

# Variation in intracellular $P^1P^4$ -bis(5'-adenosyl) tetraphosphate ( $Ap_4A$ ) in virus-infected cells

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The effect of virus infection on the intracellular concentration of the proposed stress alarmone  $P^1P^4$ -bis(5'-adenosyl) tetraphosphate ( $Ap_4A$ ) has been examined in Vero cells. Compared with exposure to 0.8 mM- $Cd^{2+}$ , which causes a 30-fold increase in  $Ap_4A$ , infection with simian virus 40 and poliovirus causes only a 2-fold increase, whereas herpes simplex virus type 1 results in a decrease in  $Ap_4A$  during the course of the infection.

## INTRODUCTION

The dinucleotide  $P^1P^4$ -bis(5'-adenosyl) tetraphosphate ( $Ap_4A$ ) is present in prokaryotic and eukaryotic cells in submicromolar to micromolar concentrations and may be involved in a number of intracellular processes (Zamecnik, 1983; Grummt, 1988). Despite its ability to bind to and prime eukaryotic DNA polymerase- $\alpha$  *in vitro* (Baril *et al.*, 1985), the original proposal that it is involved in the initiation of DNA replication in eukaryotes is not supported by more recent observations: the 100–1000-fold increases in its intracellular concentration which precede the onset of S-phase in some situations have not been universally observed (McLennan & Prescott, 1984; Weinmann-Dorsch *et al.*, 1984; Garrison *et al.*, 1986; Moris *et al.*, 1987).

An alternative role for  $Ap_4A$  and related nucleotides, as alarmones signalling the onset of metabolic stress, was proposed following the observation that, in *Escherichia coli* and *Salmonella typhimurium*, the levels of these nucleotides increased dramatically following exposure to heat shock, heavy metals and oxidizing agents (Lee *et al.*, 1983*a,b*; Bochner *et al.*, 1984). However, in eukaryotes, the changes in the concentrations of  $Ap_4A$  and other dinucleotides after such treatments are generally of the order of 2–3-fold (Miller & McLennan, 1986; Guédon *et al.*, 1986); the 100–1000-fold increases typical of *E. coli* and *S. typhimurium* are only observed after treatment with lethal doses of stressors and are therefore not thought to be of physiological importance (Baltzinger *et al.*, 1986). Nevertheless, a 2–3-fold change in the steady state concentration of an intracellular effector molecule could still be of great significance, particularly if the molecule is part of a signal amplification mechanism such as a cyclic cascade.

No information exists on the behaviour of  $Ap_4A$  during virus infections; however, certain considerations would appear to justify such an investigation. First, any molecule which plays an important role in cellular metabolism may be required to a greater or lesser extent by certain viruses for successful infection and therefore deserves consideration as a potential target for new anti-viral compounds. Secondly, the possible involvement of  $Ap_4A$  in cellular DNA replication suggests that it may also be required by viruses such as simian virus 40 (SV40) which rely extensively on host cell DNA replication mechanisms; on the other hand, RNA viruses and large DNA viruses such as herpes simplex virus type 1 (HSV-1) that replicate largely independently

of host cell factors may have no requirement for  $Ap_4A$ . Finally, as viral infection may be regarded as an extreme form of cellular stress, this may be reflected in changes in the concentrations of  $Ap_4A$  and other dinucleotides after infection. Therefore, as part of our studies into the function of  $Ap_4A$ , we have determined the intracellular level of this compound in Vero cells at various times after infection with SV40, HSV-1 and poliovirus.

## MATERIALS AND METHODS

### Materials

Vero African green monkey kidney cells, medium 199 (Earle's salts) and foetal calf serum were from Gibco U.K. [*methyl-<sup>3</sup>H*]Thymidine (40–60 Ci/mmol) and [*5-<sup>3</sup>H*]uridine (25–30 Ci/mmol) were from Amersham International. Calf intestine alkaline phosphatase and snake venom phosphodiesterase were obtained from Boehringer. Phosphate-buffered saline (PBS) contained 100 g of NaCl, 2.5 g of KCl, 14.375 g of  $Na_2HPO_4$  and 2.5 g of  $KH_2PO_4$  per litre. All other materials were as previously described.

### Viruses

SV40, Toronto strain, was a gift from the Institute of Virology, University of Glasgow. HSV-1 (strain HFEM) and poliovirus (Sabin: type 1) were from stocks held in the Department of Medical Microbiology, University of Liverpool, that had been stored at  $-70^\circ C$  until use.

