Assay of adenosine 5'-P¹-tetraphospho-P⁴-5'''-adenosine and adenosine 5'-P¹-tetraphospho-P⁴-5'''-guanosine in *Physarum polycephalum* and other eukaryotes

An isocratic high-pressure liquid-chromatography method

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A5'pppp5'A has been proposed to serve as a molecular signal that triggers DNA replication. When published methods proved to be inadequate for the assay of A5'pppp5'A in *Physarum polycephalum* by h.p.l.c. (high-pressure liquid chromatography), a set of purification procedures was developed that allowed assay of as little as 2 pmol of A5'pppp5'A. A5'pppp5'A was purified from cellular extract by covalent boronate chromatography, treated with alkaline phosphatase to hydrolyse residual mononucleotides and analysed by isocratic ion-exchange h.p.l.c. The analysis was facilitated by a pre-column switching procedure that allowed early-eluted species to be diverted from the analytical column. By using this procedure A5'pppp5'A has been detected in *Physarum polycephalum* (1.4 pmol/mg of protein), *Saccharomyces cerevisiae* (3.6 pmol/mg of protein) and rat liver (3.3 pmol/mg of protein). In each case a minor peak was also seen, which was identified as A5'pppp5'G. The identity of both peaks was confirmed by co-elution with standards on isocratic and gradient h.p.l.c. and treatment with enzymes, including a dinucleoside polyphosphate pyrophosphohydrolase from *Physarum polycephalum*.

Ap₄A was first described as a compound of biochemical significance when it was observed as a side reaction in the activity *in vitro* of lysyl-tRNA synthetase from *Escherichia coli* (Zamecnik *et al.*, 1966; Randerath *et al.*, 1966). The Zn²⁺-stimulated synthesis of Ap₄A and other dinucleoside polyphosphates by some tRNA synthetases from *E. coli*, yeast and *Physarum polycephalum* has been reported (Plateau *et al.*, 1981; Plateau & Blanquet, 1982; Goerlich *et al.*, 1982). A regulatory role for Ap₄A was suggested by Zamecnik and his colleagues, on the basis of measurements of widely varied intracellular amounts in cultured cells, dependent on proliferative activity (Rapaport & Zamecnik, 1976). This proposal was supported by Grummt's (1978) report of stimulation of initiation of DNA replication in permeabilized quiescent baby-hamster kidney cells treated with Ap₄A. It

was later reported that Ap₄A binds to purified DNA polymerase α from thymus (Grummt *et al.*, 1979) and HeLa cells (Rapaport *et al.*, 1981a). The significance of this binding activity for DNA synthesis is not clear, since Ap₄A was found to have no effect on the activity *in vitro* of DNA polymerase α , β or γ (Ono *et al.*, 1980). A novel possibility is suggested by the observation that Ap₄A can serve as a primer for the synthesis of oligodeoxyribonucleotides with either poly(dT) (Rapaport *et al.*, 1981b) or a double-stranded synthetic fragment of the simian-virus-40 replication origin as template (Zamecnik *et al.*, 1982).

Since it appears that Ap₄A may be involved in regulation of cell-cycle events, we are interested in looking for changes in its concentration during the cell cycle and differentiation processes of the slime mould *Physarum polycephalum*. We have already reported on a new enzyme from this organism that is specific for the nucleoside diphosphate-yielding hydrolysis of dinucleoside polyphosphates in which the phosphate number is greater than 2 (Barnes & Culver, 1982; Garrison *et al.*, 1982). We were able to detect the synthesis of Ap₄A in *P. polycephalum* (Garrison *et al.*, 1981) by using [³H]-

Abbreviations used: Ap₄A, A5'pppp5'A, adenosine 5'-P¹-tetraphospho-P⁴-5'''-adenosine; Ap₄G, A5'pppp5'G, adenosine 5'-P¹-tetraphospho-P⁴-5'''-guanosine; other dinucleoside 5',5'''-polyphosphates are abbreviated in a similar fashion; h.p.l.c., high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

adenosine in an adaption of the method of Rapaport & Zamecnik (1976). However, this method is tedious and expensive. Two isocratic h.p.l.c. methods for measurement of Ap₄A in cellular extracts have been reported (Plesner & Ottesen, 1980; Nierenberg *et al.*, 1980), but neither provides adequate separation of Ap₄A from other u.v.-absorbing components in extracts of *P. polycephalum*.

