferation [23,26] requires further investigation. Where comparable data exist for other *asymmetrical*-Np_4Nases, similar specificities towards bis(5′-nucleosidyl) oligophosphates are observed. The preference for tetraphosphates is a general characteristic of the *asymmetrical*-Np_4Nases while the symmetrically-cleaving enzymes appear to be less stringent in this respect. For example, the *E. coli* enzyme hydrolyses Ap_3G at 25% of the rate of Ap_4A [17], and the *Physarum* enzyme degrades Ap_5A and Ap_6A at 200% and 125% respectively of the rate of Ap_4A [20].

The ability of asymmetric nucleotides of general structure Np_nN' to act as substrates for Np_4Nases has only been reported for the lupin [16] and *E. coli* [17] enzymes. The lupin Np_4Nase hydrolyses Ap_4G, Ap_4C and Ap_4U at 27%, 67% and 73% of the rate of Ap_4A respectively, and in each case all four expected products have been detected, but their relative amounts were not reported. With substrates of the form Np_3N, the behaviour of the *E. coli* enzyme depends on the nature of N. Ap_3C yields similar amounts of AMP, ADP, CMP and CDP whereas Ap_3G and Gp_3C give only ADP+GMP and CDP+GMP respectively [17]. A comparison of the kinetic parameters of Ap_4G hydrolysis with those of Ap_4A and Gp_4G shows that the *Artemia* Np_4Nase can accept Ap_4G into its active site in either of two orientations with the affinities and resultant reaction rates characteristic of Ap_4A and Gp_4G. This result, and the fact that an NTP is always a product of Np_nN hydrolysis, support a model in which the enzyme recognizes the pppN moiety of a potential substrate [16]. Thus, according to this model, Ap_4G is more likely to bind with the higher affinity pppG end in the active site (K_m for $Gp_4G = 5\ \mu M$, $K_m{}^a$ for $Ap_4G = 4.7\ \mu M$) and be cleaved at the lower rate ($k_{cat.}$ for $Gp_4G = 6.2\ s^{-1}$, $K_{cat.}{}^a$ for $Ap_4G = 6.5\ s^{-1}$) than it is to bind with the lower affinity Appp end in the binding site (K_m for $Ap_4A = 33\ \mu M$, $K_m{}^b$ for $Ap_4G = 34\ \mu M$) when it is cleaved at the higher rate ($k_{cat.}$ for $Ap_4A = 12.7\ s^{-1}$, $k_{cat.}{}^b$ for $Ap_4G = 11.9\ s^{-1}$). This differential binding of Ap_4G in two orientations would therefore account for the overall rate of hydrolysis at saturating substrate concentration of $7.6\ s^{-1}$ which results in the observed 4.5-fold excess of AMP+GTP over ATP+GMP. This pattern of cleavage appears to be inconsistent with the model of Van Denbos & Finamore for Gp_4G catabolism which, in its simplest form, requires preferential production of ATP and GMP from an Ap_4G intermediate [9].

The data which we have provided here confirm that the *Artemia* Np_4Nase is indeed a member of a widespread family of enzymes with similar properties (although the mouse liver Np_4Nase does appear to be different) [13]. Therefore, as this enzyme cannot be regarded as unique to *Artemia*, its role in the metabolism of Gp_4G in this organism must be reconsidered. Ap_4A breakdown must be regarded as the likeliest function of this enzyme in other organisms and therefore also in *Artemia*. The similarity between the developmental changes in Np_4Nase activity and Ap_4A concentration may indicate a primary role for this enzyme in Ap_4A metabolism. A rise in the activity of Np_4Nase is not necessarily in conflict with a concomitant increase in the Ap_4A pool, as this nucleotide has a high turnover rate [25] and a rise in its concentration may be accompanied by unequal increases in its rates of both synthesis and degradation. Although Gp_4G and several other non-adenylated bis(5′-nucleosidyl) oligophosphates have recently been detected at low concentrations in yeast and in *E. coli* [43], *Artemia* is unique in possessing levels of this nucleotide which, if accessible to the Np_4Nase, might seriously compromise Ap_4A degradation. Therefore, if this enzyme is indeed responsible for the catabolism of Ap_4A, or even both nucleotides, there must exist some form of control of its activity towards these substrates, e.g. compartmentalization of enzyme and the alternative substrates or differential regulation by ligands or other proteins. The precise nature of such controls remains to be established.

The financial support of the Science and Engineering Research Council (Grant GR/E/26358), The Wellcome Trust (Grant 17218/1.5) and The Royal Society is gratefully acknowledged.

