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Reversed-phase high-performance liquid chromatography of dinucleoside polyphosphates

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ABSTRACT

The reversed-phase high-performance liquid chromatographic separation of purine dinucleoside polyphosphates on octadecyl- and phenyl-bonded silica packings using phosphate-based eluents was studied. The effects of pH, ionic strength and the content of the organic modifiers methanol and acetonitrile in the mobile phase on the retention and other chromatographic parameters are reported. The data obtained were used to establish an isocratic assay for diguanosine and diadenosine polyphosphates.

INTRODUCTION

Dinucleoside polyphosphates constitute a group of "unusual" nucleotides of increasing biological interest whose general structure is Np_nN', where N and N' represent a purine or pyrimidine nucleoside and p_n a phosphate chain of n ($\geqslant 2$) members joining the 5'-nucleoside positions.

Among these compounds, the diguanosine (Gp_nG) and the hybrids guanosine adenosine (Gp_nA) polyphosphates were the first to be discovered in the encysted embryos of the crustacean *Artemia* [1–3]. The diadenosine polyphosphates (Ap_nA), initially Ap_4A, were later discovered as *in vitro* products of some aminoacyl-tRNA synthetases [4], but it is now evident that these adenylic nucleotides are ubiquitous components of both pro- and eukaryotic cells [5–10], although their precise biological functions are still poorly understood. On the other hand, some dinucleoside polyphosphates are potent inhibitors [11,12] or activators [13,14] of some enzymes involved in nucleotide and nucleic acid metabolism.

Reversed-phase high-performance liquid chromatography (HPLC) has proved to be an invaluable tool for the separation and quantification of nucleobases, nucleosides and nucleotides in biological materials [15,16], and the chromatographic behaviour of such compounds is well enough documented to achieve many kinds of separations [17,18]. However, data concerning the chromatography of dinucleoside polyphosphates are scarce and generally focused on the pairs Ap_4A–Ap_3A and Gp_4G–Gp_3G [19,20].

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The aim of this work was to obtain data concerning the chromatographic behaviour of diguanosine and diadenosine polyphosphates on some comercially available octadecyl- and phenyl-bonded stationary phases as a function of mobile phase pH, ionic strength and organic modifier content. The data so obtained will be useful in designing strategies for the determination of these compounds in biological extracts, and in the study of the enzymes involved in dinucleoside polyphosphate metabolism.

EXPERIMENTAL

Instrumentation

The HPLC equipment consisted of a Model 510 pump, a U6K injector and a Model 440 ultraviolet detector, all from Waters Assoc. (Milford, MA, U.S.A.). The reversed-phase columns used were μBondapak C_{18} (300 × 4 mm I.D., 10 μm particle size), Nova-Pak C_{18} (150 × 4 mm I.D., 5 μm particle size), μBondapak Phenyl (300 × 4 mm I.D., 10 μm particle size, all from Waters Assoc., and LiChrosorb RP-18 (250 × 4 mm I.D., 10 μm particle size) from Kontron (Zurich, Switzerland). A Guard-Pak module (Waters Assoc.) packed with C_{18} material for octadecyl columns and with cyano material for the phenyl columns was inserted before fitting the analytical columns. Chromatographic profiles were recorded using a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) or in a data module from Waters Assoc.

Chemicals

All nucleotides were obtained from Sigma (St. Louis, MO, U.S.A.). Salts and organic modifiers used for the preparation of eluents were of HPLC grade and were purchased from Merck (Darmstadt, F.R.G.). All other chemicals were of analytical reagent grade.

Preparation of eluents and standards

High-quality water was obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.) or by double distillation. Eluents were prepared daily from a stock solution of 1 *M* salt, the pH being adjusted with concentrated orthophosphoric acid, acetic acid, ammonia solution or potassium hydroxide solution as necessary, filtered and degassed through HATF filters (Millipore) under reduced pressure. Nucleotides were prepared as 1 m*M* aqueous solutions and the exact concentrations were calculated from the available molar absorptivities [21,22] by spectrophotometry. They were then appropriately diluted with water or mixed to the desired concentrations (usually 10–20 μ*M*) and frozen at −30°C until use.

Chromatographic conditions and parameters

Before use the columns were equilibrated for 1 h at a flow-rate of 1.0 ml/min or overnight at 0.1 ml/min with the selected mobile phase. The chromatograms were

recorded at 254 nm and a chart speed of 0.2–0.5 cm/min. All separations were carried out at room temperature (19–22°C). Additional details are given in the figure legends. The mean values of retention times obtained from at least five injections were used in the calculations. The dead volume was measured by injection of 5–10 μl of 3 *M* potassium chloride solution [23] and chromatographic parameters such as capacity factor (k'), selectivity (α), resolution (R_s) and column efficiency (N) were calculated according to ref. 24.

Biological extracts and enzyme assays

Procedures for the preparation of acid-soluble extracts from bovine chromaffin granules and cytosolic extracts from adrenal medulla and for peak identification were as previously described [10]. The acidic extracts were further purified by ion-exchange chromatography to eliminate purine and pyrimidine bases and nucleosides on Sep-Pak Accell QMA cartridges (Waters Assoc.). Aliquots (100–500 μl) of the neutralized acid extract were applied to the cartridge, the cartridge was washed with 5 ml of water and then the nucleotides were eluted with 2.0 ml of 0.2 *M* potassium chloride–0.1 *M* hydrochloric acid. This final extract, adjusted to the mobile phase pH, was used for HPLC analysis.