In the present paper we report a set of methods for purification of Ap₄A from extracts of *P. polycephalum* and quantitative assay by isocratic h.p.l.c. During analysis of *P. polycephalum* samples, a second peak was seen, which was identified as Ap₄G. Ap₄A and Ap₄G were also detected in rat liver and in the yeast *Saccharomyces cerevisiae*. This is the first report of Ap₄A in *P. polycephalum* and *S. cerevisiae* and of Ap₄G in any eukaryotic species.

Experimental procedures

Materials

Ap₄A was purchased from Sigma Chemical Co., labelled with tritium by Amersham International and analysed for purity as described previously (Barnes & Culver, 1982). Partisil 10 SAX h.p.l.c. resin and columns were from Whatman. An empty h.p.l.c. pre-column was from Beckman Instruments. Venom phosphodiesterase I was from Worthington Biochemicals. Bio-Rex 70 (200–400 mesh) was obtained from Bio-Rad Laboratories and conjugated with aminophenylboronic acid (Aldrich Chemical Co.) as described by Wielckens *et al.* (1981). Ap₄G was a gift from Dr. M. Morozumi, Dr. A. Kuninaka, Dr. Y. Furuichi and Dr. A. Shatkin.

Experimental organisms

P. polycephalum. Microplasmidia were grown as described previously (Barnes & Culver, 1982) and collected at a density of 20–30g wet wt./l of medium (early exponential phase).

S. cerevisiae. Strain D273-10B was grown at 30°C in 1% yeast extract/2% bactopeptone/2% glucose and collected at 2×10^7 – 6×10^7 cells/ml.

Rats. Male Sprague–Dawley rats were fed *ad libitum* and used at body weights of 135–400g.

Preparation of acid-soluble extracts

P. polycephalum. Cultures were collected by centrifugation at 250g for 10s, and the pellets were homogenized in 3 vol. of 5% (w/v) trichloroacetic acid at 0°C. The homogenate was kept on ice for 30min and then centrifuged at 31 000g for 45min at 4°C.

S. cerevisiae. Cultures were collected by centrifugation at 1700g for 5min. The pellets were

suspended in 5% (w/v) trichloroacetic acid, sonicated and kept on ice for 30min. The precipitate and debris were removed by centrifugation at 31 000g for 20min.

Rat liver. Laparotomy was performed on rats anaesthetized with Inactin. Exposed livers were freeze-clamped *in situ* with liquid-N₂-cooled tongs. The frozen material was homogenized in cold 5% (w/v) trichloroacetic acid with a Potter–Elvehjem tissue grinder. Precipitate was removed by centrifugation as described for yeast.

When recovery was to be monitored, [³H]Ap₄A (2.5 Ci/mmol) was included in the trichloroacetic acid solution at 0.7 pmol/sample.

Boronate chromatography

Supernatants had acetic acid and aq. NH₃ added to give a final acetate plus trichloroacetate concentration of 1M and a pH of 9.8. An equal volume of cold ethanol was added and any precipitate removed by centrifugation. Samples were added to 8mm × 40mm columns of aminophenylboronic acid–Bio-Rex 70 pre-equilibrated with 1.0M-ammonium acetate (pH9.8)/ethanol (1:1, v/v), and columns were washed with 10ml of the same buffer. They were then washed with 18ml of 1M-ammonium acetate, pH9.8, and Ap₄A was eluted with 5ml of 80mM-ammonium acetate, pH4.5. The designated buffer concentrations refer to the acetate component.

Sample preparation for h.p.l.c.

Column eluates were freeze-dried and dissolved in 1 ml of 10mM-Hepes/NaOH buffer, pH8.5. As a routine, they were treated with 1 unit of Sigma *E. coli* alkaline phosphatase (type III) for 2h at 25°C and frozen. Among several commercial preparations of alkaline phosphatase, only the one used was sufficiently free of phosphodiesterase I. Some samples were at the same time treated with 1 unit of phosphodiesterase I or Ap₄A pyrophosphohydrolase from *P. polycephalum* [purified up to and including the Green A resin or Sephadex G-75 step (Barnes & Culver, 1982)]. Samples were filtered and pH was adjusted to 5.75 with 1M-acetic acid before analysis.

