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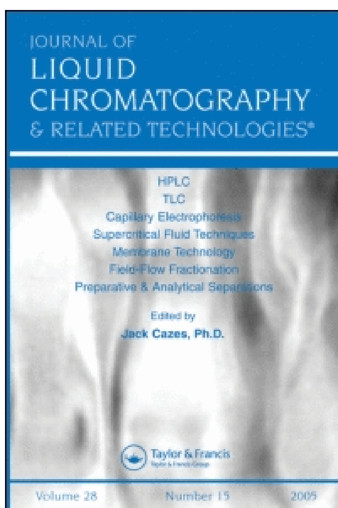

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High-Performance Liquid Chromatography of 2'-5'Oligoadenylates and Related Oligonucleotides

P. Jane Cayley[a]; Ronald E. Brown[a]; Ian M. Kerr[a]
[a] Imperial Cancer Research Fund Laboratories, London, U.K.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 5(11), 2027-2039 (1982)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 2'-5' OLIGOADENYLATES AND RELATED OLIGONUCLEOTIDES

P. Jane Cayley, Ronald E. Brown and Ian M. Kerr

Imperial Cancer Research Fund Laboratories
P.O.Box 123, Lincoln's Inn Fields,
London, WC2A 3PX, U.K.

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ABSTRACT

HPLC has been used for the analysis and separation of the components of $p_x(A2'p)_nA$ ($x = 1$ to 3, $n = 1$ to ≥ 4). Weak anion exchange columns give excellent resolution, but their instability in phosphate buffers makes them impractical for routine use. Reverse phase chromatography using C18 columns provides a satisfactory alternative method. For preliminary analysis of crude material, ammonium phosphate pH7.0 with a linear 1:1 methanol/H_2O gradient gives a good basic separation of the individual oligomers. Resolution of the 5' mono-, di- and triphosphorylated oligomers or of the nonphosphorylated components can be obtained using ammonium phosphate pH6.0 and potassium phosphate pH6.5 buffers respectively. The C18 columns are very stable and any one column will give retention times reproducible within 0.2%.

INTRODUCTION

The addition of interferon to cells causes, among other effects, an increase in the level of several proteins (1-6). The most extensively studied of these is the $ppp(A2'p)_nA$-synthetase (3,4,6) which synthesises the unusual oligonucleotide $ppp(A2'p)_nA$ where $n = 1$ to ≥ 4 (3,7). This enzyme requires double-stranded RNA, ATP and magnesium. Of the oligonucleotides synthesised those with $n = 2$ to 4 were found to activate a

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ppp(A2'p)$_n$A-dependent endonuclease which degrades mRNA (8,9) and rRNA (10,11) with a consequent inhibition of protein synthesis, both in cell extracts and the intact cell (10-14).

The purification of these oligonucleotides synthesised in cell-free systems has been achieved by chromatography using DEAE cellulose (7,15-17). This method is time consuming and unsuitable for routine analyses. Weak anion exchange HPLC columns (18) in which electrostatic interactions are predominant were the obvious choice for separation of the highly charged oligonucleotides. Initially amine columns (LiChrosorb 10 NH_2) were used with a gradient of 40-200mM ammonium phosphate at pH7.2 (19). These columns gave excellent resolution of all the components (19,20), but were found to be unstable, giving a constantly drifting base line (19-21) and decreasing retention times. An alternative approach was therefore made using a stable chemically-bonded phase composed of octadecyl groups on silica microparticles. When used in the reverse phase mode, hydrophobic interactions determine the extent of retention (22,23). Polar or ionic solutes, which favour the aqueous phase, elute fastest. Schweinsberg and Loo (24), were able to resolve ATP, ADP and AMP from other nucleotides, nucleosides and bases in the system using potassium phosphate pH6.0 buffer. Of several different buffer systems tested it was found that ammonium phosphate was the most suitable for the separation of the ppp(A2'p)$_n$A components. The nonphosphorylated oligomers of ppp(A2'p)$_n$A, i.e. (A2'p)$_n$A do not separate in this buffer system. For these the potassium phosphate pH6.5 system was more satisfactory (20).

These methods have been used for the purification of ppp(A2'p)$_n$A oligomers ($n = 1$ to 6) and related oligonucleotides on both an analytical and preparative (mg quantities) scale and for the routine analysis of mixtures of these compounds. When used in conjunction with biological (19) and radioimmune type assays (25,26), they have allowed the identification of naturally occurring ppp(A2'p)$_n$A at concentrations varying from 5-500nM in mouse and human cells which have had different combinations of interferon treatment and virus infection (19, 25,36).