The cytosolic extracts used to determine dinucleoside polyphosphate cleaving activities were subjected to dialysis against 10 m*M* Tris–HCl (pH 7.5), 0.15 *M* potassium chloride and 1 m*M* magnesium chloride to eliminate interfering purine and pyrimidine compounds. The assay conditions for following the enzymatic cleavage of dinucleoside polyphosphates were essentially as reported previously [10]. Initial substrate concentrations were fixed at 10 μ*M*. The reaction mixtures were incubated at 37°C for the required times; incubation was stopped by placing the tubes in a boiling water-bath for 2 min. The tubes were then chilled in an ice-bath, centrifuged to remove precipitated protein and 10–20-μl aliquots from the clear supernatant were used for HPLC analysis.

RESULTS AND DISCUSSION

Octadecyl columns

The effects of pH on the retention of diguanosine and diadenosine polyphosphates on two kinds of octadecyl columns using KH_2PO_4-based eluents are summarized in Figs. 1–3. Fig. 1 shows the results obtained with a μBondapak C_{18} column. At neutral or slightly acidic pH, the retention was not very influenced by pH changes and the diadenosine polyphosphates showed the highest retention. The elution order is inversely related to the number of phosphate groups in both families of compounds, as would be expected in a reversed-phase system.

A decrease in pH below 5 produced an important decrease in retention of diadenosine polyphosphates. This was especially pronounced for Ap_2A; the retention of diguanosine derivatives, however, increased at low pH. Further, a pH decrease induced some changes in the elution order observed at neutral pH (*e.g.*,

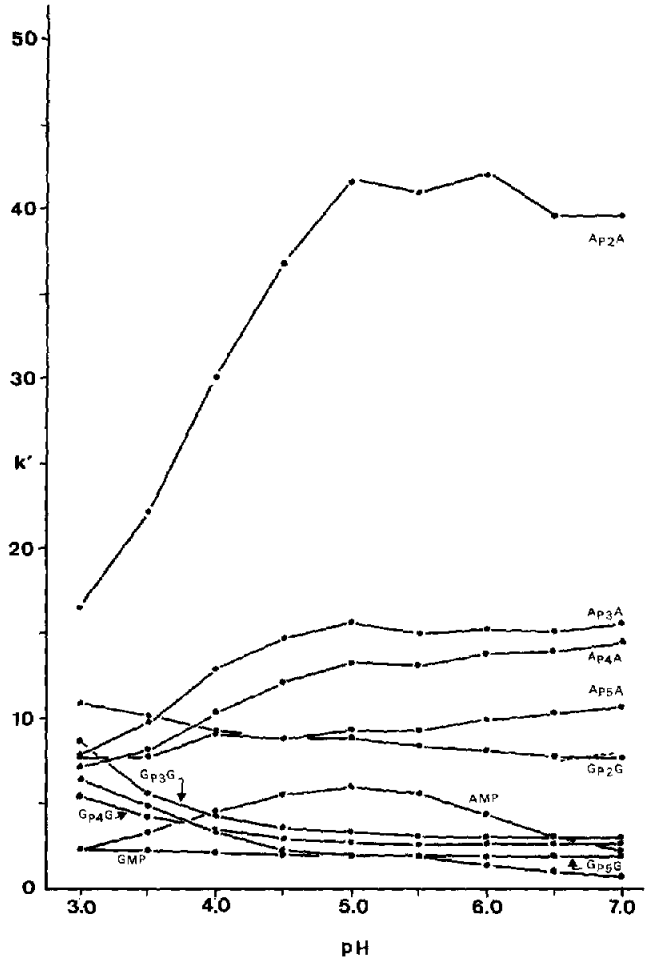

Fig. 1. Effect of mobile phase pH on the retention of diguanosine and diadenosine polyphosphates on an octadecyl-bonded phase. Column, μBondapak C_{18}; mobile phase, 0.1 *M* potassium dihydrogenphosphate; flow-rate, 1.5 ml/min. The broken line indicates the retention of Ap_6A at pH 6.5–7.0. GMP and AMP were introduced as reference compounds.

at pH 3.0 Ap_4A and Gp_4G eluted before Ap_5A and Gp_5G, respectively) and a dramatic distortion of the peak shapes; as the pH decreased, pronounced tailing was observed for the dinucleoside polyphosphates tested, except for Ap_2A and Gp_2G, which were always eluted as two well defined and symmetrical peaks (Fig. 2). Ap_6A was the compound most affected by pH decreases; this nucleotide eluted as a symmetrical peak only in the pH range 7–6.5; between pH 6.5 and 4.5, Ap_6A eluted as a peak of gradually increasing asymmetry, broadening in such a way that it virtually disappeared from the chromatograms at pH 4.5. As shown in Fig. 2, other common purine and pyrimidine nucleotides reference compounds did not