### Infection of cells and radiolabelling

Vero cells were grown in 75 mm<sup>2</sup> flasks at 37 °C in medium 199 supplemented with 5% (v/v) foetal calf serum in an atmosphere of air/CO<sub>2</sub> (19:1). For virus infections, cells were grown to confluence as above in 50 mm dishes in medium supplemented with 100 µg of streptomycin/ml and 100 units of penicillin/ml. After a further 2 days, the medium was removed and the cells were infected with 10 plaque-forming units (p.f.u.) of virus/cell in 1 ml of maintenance medium [medium 199 supplemented with 1.7% (v/v) foetal calf serum]; mock infections were with 1 ml of maintenance medium alone. After a 30 min adsorption period, the medium was replaced with 10 ml of fresh maintenance medium and the infected cells were harvested at various times thereafter for the determination of  $Ap_4A$ .

Abbreviations used:  $Ap_4A$ ,  $P^1P^4$ -bis(5'-adenosyl) tetraphosphate;  $Ap_4N$ , adenosine 5'- $P^1$ -tetraphospho- $P^4$ -5''-nucleoside; SV40, simian virus 40; HSV-1, herpes simplex virus type 1; PBS, phosphate-buffered saline; p.f.u., plaque-forming unit(s).

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For the measurement of DNA or RNA synthesis, dishes contained  $4 \times 14$  mm glass coverslips, and cells were pulse-labelled for periods of 30 min or 1 h at various times after infection by replacement of maintenance medium with 10 ml of fresh maintenance medium containing  $2 \mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine/ml for SV40 and HSV-1 or  $2 \mu\text{Ci}$  of [ $^3\text{H}$ ]uridine/ml plus  $1 \mu\text{g}$  of actinomycin D/ml for poliovirus (Zimmerman *et al.*, 1963). After labelling, the medium was removed and the plates were washed with PBS (twice), 5% (w/v) trichloroacetic acid ( $3 \times 5$  min), water and absolute ethanol (twice). The plates were left to dry for 24 h, and the coverslips were then removed and counted for radioactivity in 8 ml of toluene-based scintillant.

Cells were counted in a haemocytometer after trypsin treatment.

#### Measurement of $\text{Ap}_4\text{A}$

After removal of medium, cell monolayers were washed quickly with 5 ml of cold PBS and drained. Ice-cold 10% (w/v) trichloroacetic acid/5 mM-EDTA (5 ml) was then added to each dish and the cells were scraped from the dishes. The cell suspensions were then centrifuged at  $23\,500 g_{\text{av}}$  for 15 min and the supernatants were adjusted to pH 9.0 with conc. ammonia solution and to 1 M-ammonium acetate by the addition of 6 M-ammonium acetate, pH 9.0. Purification of a fraction containing nucleotides of the form  $\text{Ap}_4\text{N}$  from these cell extracts was performed by affinity chromatography on dihydroxyl-BioRex 70 as described by Baker & Jacobson (1984). For the determination of  $\text{Ap}_4\text{N}$ , the freeze-dried eluates were dissolved in 800  $\mu\text{l}$  of assay buffer [35 mM-Hepes/KOH (pH 7.8)/7 mM-magnesium acetate] containing 0.4 units of alkaline phosphatase and incubated for 30 min at 37 °C. ATP-monitoring reagent (200  $\mu\text{l}$ ) (Pharmacia-LKB) was then added, followed by 1.5  $\mu\text{g}$  of snake venom phosphodiesterase, and the increase in luminescence was recorded with an LKB 1250 Luminometer (McLennan & Prescott, 1984).