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ppp(A2'p)$_n$A can be synthesised enzymatically or chemically (7,16, 27,28). Open column (7,16,27) and thin layer (7,29) chromatographic methods for the separation of the individual components have been described elsewhere.

HPLC Analysis of 2'-5' oligoadenylates

(a) Weak anion exchange chromatography

Weak anion exchange columns (LiChrosorb 10 NH_2 from E. Merck Darmstadt) (19,20) which have an isopropylamine bonded phase, give excellent separations of the 5' mono-, di- and triphosphorylated components of ppp(A2'p)$_n$A and of the different chain length oligomers (n = 1 to 3) with a linear gradient of 40-200mM ammonium phosphate pH7.2 (Fig.1). This type of column obtained from several manufacturers has proved to be very unstable in the phosphate buffer used.

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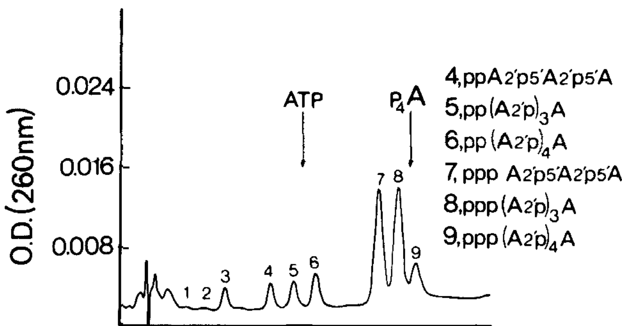

Figure 1.

HPLC analysis of ppp(A2'p)$_n$A on an amine column. Reticulocyte ppp(A2'p)$_n$A, where n = 2 to 4 containing 5' triphosphates (peaks 7-9 respectively) and lesser amounts of the corresponding 5' di- (peaks 4-6) and mono- (peaks 1-3) phosphates was analysed on a LiChrosorb 10 NH_2 column with a linear gradient of 40-200mM ammonium phosphate, pH7.2. In a parallel run ATP and p_4A were included with the above components and the positions at which they eluted are indicated by arrows. Reprinted, from Nature (19), with permission from MacMillan Journals Limited.

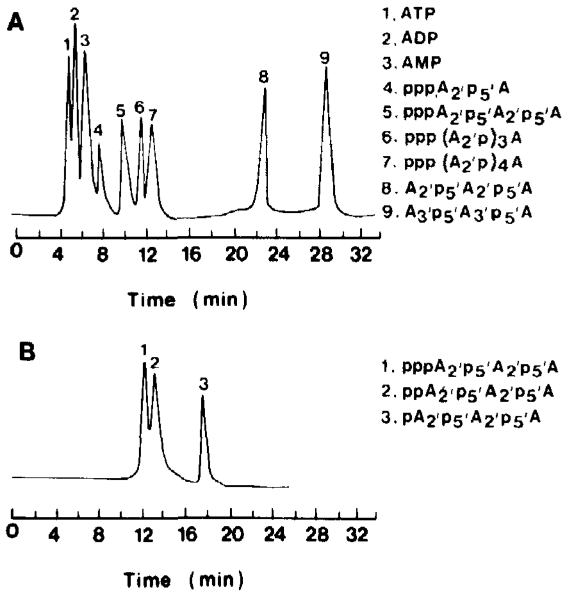

FIGURE 2A & B

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(b) Reverse phase chromatography.

Columns from two manufacturers have been found particularly suitable: μ-Bondapak C18 (dimensions 0.39 x30cm) 10μ particle size from Waters Associates, and Hypersil C18 (dimensions 0.5 x25cm) 5μ particle size from Shandon. The alkyl loading on the columns is 10% and 9% respectively and both have had their unreacted hydroxyl groups inactivated during manufacture. Chromatography in 50mM ammonium phosphate, pH7.0 is based on the method developed by Anderson and Murphy (30) for the separation of purine nucleotides. Linear gradients of 1:1 methanol/ H_2O are used to obtain optimum separation and minimum peak width. The different oligomers ppp(A2'p)$_n$A (n = 1 to 4) but not the 5' mono-, di- and triphosphorylated components are well separated in ammonium phosphate pH7.0 (Fig. 2A). The use of a 0-50% 1:1 methanol/H_2O gradient allows the elution of (A2'p)$_n$A and its separation from (A3'p)$_n$A (Fig. 2A). However, individual components of (A2'p)$_n$A cannot be separated in this solvent