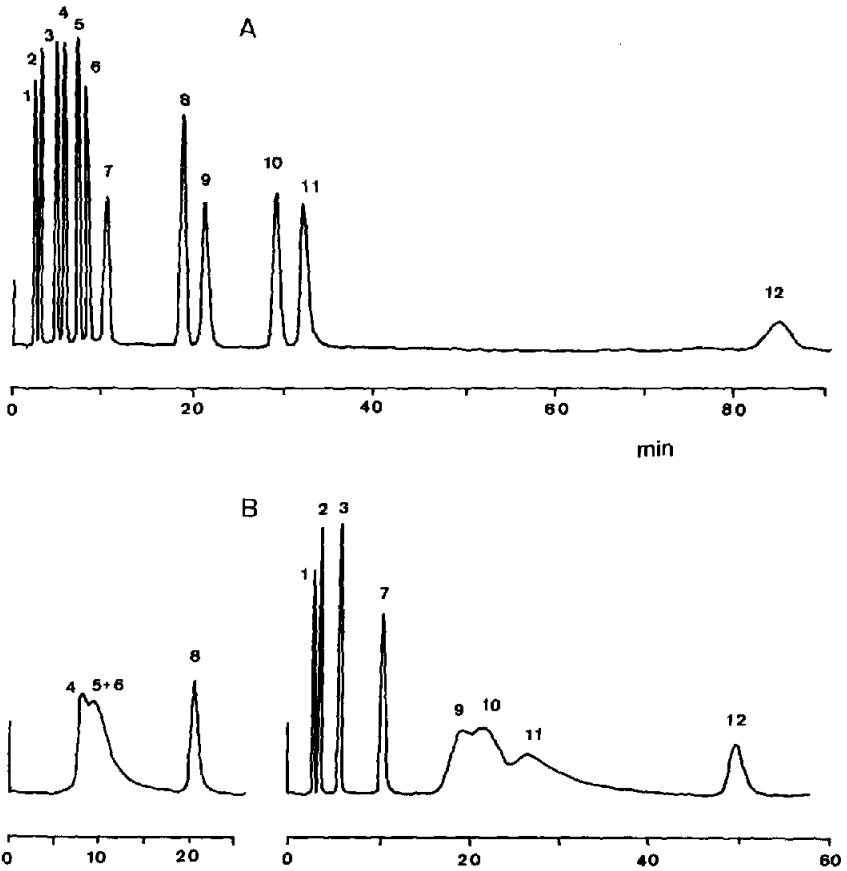

Fig. 2. Effect of mobile phase pH on the elution profiles of diguanosine and diadenosine polyphophates. Column, μBondapak C_{18}; mobile phase, 0.1 *M* potassium dihydrogenphosphate; flow-rate, 1.5 ml/min; chart speed, 0.2 cm/min. (A) pH 6.0; (B) pH 4.0. Peaks: 1 = CMP; 2 = UMP; 3 = GMP; 4 = Gp_5G; 5 = Gp_4G; 6 = Gp_3G; 7 = AMP; 8 = Gp_2G; 9 = Ap_5A; 10 = Ap_4A; 11 = Ap_3A; 12 = Ap_2A.

behave identically, although some tailing was noted for GDP and ADP peaks (results not shown). These findings were also observed on two other columns from the same and other manufacturers (LiChrosorb RP-18 from Kontron). The use of ammonium ion-based eluents (phosphate and acetate) did not prevent the peak asymmetry at low pH. Moreover, acetate buffers provided very poor separations, even at neutral pH.

The results obtained with the Nova-Pak C_{18} column are summarized in Fig. 3. They were qualitatively similar to those obtained on the μBondapak C_{18} column but with a general decrease in retention and lower efficiency for diguanosine nucleotides. The effects of low pH on retention for diguanosine nucleotides were negligible but a clear distortion of the Gp_5G, Ap_5A and specially Ap_6A peaks was evident at pH 4.0.

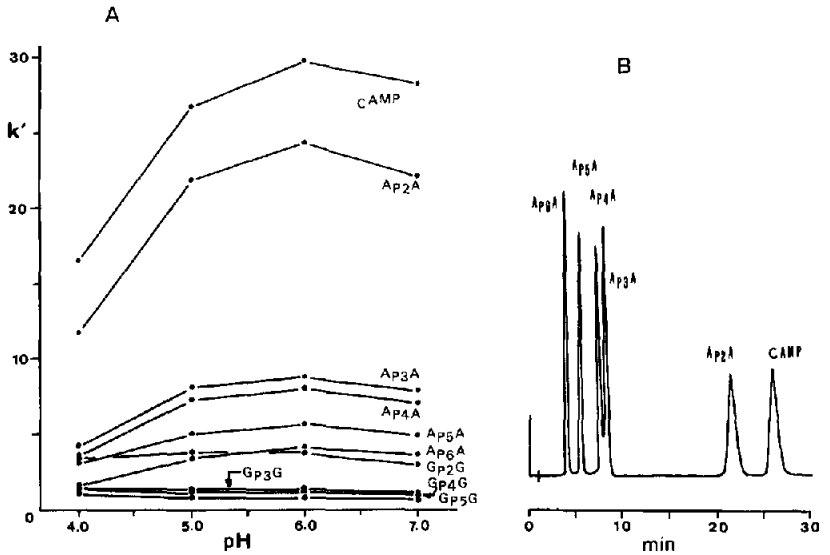

Fig. 3. (A) Effect of mobile phase pH on the retention of diguanosine and diadenosine polyphosphates. Column, Nova-Pak C_{18}; mobile phase, 0.2 *M* potassium dihydrogenphosphate; flow-rate, 1.0 ml/min. (B) Separation of a mixture of diadenosine polyphosphates. Column, Nova-Pak C_{18}; mobile phase, 0.2 *M* potassium dihydrogenphosphate (pH 6.0); flow-rate, 1.0 ml/min; chart speed, 0.25 cm/min. cAMP was introduced as a reference compound.