## RESULTS AND DISCUSSION

The assay system employed does not distinguish between bis(5'-nucleosidyl) oligophosphates in which both bases are adenine (e.g.  $\text{Ap}_4\text{A}$ ) and those containing a single adenine [e.g. adenosine 5'- $P^1$ -tetraphospho- $P^4$ -5'''-nucleoside ( $\text{Ap}_4\text{N}$ )]. However, since the major bis(5'-nucleosidyl) oligophosphate in mammalian cells is  $\text{Ap}_4\text{A}$  and the assay is twice as sensitive to  $\text{Ap}_4\text{A}$  as to  $\text{Ap}_4\text{N}$ , the level of  $\text{Ap}_4\text{N}$  can be taken to reflect that of  $\text{Ap}_4\text{A}$  (Moris *et al.*, 1987). In order to show that the extraction and assay procedures employed were able to detect differences in the cellular levels of nucleotides, a positive control system was required. The most satisfactory and reproducible control, which was included with each viral infection, involved treatment of confluent monolayers of Vero cells with 0.8 mM-cadmium acetate. Fig. 1 shows that this resulted in a time-dependent increase in the level of  $\text{Ap}_4\text{N}$ , with the maximum 30-fold increase being reached after 4 h of exposure. By 6 h the level had declined substantially, reflecting the fact that the cells had been killed by the toxic  $\text{Cd}^{2+}$  ions. This result is similar to that described by others (Baker & Jacobson, 1986; Baltzinger *et al.*, 1986; Coste *et al.*, 1987).

For all viral infections, cells which had been in a confluent state for 2 days were used. These yielded values of  $0.40 \pm 0.10$  pmol of  $\text{Ap}_4\text{N}/10^6$  cells. Upon infection with SV40, a time-dependent increase in DNA synthesis was observed (Fig. 2). Although separate measurements of host cell and viral DNA synthesis were not made, it is likely that the early increase (10–20 h) is due predominantly to the induction of host cell DNA replication, whereas later thymidine incorporation (20–36 h) represents mainly viral DNA synthesis (Ritzi & Levine,

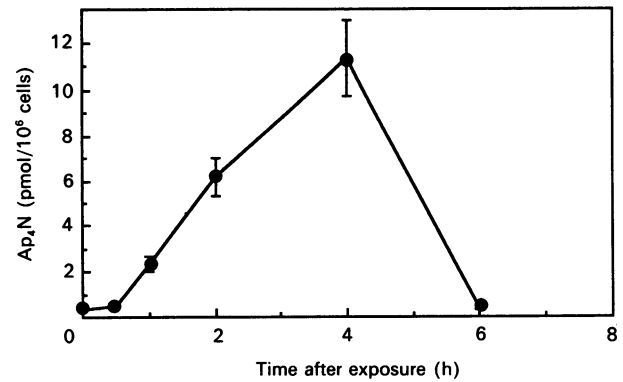


Fig. 1. Effect of  $\text{Cd}^{2+}$  on the  $\text{Ap}_4\text{N}$  content of Vero cells

Cell monolayers were treated with 10 ml of maintenance medium containing 0.8 mM-cadmium acetate for various times and the  $\text{Ap}_4\text{N}$  content was measured as described in the Materials and methods section. Values are means  $\pm$  S.D. derived from three independent experiments.

1970; DePamphilis & Wassarman, 1982). During this time, the intracellular concentration of  $\text{Ap}_4\text{N}$  showed a small but significant ( $P = 0.01$ ) 2-fold increase up to 16 h compared with mock-infected cells, with a subsequent decline during the period of peak viral DNA synthesis.

In contrast to SV40, which induces host DNA replication, infection with HSV-1 causes a rapid cessation of host DNA synthesis (Roizman, 1969). Fig. 3 shows that viral DNA synthesis in Vero cells commences about 4 h post-infection with maximum synthesis around 10–12 h (somewhat later than in other host cell systems). In this case,  $\text{Ap}_4\text{N}$  levels show little change, although there is some indication of a gradual loss ( $P = 0.1$ ) throughout the growth cycle. At later times this may be due in part to the loss of detached cells during washing of the monolayers, though this could account for only about 10–15% of the decrease.

In view of the previously suggested association of  $\text{Ap}_4\text{A}$  with DNA replication, it might be concluded that the changes in  $\text{Ap}_4\text{N}$  observed above are related to the levels of host cell DNA synthesis. However, poliovirus infection, which inhibits the low