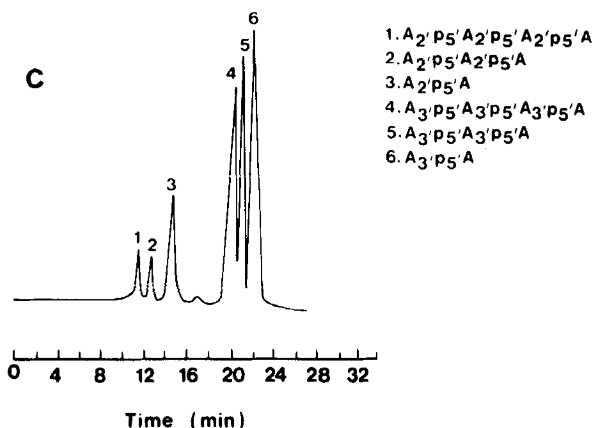

Figure 2.

Reverse phase HPLC analysis of (A) ppp(A2'p)$_n$A (B) the 5' mono-, di- and triphosphates of (A2'p)$_2$A and (C) (A2'p)$_n$A and (A3'p)$_n$A. (A) Reticulocyte ppp(A2'p)$_n$A, where n = 1 to 4 containing lesser amounts of the corresponding 5' di- and monophosphates was analysed together with ATP, ADP, AMP, A2'p5'A2'p5'A and A3'p5'A3'p5'A on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-50% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1.0ml/min. (B) Chemically synthesised (28) 5' tri-, di- and monophosphorylated A2'p5'A2'p5'A components (peaks 1-3, respectively) were separated on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH6.0. A linear gradient of 0-50% 1:1 methanol H_2O was applied in 25 min. The flow rate was 1ml/min. (C) Enzymatically synthesised ppp(A2'p)$_n$A from which the terminal phosphates had been removed by digestion with bacterial alkaline phosphatase ((A2'p)$_n$A, n = 1 to 3) were analysed together with commercial (A3'p)$_n$A, n = 1 to 3 on a μ-Bondapak C18 column run in 4mM potassium phosphate pH6.5. A linear gradient of 0-30% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1ml/min. Reprinted from Methods in Enzymol (20) with permission from Academic Press.

system. Separation of the 5' mono-, di- and triphosphorylated components of the individual oligomers can be achieved if the ammonium phosphate buffer is pH6.0. This is shown for the trimer in Fig.2B. The same basic separation (Fig. 2A) of the ppp(A2'p)$_n$A components is obtained at pH7.0 with both the μ-Bondapak and Hypersil C18 columns. With the latter

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column, no separation of the 5'phosphorylated components of the type shown in Fig.2B is obtained.

Separation of the nonphosphorylated components $(A2'p)_nA$ (n = 1 to 3) can be obtained in 4mM potassium phosphate pH6.5 on elution with a 0-30% linear gradient of 1:1 methanol/H_2O (Fig. 2C). This buffer system containing 10% v/v methanol was first brought to our attention by Harkness (31) as a suitable system for the separation of $(A2'p)_nA$ oligomers. The $(A3'p)_nA$ oligomers n = 1 to 3 can also be separated in this way (Fig. 2C).

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Biochemical Applications

(a) Separation of enzymatically synthesised $ppp(A2'p)_nA$

Analytical C18 columns have been used for the routine analysis of $ppp(A2'p)_nA$ synthetase reaction products. The technique provides both a quantitative estimate and a component analysis.

Preparative HPLC can be used to purify individual components from enzymatically synthesised $ppp(A2'p)_nA$. Using a column 25cm long and 2.25cm internal diameter packed with Hypersil C18 approximately 30mg of crude $ppp(A2'p)_nA$ can be separated in one run. A typical separation using 50mM ammonium phosphate pH7.0 buffer, elution with a linear gradient of 0-20% 1:1 methanol/H_2O and a flow rate of 16.8ml/min is shown in Fig. 3. The flow rate has been found to be the most critical factor in the resolution obtained with these preparative columns.