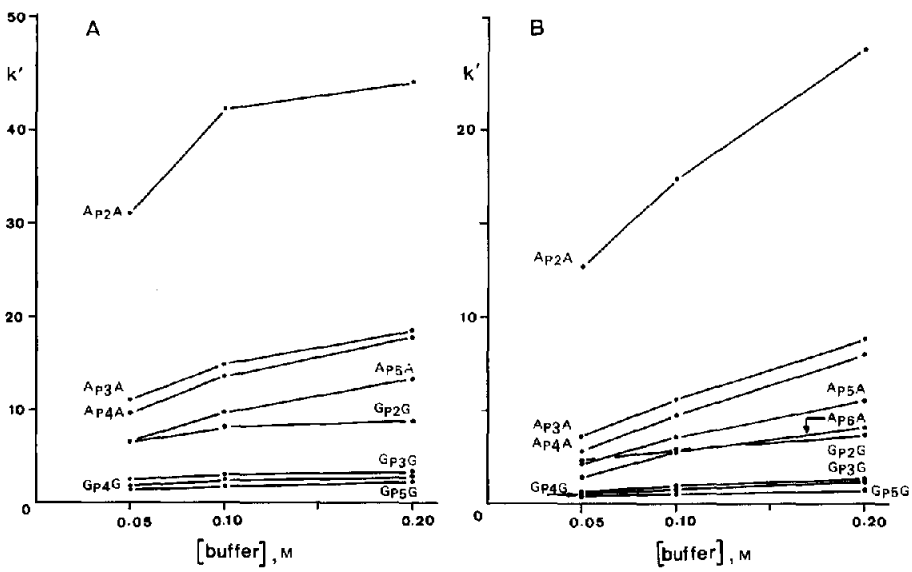

Fig. 4. Effects of buffer concentration on the retention of diguanosine and diadenosine nucleotides. Mobile phase, potassium dihydrogenphosphate (pH 6.0). (A) μBondapak C_{18} column; flow-rate, 1.5 ml/min. (B) Nova-Pak C_{18} column; flow-rate, 1.0 ml/min.

Increasing phosphate buffer concentration increased the retention on both columns, diadenosine nucleotides being the most sensitive (Fig. 4). The best separations were achieved with 0.1 *M* phosphate buffer (pH 6.0–7.0) using the μBondapak C_{18} column, and with 0.2 *M* phosphate buffer (pH 6.0–7.0) using the Nova-Pak C_{18} column (Figs. 2 and 3).

The first column provided a virtually baseline separation of diguanosine and diadenosine polyphosphates, but with Nova-Pak C_{18} considerable overlap of Gp_5G, Gp_4G and Gp_3G was noted. Higher phosphate concentrations improved the separation between Ap_6A and Gp_2G but increased the degree of overlap for the critical peaks Ap_4A–Ap_3A and Gp_4G–Gp_3G.

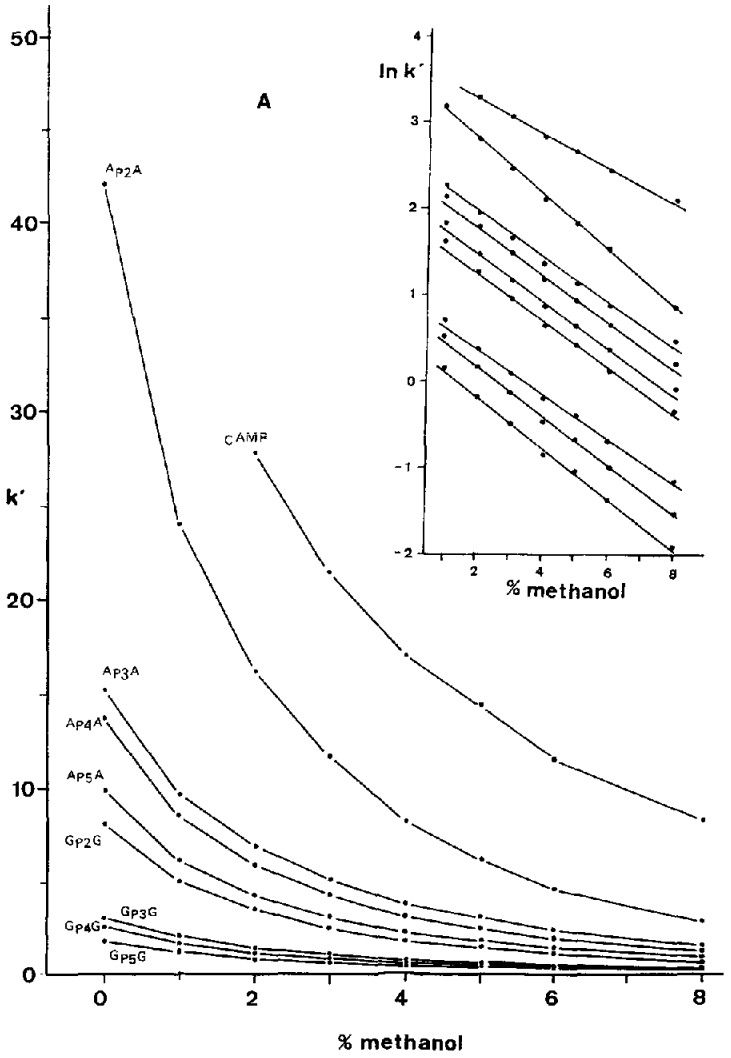

Fig. 5.

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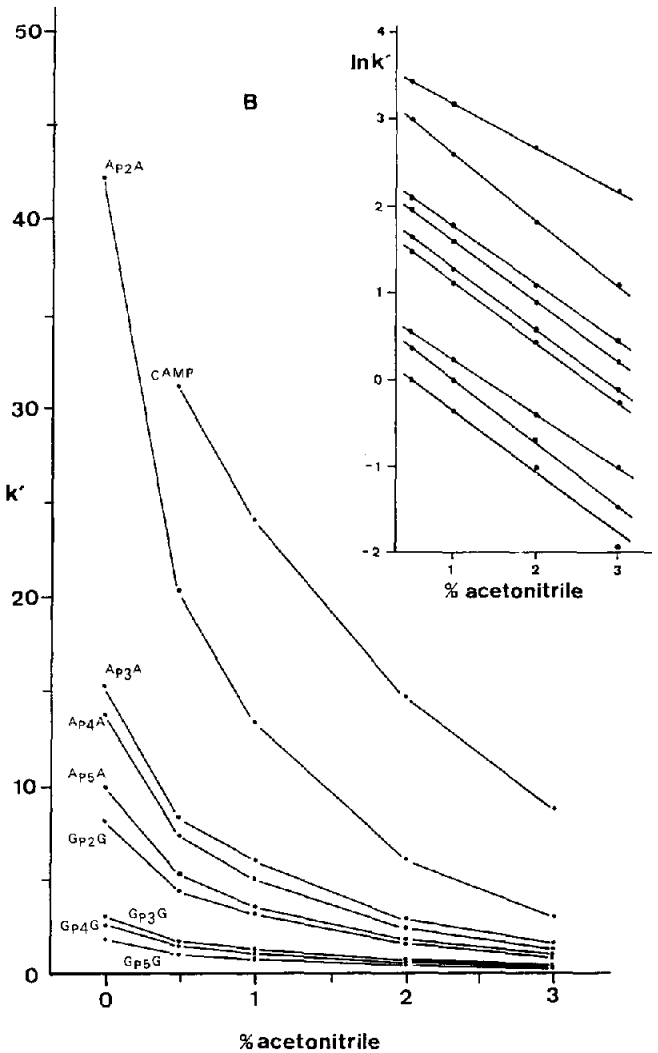