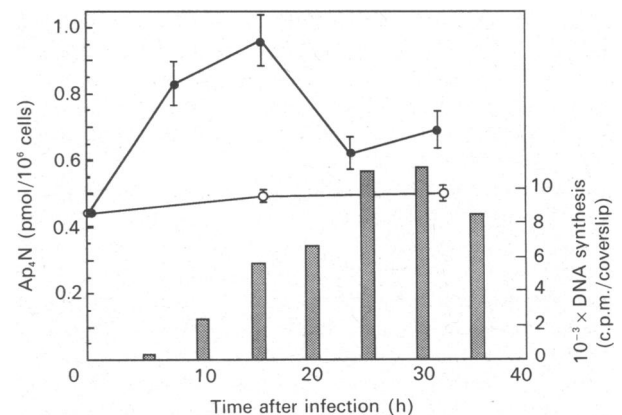
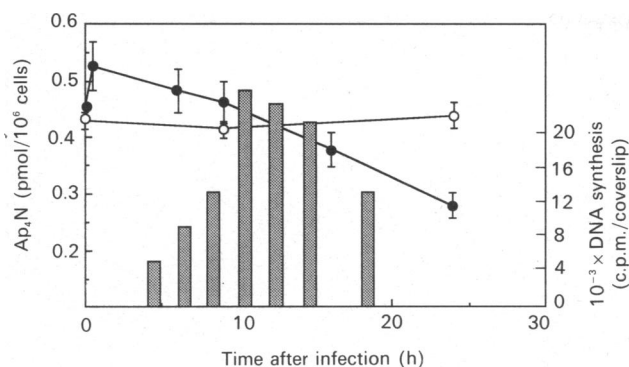


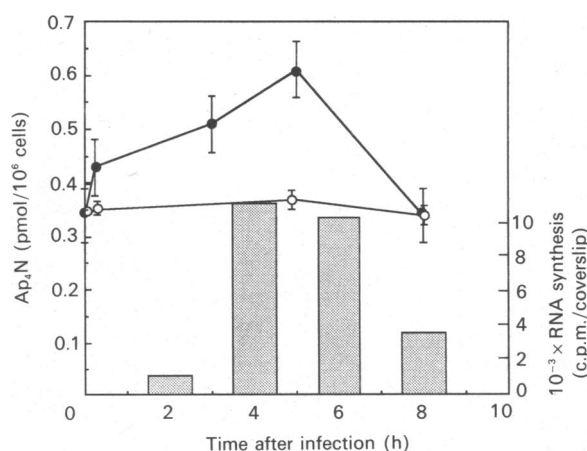
Fig. 2. Effect of infection by SV40 on the  $\text{Ap}_4\text{N}$  content of Vero cells

Cell monolayers were infected (●) or mock-infected (○) and  $\text{Ap}_4\text{N}$  was extracted and measured as described in the Materials and methods section. Values are means  $\pm$  S.D. derived from three independent experiments. For each time point, duplicate dishes containing four glass coverslips were used for the determination of [ $^3\text{H}$ ]thymidine incorporation as described in the Materials and methods section (bars).



**Fig. 3. Effect of infection by HSV-1 on the Ap<sub>4</sub>N content of Vero cells**

Cell monolayers were infected (●) or mock-infected (○) and Ap<sub>4</sub>N was extracted and measured as described in the Materials and methods section. Values are means ± s.d. derived from three independent experiments. For each time point, duplicate dishes containing four glass coverslips were used for the determination of [<sup>3</sup>H]thymidine incorporation as described in the Materials and methods section (bars).



**Fig. 4. Effect of infection by poliovirus on the Ap<sub>4</sub>N content of Vero cells**

Cell monolayers were infected (●) or mock-infected (○) and Ap<sub>4</sub>N was extracted and measured as described in the Materials and methods section. Values are means ± s.d. derived from three independent experiments. For each time point, duplicate dishes containing four glass coverslips were used for the determination of actinomycin D-resistant [<sup>3</sup>H]uridine incorporation as described in the Materials and methods section (bars).

level of host DNA replication remaining in the confluent cell monolayers as a consequence of the inhibition of host protein synthesis (results not shown), causes a 2-fold increase ( $P = 0.02$ ) in Ap<sub>4</sub>N during the period of viral RNA synthesis (Fig. 4). In no case is there an obvious correlation with host cell RNA or protein synthesis, both of which are inhibited by infection with HSV-1 and poliovirus but stimulated by SV40, or with known patterns of synthesis of virus-specific macromolecules.

In conclusion, small but significant changes in the intracellular concentration of Ap<sub>4</sub>N (Ap<sub>4</sub>A) have been observed upon infection of monkey kidney cells with different viruses. Until such time as a clearer picture emerges of the function of Ap<sub>4</sub>A in uninfected cells, the significance of these observations remains uncertain. However, they are consistent with the view that the level of Ap<sub>4</sub>A does not readily correlate with that of DNA synthesis.

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