(b) Separation of enzymatically synthesised $ppp(A2'p)_nA$ analogues

The possible wider significance of the $ppp(A2'p)_nA$ synthetase was emphasised by the initial observation by Ball (32,33) that an enzyme which is almost certainly the synthetase can join AMP in 2'-5' linkage to important metabolites having a terminal adenosine with a free 2'-hydroxyl group, for example NAD^+, ADP-ribose and $A5'p_45'A$. With $ppp(A2'p)_nA$, as the oligomer length increases there is a corresponding increase in retention time of the oligonucleotide (Fig. 2A). The addition of AMP to NAD^+ also increases the retention time although to a smaller extent than with $ppp(A2'p)_nA$. Nevertheless, using a 0-20% 1:1 methanol/H_2O linear

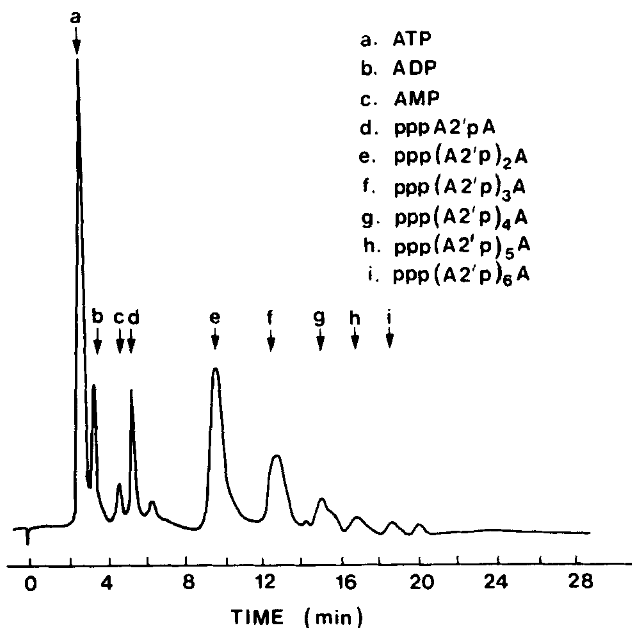

Figure 3.

Preparative reverse phase HPLC purification of enzymatically synthesised ppp(A2'p)$_n$A. The column (25cm x 2.25cm i.d.) was packed with 5μ Hypersil and run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied in 25min. The flow rate was 16.8ml/min.

gradient NAD^+ can be separated from NAD^+2'pA and NAD^+2'pA2'pA (Fig. 4, 34). These conditions also allow the separation of the most interesting analogues NAD^+2'pA2'pA, A5'p$_4$5'A2'pA2'pA and ADP-ribose 2'pA2'pA from each other and from the ppp(A2'p)$_n$A series of components where n = 1 to 3 (Fig.5). HPLC has been invaluable in the analysis and identification of the complex mixtures of these analogues obtained on enzymic synthesis (34).

A high specific activity (1-3 x 10^6 Ci/mol) radioactive analogue of ppp(A2'p)$_n$A has been synthesised for use in radioimmune type assays, by ligating [^{32}P] pCp to the 3' terminus of ppp(A2'p)$_3$A using T4 RNA ligase (35). The product ppp(A2'p)$_3$A[^{32}P] pCp is well separated from the

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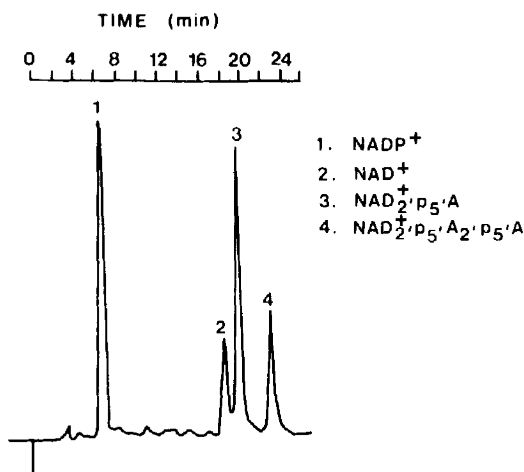

Figure 4.

Reverse phase analysis of NAD^+, $NAD^+2'pA$, $NAD^+2'pA2'pA$ and $NADP^+$. Chromatography was on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% was applied in 25 min. The flow rate was 1ml/min. Reprinted from Methods in Enzymol (20) with permission from Academic Press.

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acceptor molecule $ppp(A2'p)_3A$ and $[^{32}P]$pCp using a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0 with a linear 0-20% gradient of 1:1 methanol/H_2O (Fig.6). The recovery of the product is >95%.