Fig. 5. Effect of (A) methanol and (B) acetonitrile content on the retention of dinucleoside polyphosphates. Column, μBondapak C_{18}; mobile phase, 0.1 *M* potassium dihydrogenphosphate (pH 6.0); flow-rate, 1.5 ml/min.

The organic modifiers methanol and acetonitrile were used to reduce retention and their effects were studied using 0.1 *M* phosphate buffer (pH 6.0) as the basic mobile phase on a μBondapak C_{18} column. As depicted in Fig. 5, both modifiers reduced the retention considerably and gave linear decreases in ln *k'* as a function of the percentage of organic modifier in the mobile phase. The selectivity remained within a favourable range, even for the critical pairs of compounds, as shown in Table I. The optimum concentrations of methanol and acetonitrile in

TABLE I

EFFECT OF METHANOL AND ACETONITRILE ON k' AND α FOR THE CRITICAL PAIRS OF DINUCLEOSIDE POLYPHOSPHATES Gp_3G–Gp_4G, Ap_5A–Gp_2G AND Ap_3A–Ap_4A

Column, μBondapak C_{18}; mobile phase, 0.1 *M* KH_2PO_4 (pH 6.0); flow-rate, 1.5 ml/min.

Compound	No organic modifier		3% methanol		1% acetonitrile	
	k'	α	k'	α	k'	α
Gp_3G	3.09	1.16	1.10	1.25	1.26	1.24
Gp_4G	2.63		0.88		1.02	
Ap_5A	9.86	1.21	3.23	1.24	3.57	1.15
Gp_2G	8.14		2.60		3.11	
Ap_3A	15.29	1.11	5.21	1.18	5.80	1.17
Ap_4A	13.82		4.42		4.94	

the eluent were found to be 3–4% and 1–2%, respectively (Fig. 6A and B), although higher organic modifier contents can be used for the separation of diadenosine polyphosphates only. Slightly modified conditions also provided an excellent separation of pyridine dinucleotides, molecules that can also be considered as hybrid dinucleoside polyphosphates (Fig. 6C and D). The use of organic modifiers at low pH did not solve the problem of peak distortion on any of the 10-μm particle columns tested.

The origin of the marked distortion of the peak shapes of some dinucleoside polyphosphates with mobile phases of low pH is unclear. However, it is worth mentioning that these compounds exhibit different conformational states depending on the mobile phase pH; at neutral pH they present a compact conformation, maintained through a unique intramolecular stacking of purine rings [21], but below pH 5 they produce folded, unstacked and extended conformations [25], probably owing to the protonation of purine rings. It remains to be investigated whether peak distortion indicates changes in the chromatographic behaviour as a consequence of low pH-induced conformational changes and why Ap_2A and Gp_2G were unaffected. Further, protonation of purine rings probably causes the decrease in retention of diadenosine polyphosphates at low pH. However, diguanosine polyphosphates do not behave in the same manner.

On the other hand, at neutral pH, ionized phosphate groups, which are important for decreasing the retention of common 5′-nucleotides [17,26], appear to have little effect on the retention of dinucleoside polyphosphates. We therefore conclude that functional group ionization is not the only factor which affects the retention of these dinucleotides.