H.p.l.c. procedures

Samples were analysed on a Beckman 332 gradient chromatography system with a Beckman 160 UV detector and CR1A integrator. The system was used in a special configuration employing a 3.2mm × 40.0mm pre-column and a Rheodyne 7000 valve (described in the Results section). The pre-column was packed with Whatman Partisil 10 SAX resin in water at 10ml/min by using an h.p.l.c. pump and a 3.2mm × 250mm column

Fig. 1. Boronate chromatography of $[^3\text{H}]\text{Ap}_4\text{A}$ added to an extract of *P. polycephalum*

$[^3\text{H}]\text{Ap}_4\text{A}$ was added to a sample of extract from *P. polycephalum* prepared as described in the Experimental procedures section. The sample was then added to an 8 mm $\times$ 40 mm column of aminophenylboronic acid-Bio-Rex 70 and chromatographed as described in the Experimental procedures section. The arrows represent, from left to right, the beginning of the washes with 1M-ammonium acetate (pH 9.8)/ethanol (1:1, v/v), 1M-ammonium acetate, pH 9.8, and 80mM-ammonium acetate, pH 4.5. $\bigcirc$ --- $\bigcirc$, $[^3\text{H}]\text{Ap}_4\text{A}$ radioactivity; $\bullet$ --- $\bullet$, A_{260} .

Fig. 2. System for the pre-column purification of Ap_4A during h.p.l.c.

A high-pressure valve was used to switch a 3.2 mm $\times$ 40 mm column of Partisil 10 SAX resin between high-strength and low-strength elution buffers. Sample was loaded into a 2 ml sample loop and concentrated on the pre-column with 0.2M- $\text{NH}_4\text{H}_2\text{PO}_4$, pH 5.05 (A pump). This wash was continued until Ap_4A was almost eluted from the pre-column (4–5 min). The valve was then switched to the B position and Ap_4A was diverted to the analytical column with 0.4M- $\text{NH}_4\text{H}_2\text{PO}_4$, pH 5.05 (B pump), for isocratic chromatography. The pre-column was re-equilibrated with the A buffer for 1.5 min between samples. All flow rates were 1.0 ml/min.

extension as a reservoir. The analytical column (4.6 mm $\times$ 25 cm) was Whatman Partisil 10/25 SAX. Whatman Solvecon Pre-Conditioning columns were in line between each pump and the rest of the system. Buffers were prepared by mixing solutions of $\text{NH}_4\text{H}_2\text{PO}_4$ and $(\text{NH}_4)_2\text{HPO}_4$ at equal concentrations to achieve the desired pH. The elution procedure is described in the Results section. Peaks were detected by A_{254} and measured quantitatively by area integration. In some cases recovery was determined by counting the $[^3\text{H}]\text{Ap}_4\text{A}$ radioactivity in a portion of the collected peak.

Protein determination

Trichloroacetic precipitates were extracted twice with 95% (v/v) ethanol, dissolved in 2M-NaOH and assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Boronate chromatography

Ap_4A was separated from most u.v.-absorbing materials in cellular extracts by chromatography on a covalent chromatography resin, aminophenylboronic acid-Bio-Rex 70. The resin was found to bind Ap_4A and NADH (compounds with two *cis*-diol groupings) at high salt concentration and pH greater than 8.8, whereas mononucleotides were

eluted under the same conditions (Alvarez-Gonzales *et al.*, 1983). Ethanol was initially added to extracts of *P. polycephalum* to precipitate slime. However, we found that ethanol also resulted in the binding of mononucleotides and a lower salt requirement for binding. Most u.v.-absorbing species in extracts, including mononucleotides, were removed by washing with 1M-ammonium acetate, pH 9.8, and Ap_4A was eluted with 80mM-ammonium acetate, pH 4.5 (Fig. 1).