(c) Analysis of naturally occurring $ppp(A2'p)_nA$

Several cell lines (mouse L-cells and human HeLa and Daudi cells) with or without interferon treatment and/or virus infection, have been analysed for the presence of $ppp(A2'p)_nA$ (19, 25, 36). The acid soluble material from the cell pellets was fractionated on either a Hypersil or μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. The $ppp(A2'p)_nA$ components were eluted with a 0-20% linear gradient of 1:1 methanol/H_2O and the nonphosphorylated components were eluted with 50% 1:1 methanol/H_2O (Fig. 7A & B). Although the majority of the acid soluble material is separated from the $ppp(A2'p)_nA$ components, the latter are present at very low concentrations ($1\text{-}50 \times 10^{-8}$M) and cannot be

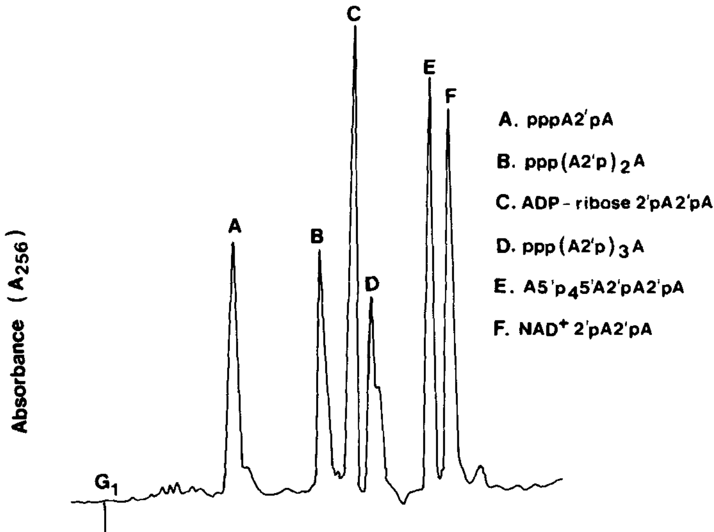

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Figure 5.

HPLC analysis of pppA2'pA, ppp$(A2'p)_2$A, ppp$(A2'p)_3$A, ADP-ribose-2'pA2'pA, A5'$p_4$5'A2'pA2'pA and NAD^+2'pA2'pA. Chromatography was on a µ-Bondapak C18 column run in 50mM ammonium phosphate, pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1.0ml/min. Reprinted from Eur. J. Biochem. (34) with permission from Springer-Verlag.

observed directly from the absorbance profile (Fig.7B). The column fractions were assayed for ppp$(A2'p)_n$A using either radioimmune and radiobinding assays (Fig.7C and 25,26) or biological assay (19). The individual oligomers were identified by their retention times compared with standards run under the same conditions (Fig. 7A). The individual components of the nonphosphorylated oligomers $(A2'p)_n$A not separated by the ammonium phosphate system were further analysed in the 4mM potassium phosphate pH6.5 system described in Fig.3C.

When fractionating a series of crude trichloroacetic acid/ether extracts from cells the C18 columns rapidly deteriorate causing a reduction in the retention times and a broadening of the 5' triphosphorylated ppp$(A2'p)_n$A peaks. Deterioration may be partially prevented by the in-

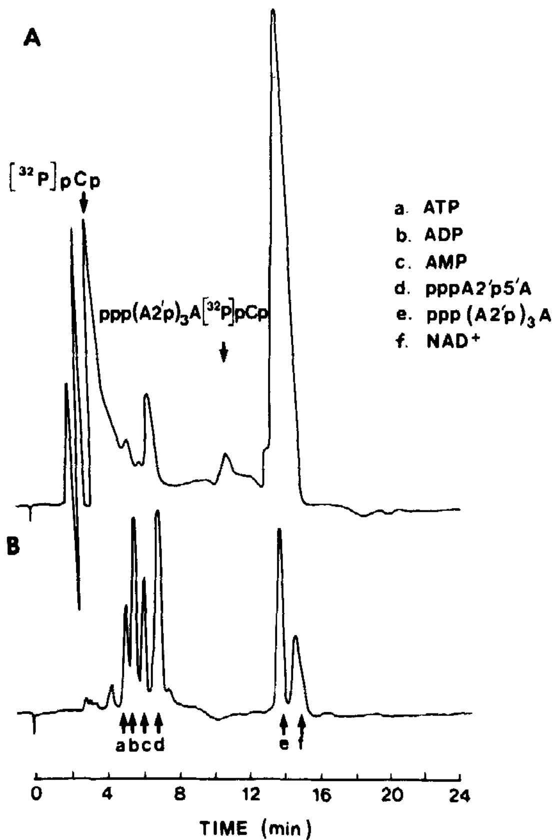

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Figure 6.