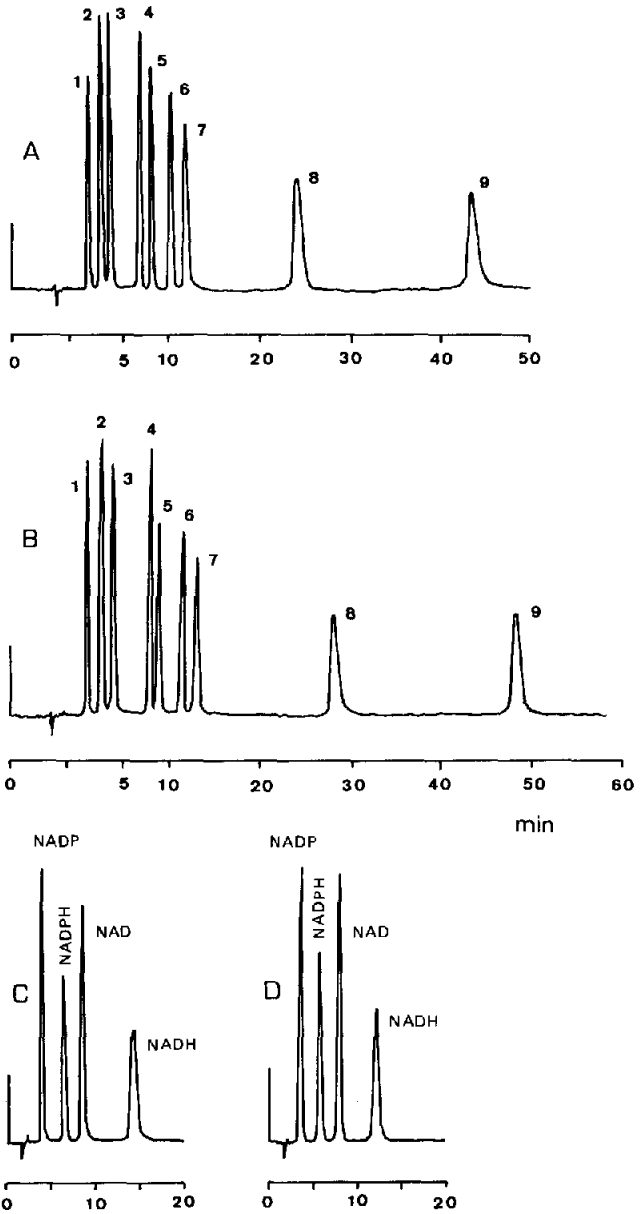

Fig. 6. Isocratic reversed-phase separation of a mixture of (A, B) dinucleoside polyphophates and (C, D) pyridine dinucleotides. Column, μBondapak C_{18}; mobile phase, 0.1 *M* potassium dihydrogenphosphate (pH 6.0); flow-rate, 1.5 ml/min. (A) 3% methanol; (B) 1% acetonitrile; (C) 4% methanol; (D) 2% acetonitrile. Chart speed, 0.5 cm/min during the first 5 min and 0.2 cm/min subsequently in A and B and 0.2 cm/min in C and D. cAMP was introduced as reference compound. Peaks: 1 = Gp_5G; 2 = Gp_4G; 3 = Gp_3G; 4 = Gp_2G; 5 = Ap_5A; 6 = Ap_4A; 7 = Ap_3A; 8 = Ap_2A; 9 = cAMP.

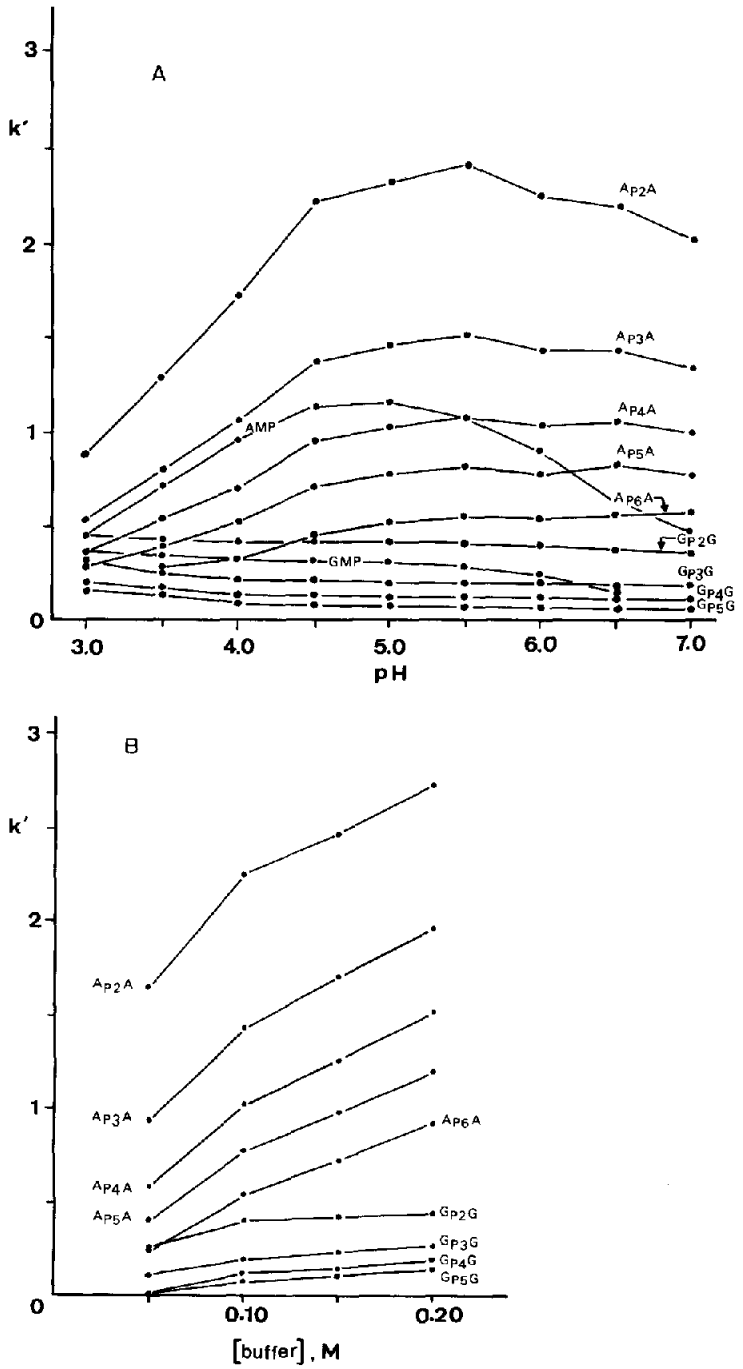

Fig. 7. Effect of (A) mobile phase pH and (B) buffer concentration on the retention of dinucleoside polyphosphates on a phenyl-bonded stationary phase. Column, μBondapak Phenyl; flow-rate, 1.0 ml/min. Mobile phase: (A) 0.1 *M* potassium dihydrogenphosphate; (B) potassium dihydrogenphosphate (pH 6.0).