H.p.l.c. method

The Ap_4A fraction from the boronate columns contained relatively large amounts of NADH and other unidentified components that trailed and interfered with detection of Ap_4A . There were also trace amounts of ATP and GTP, which are eluted later than Ap_4A with the h.p.l.c. system used. In order to facilitate rapid isocratic analysis, two simple purification procedures were used. Alkaline phosphatase treatment removed all later-eluted peaks. Interference from early-eluted material was eliminated by a pre-column wash procedure, as shown in Fig. 2. Samples (1 ml) were injected and sample constituents concentrated on the pre-column with a low-strength mobile phase (A pump). The pre-column was eluted to waste with

Fig. 3. Assay of endogenous Ap_4A from *P. polycephalum*

Samples of extract corresponding to 20 mg of protein were purified and analysed as described in the text and Fig. 2 legend. (a) Treated with 1.0 unit of alkaline phosphatase for 8 h; the Ap_4A (9.74 min) assayed is 18 pmol; (b) same as (a) with 20 pmol of Ap_4A added; (c) same as (a) with 5 μ g of Ap_4A pyrophosphohydrolase added 4 h after addition of alkaline phosphatase.

the low-strength buffer for a time predetermined to leave Ap_4A just short of elution. Ap_4A was then diverted to the analytical column and eluted with high-strength mobile phase (B pump). By this method, samples could be analysed rapidly with good backgrounds for integration (Fig. 3). To avoid loss of Ap_4A from the pre-column, it was essential that samples be at low ionic strength and pH less than 6.0 to inhibit complex-formation of Ap_4A with trace bivalent cations.

Peak area of standard Ap_4A was linear with mass with a lower limit of detection of 1–2 pmol (Fig. 4). Recoveries determined by adding [3H] Ap_4A to extracts and counting the radioactivity of collected h.p.l.c. peaks were generally 45–60%.

Identification of cellular Ap_4A and Ap_4G

A peak of endogenous material from *P. polycephalum* co-migrated with Ap_4A , was resistant to alkaline phosphatase and was removed by an enzyme preparation from the same organism that is specific for dinucleoside polyphosphates (Fig. 3) (Garrison *et al.*, 1982). The peak was also completely removed by venom phosphodiesterase I and co-migrated with Ap_4A on a gradient h.p.l.c. system (Garrison *et al.*, 1982) (results not shown). Samples that were analysed on the gradient system with and without prior treatment with alkaline phosphatase showed the same amount of Ap_4A , indicating that the enzyme treatment neither generated nor removed Ap_4A (results not shown). Similar controls confirmed the identification of Ap_4A in rat liver and *S. cerevisiae* (results not shown).

A minor peak with a retention time slightly longer than that of Ap_4A was consistently present

Fig. 4. Quantitative determination of Ap_4A by peak area. Ap_4A samples were prepared in 1 ml of 10 mM-Hepes/acetate buffer, pH 5.75, and analysed by using the two-pump method described in Fig. 2 legend. The sample was washed on to the pre-column for 3.0 min at 1.0 ml/min by pump A, conditions determined to give complete recovery of Ap_4A . The Ap_4A peak area was determined by electronic integration.

in extracts of *P. polycephalum* and rat liver (Figs. 3 and 5). It was removed by dinucleoside polyphosphate pyrophosphohydrolase (Fig. 3) and phosphodiesterase I (not shown), and was co-eluted with authentic Ap_4G on isocratic (Fig. 5) and gradient (not shown) h.p.l.c. systems. A peak with the same retention time and enzyme-sensitivity was also present in *S. cerevisiae* samples (not shown).

Fig. 5. Detection of Ap_4G in *P. polycephalum* and rat liver

Samples were purified and analysed as described in the text and Fig. 2 legend. All were treated with 1.0 unit of alkaline phosphatase for 2 h. (a) *P. polycephalum*; (b) *P. polycephalum* with 7.5 pmol of Ap_4G added; (c) rat liver; (d) rat liver with 7.5 pmol of Ap_4G added. Identified peaks are Ap_4A , 11.91–11.98 min, and Ap_4G , 13.83–13.87 min. The difference in retention times from Fig. 3 is the result of variation between columns.

Table 1. Cellular concentrations of Ap_4A and Ap_4G

Ap_4A was assayed as described in the text and Fig. 2 legend. Ap_4G was determined quantitatively by using the Ap_4A standard curve and corrected for a lower ϵ_{254} by multiplying by 1.10. This factor was calculated by adding the ϵ_{254} values of ATP and GTP and assuming the same hypochromicity as Ap_4A (Randerath *et al.*, 1966). Ap_4A and Ap_4G were corrected for recovery, based on the recovery of added $[^3H]Ap_4A$. Values for Ap_4A , Ap_4G and the ratio are means $\pm$ S.E.M.