Purification of ppp(A2'p)$_3$A [^{32}P] pCp. (A) Analysis of the products of the enzymic synthesis of ppp(A2'p)$_3$A [^{32}P] pCp from ppp(A2'p)$_3$A and [^{32}P] pCp with T4 RNA ligase (36). The profile represents the U.V. absorbance of the separated reaction components at 254nM. The position of elution of the ppp(A2'p)$_3$A [^{32}P] pCp was confirmed by monitoring the radioactivity of the eluate. The product was characterised as described previously (35). (B) Analysis of the standards ATP, ADP, AMP, pppA2'pA and ppp(A2'p)$_3$A (arrowed a to e respectively was carried out under the same conditions. Chromatography was on a μ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied in 25 min. The flow rate was 1ml/min.

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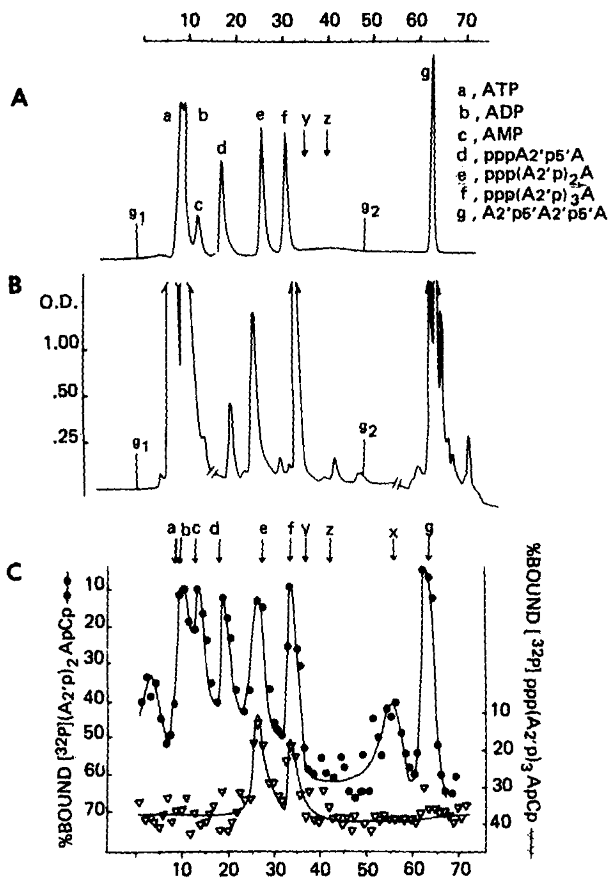

Figure 7.

HPLC analysis of $ppp(A2'p)_nA$ from interferon-treated encephalomyocarditis virus infected L-cells. (A) A mixture of standards containing ATP, ADP, AMP, pppA2'pA, $ppp(A2'p)_2A$, $ppp(A2'p)_3A$ and $(A2'p)_2A$ (peaks a-g). The positions y and z at which $A5'p_45'A(2'pA)_2$ and $NAD^+(2'pA)_2$ respectively, eluted in parallel runs are also indicated on the figure. (B) and (C) Cell extract (150µl) from 0.75ml of packed cells. (B) Absorbance profile:absorbance was measured at 256nM and at fraction 17 and fraction 57 the full-scale deflection (2.56) was reduced 2-fold (to 1.28) and 32-fold (to 0.08). (C) Radiobinding assay (▽): column fractions (5µl) were assayed for their ability to displace $ppp(A2'p)_3A[^{32}P]$ pCp and radioimmune assay (●): column fractions (15µl) were assayed after bacterial alkaline phosphatase digestion for their ability to displace $(A2'p)_2A[^{32}P]$ pCp (25). HPLC analysis was on µ-Bondapak C18 column run in 50mM ammonium phosphate pH7.0. A linear gradient of 0-20% 1:1 methanol/H_2O was applied at g_1 in 25 min, at g_2 a 20-50% 1:1 methanol/H_2O gradient was applied in 5 min. Reprinted from Nature (25) with permision from MacMillan Journals Limited.

sertion of a dry packed pre-column to protect the main column. With commercial column packers (i.e.Shandon) now available,C18 columns can be packed for less than the cost of a commercially packed column. In our experience these columns give equally good resolution and peak shape.

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