TABLE II

CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF DINUCLEOSIDE POLYPHSOPHATES ON THE THREE STATIONARY PHASES INVESTIGATED UNDER SPECIFIED CONDITIONS

Compound	μBondapak C_{18}[a]				Nova-Pak C_{18}[b]				μBondapak Phenyl[c]			
	k'	α	N	H[d]	k'	α	N	H	k'	α	N	H
Gp_5G	1.81	1.45	2098	0.14	0.77	1.56	256	0.59	0.14	1.36	274	1.09
Gp_4G	2.63	1.16	2165	0.14	1.20	1.08	289	0.52	0.19	1.37	1330	0.23
Gp_3G	3.03	2.66	2199	0.14	1.30	2.92	711	0.21	0.26	1.58	1496	0.20
Gp_2G	8.14		2682	0.11	3.80		1086	0.14	0.41		1318	0.23
Ap_6A	8.04	1.23	879	0.34	4.12	1.36	864	0.17	0.92	1.30	2126	0.14
Ap_5A	9.86	1.40	2538	0.12	5.60	1.43	2043	0.07	1.20	1.26	2176	0.14
Ap_4A	13.82	1.11	2492	0.12	8.00	1.09	2662	0.06	1.51	1.29	2319	0.13
Ap_3A	15.29	2.76	2181	0.14	8.70	2.79	2749	0.05	1.95	1.40	2449	0.12
Ap_2A	42.15	1.64[e]	3265	0.09	24.30	1.22	2780	0.05	2.73	2.50	2496	0.12
cAMP	ND		4257[e]	0.07[e]	29.70		4200	0.04	6.83		3350	0.09

[a] 0.1 *M* KH_2PO_4 (pH 6.0), 1.5 ml/min.
[b] 0.2 *M* KH_2PO_4 (pH 6.0), 1.0 ml/min.
[c] 0.1 *M* KH_2PO_4 (pH 6.0), 1.0 ml/min.
[d] H = height equivalent to a theoretical plate (mm).
[e] Data obtained with 2% methanol in the mobile phase.

Phenyl column

A similar set of experiments was carried out on a μBondapak Phenyl column. The effects of pH and buffer concentration are shown in Fig. 7; this column gave elution patterns similar to those found with C_{18} columns but with a marked decrease in retention. The chromatographic behaviour of dinucleoside polyphosphates on the phenyl stationary phase displayed many similar features to the behaviour of the corresponding adenosine and guanosine nucleotide moieties (unpublished results). As with Nova-Pak C_{18}, the phenyl column also did not satisfactorily resolve the highly phosphorylated diguanosine nucleotides but provided a rapid separation of diadenosine nucleotides. The effect of low pH on peak shapes observed on C_{18} columns was not observed using the phenyl column, except for Ap_6A at pH 3.0–3.5. Better separations were achieved in the range 0.1–0.2 *M* phosphate and pH 5.0–7.0 (Fig. 8). Low buffer concentrations resulted in overlap of Ap_6A and Gp_2G and Gp_5G and Gp_4G eluted close to the void volume.

Comparison of columns and applications

Table II gives the values of the capacity factor (k'), selectivity (α), efficiency (N) and plate height (H) for the separation of dinucleoside polyphosphates on the three stationary phases investigated. The three columns displayed similar and higher efficiencies for diadenosine polyphosphates. For diguanosine polyphosphates the efficiencies were lower and some marked differences between columns were noted. The column efficiencies may be ranked as μBondapak C_{18} > μBondapak Phenyl > Nova-Pak C_{18}. The low retention measured on the phenyl phase must be due mainly to the reduced size of the phenyl-bonded ligand relative to the longer octadecyl chains.

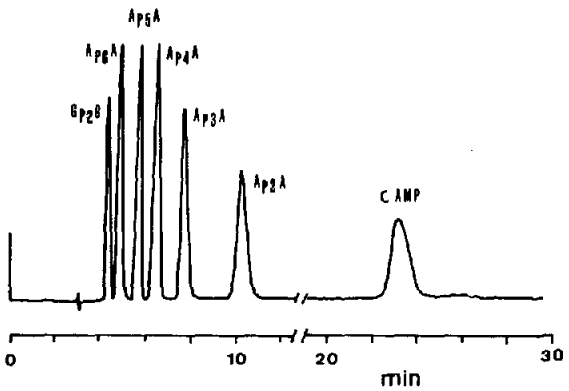

Fig. 8. Isocratic reversed-phase separation of a mixture of dinucleoside polyphosphates on a phenyl-bonded stationary phase. Column, μBondapak Phenyl; mobile phase, 0.1 *M* potassium dihydrogenphosphate (pH 6.0); flow-rate, 1.0 ml/min; chart speed, 0.5 cm/min.

Some interesting differences in selectivities were observed between phenyl and octadecyl phases: the phenyl phase discriminated best between dinucleoside tetra- and triphosphates (α = 1.37 and 1.29 for the pairs Gp_4G–Gp_3G and Ap_4A–Ap_3A, respectively, relative to octadecyl phases). However, octadecyl phases displayed very high selectivity for the pairs of dinucleoside tri- and diphosphates (α > 2.5) relative to the phenyl phase (α = 1.58 and 1.40 for the pairs Gp_3G–Gp_2G and Ap_3A–Ap_2A, respectively). A very high selectivity was also noted for the pair Ap_2A–cAMP (α = 2.5) on the phenyl phase over the C_{18} phases.

From the results reported here, it is evident that the μBondapak C_{18} and LiChrosorb RP-18 columns, owing to their high retention and selectivity, can provide good results when the analyses of complex mixtures are desired. The Nova-Pak C_{18} column provided a good separation of diadenosine polyphosphates in a shorter time than the 10-μm particle columns, but failed to resolve Gp_5G, Gp_4G and Gp_3G. However, good separations of both diguanosine and diadenosine dinucleotides can be achieved by operating this column under ion-pairing conditions (unpublished results). Under reversed-phase conditions μBondapak Phenyl or Nova-Pak C_{18} columns can result in very useful and rapid separations of substrates and products of enzymes acting on dinucleoside polyphosphates such as phosphodiesterases and specific dinucleoside polyphosphate hydrolases or phosphorylases, thus allowing measurements of enzyme activity or even kinetic studies (Fig. 9).