REFERENCES

1. Finamore, F. J. & Warner, A. H. (1963) J. Biol. Chem. **238**, 344–348
2. Warner, A. H. & Finamore, F. J. (1965) Biochemistry **4**, 1568–1575

3. Vallejo, C. G., Sillero, M. A. G. & Sillero, A. (1974) Biochim. Biophys. Acta **358**, 117–125
4. Vallejo, C. G., Lobaton, C. D., Quintanilla, M., Sillero, A. & Sillero, M. A. G. (1976) Biochim. Biophys. Acta **438**, 304–309
5. Warner, A. H. & Finamore, F. J. (1965) Biochim. Biophys. Acta **108**, 525–530
6. Warner, A. H. (1969) in Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators (Koch, G. & Richter, D., eds.), pp. 161–177, Academic Press, New York
7. Warner, A. H. (1980) in The Brine Shrimp *Artemia*, Vol. 2, Physiology, Biochemistry, Molecular Biology (Persoone, G., Sorgeloos, P., Roels, O. & Jaspers, E., eds.), pp. 105–109, Universa Press, Wetteren, Belgium
8. Sillero, A. & Günther Sillero, M. A. (1987) in *Artemia* Research and its Applications, vol. 2, Physiology, Biochemistry, Molecular Biology (Decleir, W., Moens, L., Slegers, H., Jaspers, E. & Sorgeloos, P., eds.), pp. 289–308, Universa Press, Wetteren, Belgium
9. Van Denbos, G. & Finamore, F. J. (1974) J. Biol. Chem. **249**, 2816–2818
10. Lobaton, C. D., Vallejo, C. G., Sillero, A. & Sillero, M. A. G. (1975) Eur. J. Biochem. **50**, 495–501
11. Cameselle, J. C., Costas, M. J., Günther Sillero, M. A. & Sillero, A. (1984) J. Biol. Chem. **259**, 2879–2885
12. Cameselle, J. C., Costas, M. J., Günther Sillero, M. A. & Sillero, A. (1982) Biochem J. **201**, 405–410
13. Höhn, M., Albert, W. & Grummt, F. (1982) J. Biol. Chem. **257**, 3003–3006
14. Moreno, A., Lobaton, C. D., Günther Sillero, M. A. & Sillero, A. (1982) Int. J. Biochem. **14**, 629–634
15. Ogilvie, A. & Antl, W. (1983) J. Biol. Chem. **258**, 4105–4109
16. Jakubowski, H. & Guranowski, A. (1983) J. Biol. Chem. **258**, 9982–9989
17. Plateau, P., Fromant, M., Brevet, A., Gesquière, A. & Blanquet, S. (1985) Biochemistry **24**, 914–922
18. Guranowski, A., Jakubowski, H. & Holler, E. (1983) J. Biol. Chem. **258**, 14784–14789
19. Barnes, L. D. & Culver, C. A. (1982) Biochemistry **21**, 6123–6128
20 Garrison, P. N., Robberson, G. M., Culver, C. A. & Barnes, L. D. (1982) Biochemistry **21**, 6129–6133
21. Zamecnik, P. C. (1983) Anal. Biochem. **134**, 1–13
22. Baril, E. F., Coughlin, S. A. & Zamecnik, P. C. (1985) Cancer Invest. **3**, 465–471
23. Grummt, F. (1988) in Modern Cell Biology (Satir, B. H., ed.), vol. 6, pp. 29–64, Alan R. Liss Inc., New York
24. Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W. & Ames, B. N (1984) Cell **37**, 225–232
25. Rapaport, E. & Zamecnik, P. C. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 3984–3988
26. Weinmann-Dorsch, C., Hedl, A., Grummt, I., Albert, W., Ferdinand, F.-J., Friis, R. R., Pierron, G., Moll, W. & Grummt, F. (1984) Eur. J. Biochem. **138**, 179–185
27. McLennan, A. G. & Prescott, M. (1984) Nucleic Acids Res. **12**, 1609–1619
28. Renart, M.-F., Renart, J., Sillero, M. A. G. & Sillero, A. (1976) Biochemistry **15**, 4962–4966
29. Ezquieta, B. & Vallejo, C. G. (1986) Biochim. Biophys. Acta **883**, 380–382
30. Ng, K. E. & Orgel, L. E. (1987) Nucleic Acids Res. **15**, 3573–3581
31. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Anal. Biochem. **150**, 786–793
32. Krystal, G. (1987) Anal. Biochem. **167**, 86–96
33. Laemmli, U.K. (1970) Nature (London) **227**, 680–685
34. Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) Anal. Biochem. **118**, 197–203
35. Warner, A. H., Puodziukas, J. G. & Finamore, F. J. (1972) Exp. Cell Res. **70**, 365–375
36. Grummt, F., Weinmann-Dorsch, C., Schneider-Schaulies, J. & Lux, A. (1986) Exp. Cell Res. **163**, 191–200
37. Cameselle, J. C., Costas, M. J., Günther Sillero, M. A. & Sillero, A. (1983) Biochem. Biophys. Res. Commun. **113**, 717–722
38. Morioka, M. & Shimada, H. (1987) Exp. Cell Res. **169**, 57–62
39. Blackburn, G. M., Taylor, G. E., Thatcher, G. R. J., Prescott, M. & McLennan, A. G. (1987) Nucleic Acid Res. **15**, 6991–7004
40. Costas, M. J., Montero, J. M., Cameselle, J. C., Günther Sillero, M. A. & Sillero, A. (1984) Int. J. Biochem. **16**, 757–762
41. Warner, A. H. & Finamore, F. J. (1967) J. Biol. Chem. **242**, 1933–1937
42. Beers, P. C. (1971) Ph.D. Dissertation, University of Windsor, Ontario, Canada
43. Coste, H., Brevet, A., Plateau, P. & Blanquet, S. (1987) J. Biol. Chem. **262**, 12096–12103

Received 4 November 1988/22 December 1988; accepted 10 January 1989