Organism	No. of samples analysed	Cellular concentration (pmol/mg of protein)		Ap_4G/Ap_4A ratio
		Ap_4A	Ap_4G	
<i>P. polycephalum</i> (exponential phase)	5	1.4 ± 0.1	0.44 ± 0.03	0.31 ± 0.005
Rat liver	5	3.3 ± 0.5	0.12 ± 0.02	0.038 ± 0.004
<i>S. cerevisiae</i> (exponential phase)	4	3.6 ± 0.6	1.03 ± 0.25	0.28 ± 0.02

Standard Ap_4G was found to purify on amino-phenylboronic acid–Bio-Rex 70 with a recovery similar to that of Ap_4A , and it was hydrolysed by dinucleoside pyrophosphohydrolase at about the same rate as Ap_4A (results not shown).

Cellular concentrations of Ap_4A and Ap_4G

Cellular Ap_4A and Ap_4G concentrations in several eukaryotic systems are shown in Table 1. The concentrations measured for *P. polycephalum* and yeast may represent minimum values, since Ap_4A has been shown to decrease rapidly under conditions, such as centrifugation, that lower the cellular energy charge (Rapaport & Zamecnik, 1976; Plesner *et al.*, 1979).

Discussion

Several h.p.l.c. procedures based on anion-exchange or reversed-phase resins have been described that are effective for analysis of dinucleoside polyphosphates in simple mixtures (Nierenberg *et al.*, 1980; Hagemeyer *et al.*, 1982; Block *et al.*, 1982). Some gradient procedures may be capable of resolving Ap_4A or other dinucleoside polyphosphates in cellular extracts (McKeag & Brown, 1978; Garrison *et al.*, 1982; Payne & Ames, 1982; Reinhart & Koroly, 1982; Flodgaard & Klenow, 1982), but generally lack the sensitivity possible with isocratic methods and are time-consuming. We were unable to detect Ap_4A in slime-

mould extracts by the gradient method of McKeag & Brown (1978) without radioactive labelling and sample pre-purification (Garrison *et al.*, 1981).

The two isocratic h.p.l.c. methods previously published for assay of cellular Ap₄A were based on treatment of acid-soluble extracts with alkaline phosphatase followed by chromatography on a Whatman Partisil 10 SAX column (Plesner & Otteson, 1980; Nierenberg *et al.*, 1980). With these methods, Ap₄A in *P. polycephalum* extracts could not be resolved on the h.p.l.c. from a large amount of early-eluted material. Treatment of crude acid-soluble extracts with alkaline phosphatase was relatively ineffective, presumably owing to P_i inhibition of the enzyme (P. N. Garrison, unpublished work). The methods of purification described in the present paper allowed analysis of cellular samples with a sensitivity and accuracy equal to that possible for standard Ap₄A and Ap₄G. The effectiveness of these methods for analysis of samples from three organisms suggests that they may be generally applicable.

A sensitive assay for Ap₄A based on luciferin/luciferase determination of ATP released by phosphodiesterase I was described by Ogilvie (1981). With a lower limit of detection of 20 fmol, the luminescence assay will be useful when sample size is limited. However, the method requires near-quantitative removal of ATP from samples before analysis, which is impractical with more than a few samples with the low-pressure gradient chromatography described (Ogilvie, 1981). The boronate chromatography described in the present paper would facilitate sample preparation for the luminescence assay. With the identification of Ap₄G and possibly other dinucleoside polyphosphates in cells (see below), care will have to be taken with the luminescence assay to identify the source of released ATP. H.p.l.c. methods, despite their lower sensitivity, offer the control of identification by retention time and the possibility of assaying multiple components.