Fig. 10 illustrates the presence of Ap_5A and Ap_4A in a chromaffin granule extract, thus confirming a recent report [10] using ion-pair chromatography and gradient elution. Also in that report, the presence of cleaving activities on Ap_5A and Ap_4A in cytosolic extracts from adrenal medulla was established; now and as shown in Fig. 11, such observations can be extended to Ap_3A, this nucleotide

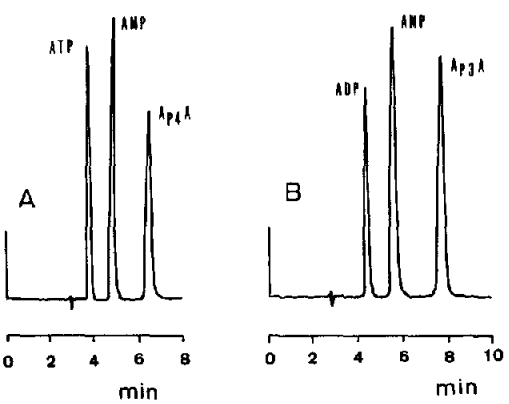

Fig. 9. Isocratic reversed-phase separations of substrates and products of dinucleoside polyphosphate hydrolases acting on (A) Ap_4A ($Ap_4A + H_2O \rightleftharpoons ATP + AMP$) or (B) Ap_3A ($Ap_3A + H_2O \rightleftharpoons ADP + AMP$). Column, μBondapak Phenyl; mobile phase, 0.1 *M* potassium dihydrogenphosphate (A, pH 6.5 and B, pH 6.0). Flow-rate, 1.0 ml/min; chart speed, 0.5 cm/min.

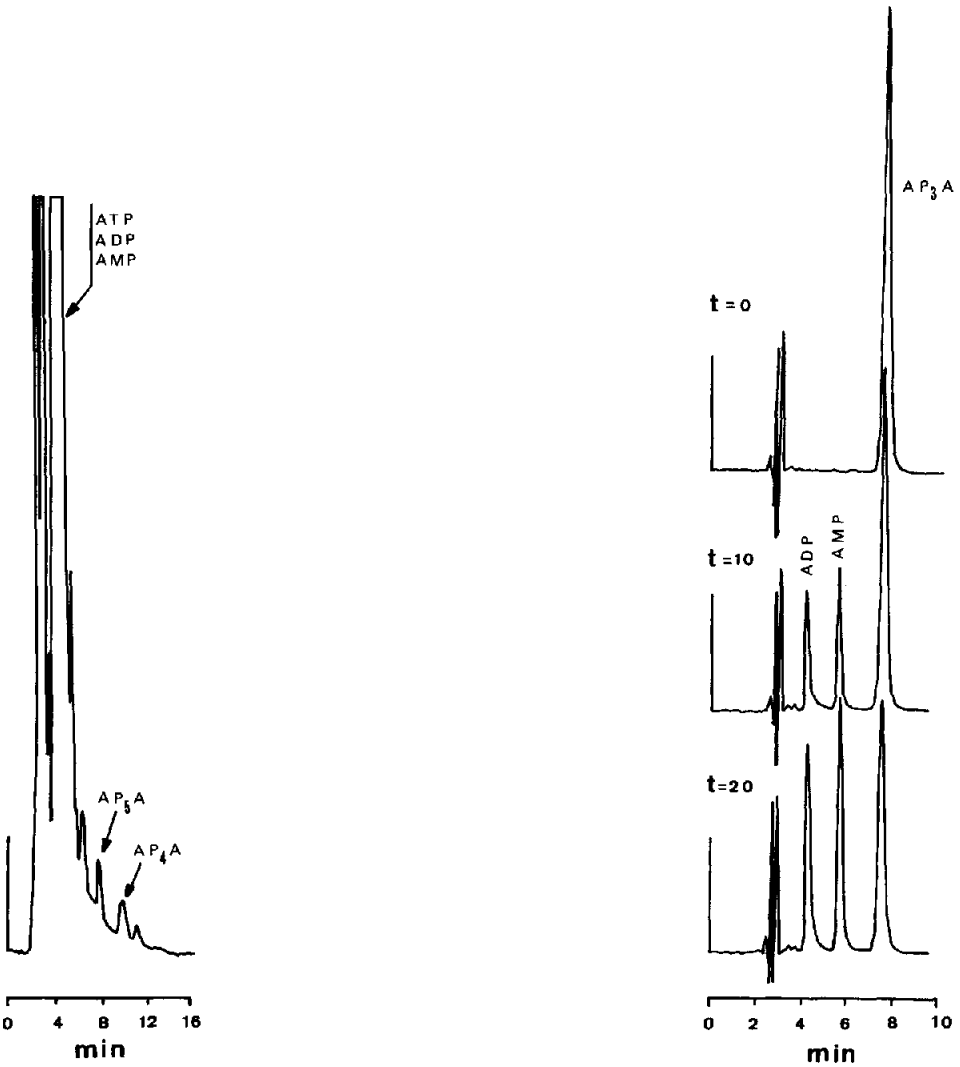

Fig. 10. Chromatographic profile of an acid extract from bovine chromaffin granules. Chromatographic conditions as in Fig. 6A, except that the chart speed was 0.25 cm/min.

Fig. 11. Enzymic conversion of Ap_3A into ADP and AMP by a cytosolic extract from bovine adrenal medulla as a function of time. Reaction mixtures were incubated for 0, 10 and 20 min. Chromatographic conditions as in Fig. 9B.

being cleaved into ADP and AMP. Work in progress is directed towards the characterization of these enzymes and the search for other dinucleoside polyphosphates in secretory granules.

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