Detection of Ap₄A has been reported in a number of cultured mammalian cell lines (Rapaport & Zamecnik, 1976; Plesner *et al.*, 1979; Nierenberg *et al.*, 1980; Ogilvie, 1981), rat liver and muscle (Zamecnik & Stephenson, 1968; Plesner *et al.*, 1979), mouse liver (Rapaport & Zamecnik, 1976), *E. coli* (Plesner *et al.*, 1979), platelets (Flodgaard & Klenow, 1982), *Tetrahymena pyriformis* (Plesner *et al.*, 1979; Plesner & Kristiansen, 1979) and *Salmonella typhimurium* (Lee *et al.*, 1983). The apparently general occurrence of Ap₄A, extended in the present paper to *P. polycephalum* and *S. cerevisiae*, supports the idea that it has a significant biological role. Measurements of Ap₄A in cultured cells of different proliferative activity (Rapaport & Zamecnik, 1976) and in the *E. coli*

growth curve (Plesner *et al.*, 1979) indicate a correlation between Ap₄A concentrations and cell growth. However, the data available at this time are too limited to tell how universal this correlation may be.

Dinucleoside polyphosphates other than Ap₄A have been reported in connection with several areas of study. Several such compounds, including Gp₄G, Gp₃G, Gp₃A and Gp₂G, are constituents of the brine shrimp *Artemia salina*, and similar dinucleotides have been reported in related species [reviewed by Warner (1979) and Silverman & Atherly (1979)]. Gp₄G is the major nucleotide in encysted embryos of *A. salina* and apparently serves as a reservoir for the synthesis of purine nucleotides during development (Warner, 1979). Ap₄G was proposed as a possible intermediate in a novel synthesis of ATP from Gp₄G in that system (Van Denbos & Finamore, 1974), but its presence in the brine shrimp has not been demonstrated. However, Ap₄G has been detected in *Salm. typhimurium* (Lee *et al.*, 1983). In the present paper we report for the first time Ap₄G as a component of eukaryotic cells. The only other instance of a dinucleoside polyphosphate besides Ap₄A as a cellular constituent of which we are aware is the statement by Zamecnik *et al.* (1967) that Ap₃A was found in commercial yeast nucleotide preparations.

Compounds with the structure Gp₄N and Gp₃N, where N is A, G, C or U, were found to be synthesized during transcription *in vitro* by virions of cytoplasmic polyhedrosis virus (Smith & Furuichi, 1982). These structures were synthesized in a large molar excess over mRNA production, apparently by an uncoupling of RNA capping from methylation and elongation. The viral synthesis of Gp₄N or Gp₃N requires GTP as donor of a GMP moiety, with the other two or three phosphate groups supplied by NDP or NTP (Smith & Furuichi, 1982). PP_i is also a product. This reaction is similar to the synthesis of Gp₄G and Gp₃G by an enzyme from the brine shrimp (Warner *et al.*, 1974; Warner & Huang, 1974). It is possible that the cellular Ap₄G reported here is synthesized by a GTP-specific enzyme similar to these. However, with our h.p.l.c. method, we have been unable to detect any cellular Gp₄G or Gp₃G in *P. polycephalum* or rat liver (P. N. Garrison & L. D. Barnes, unpublished work), making this seem unlikely.

Synthesis *in vitro* of Ap₄A and other Ap₄N compounds has been demonstrated for several aminoacyl-tRNA synthetases (Zamecnik *et al.*, 1966; Randerath *et al.*, 1966; Plateau *et al.*, 1981; Plateau & Blanquet, 1982; Goerlich *et al.*, 1982). The synthesis of Ap₄N and Ap₃N by these enzymes is apparently similar to those described

above for Gp₄N and Gp₃N except for the requirement for ATP as donor of one nucleoside monophosphate and the required formation of aminoacyl-adenylate. If Ap₄A and Ap₄G arise by this mechanism *in vivo*, the occurrence of other dinucleoside polyphosphates might be expected. In this regard, if the proposal by Zamecnik *et al.* (1982) that Ap₄A serves as a primer for initiation of DNA replication is correct, base-pairing requirements at different initiation sites might require a full complement of Ap₄N structures. In fact, when *P. polycephalum* and rat liver samples were analysed on h.p.l.c. gradients, several unidentified peaks showed the same enzyme-sensitivities as Ap₄A and Ap₄G (P. N. Garrison & L. D. Barnes, unpublished work). If further work demonstrates the presence of other dinucleoside polyphosphates in cells, the methods described here should be useful in assaying this family of compounds.

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