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HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF MODIFIED PURINE MONONUCLEOTIDES AND NUCLEOSIDES

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SUMMARY

The useful application of high-pressure liquid chromatography in biochemical research with adenine nucleotide analogues is demonstrated by using a glass-walled separating column at inlet pressures between 100 and 200 atm. Three different aspects are highlighted:

(1) purity control of the adenine nucleotide analogues;

(2) direct reaction monitoring by high-pressure liquid chromatography;

(3) high-pressure liquid chromatography data and mononucleotide conformation.

INTRODUCTION

Recently, efforts have been directed towards the study of the biochemiphoric pattern of adenine nucleotide (AdN) analogues[1–3]. We have tested modified adenine nucleotides in order to obtain information about the nature of the protein receptor, which may be a kinase, the AdN translocator of the mitochondrial membrane, or the mitochondrial ATP synthetase[4–7]. All biochemical tests with these modified nucleotides necessitate a critical and direct purity control[8,9]. Modern high-pressure liquid chromatography (HPLC), besides being a very powerful analytical tool for this purpose, was also used for the rapid and sensitive progress control of a modification reaction of mononucleotides. We were interested in monitoring a Cu^{2+}-induced *in vitro* alkylation of AdN, since *in vivo* experiments showed[10,11] that the toxicity of ethylnitrosourea (ENU) in rats could be increased by the addition of Cu^{2+} ions.

EXPERIMENTAL

The cation-exchange HPLC of nucleosides has been described earlier[8].

The HPLC apparatus used for anion-exchange chromatography was manufactured in our workshop and is shown schematically in Fig. 1. The mobile phase (0.1 N KNO_3 + 0.02 N KH_2PO_4, pH 2.6) is pumped from reservoir 1 by an Orlita high-pressure diaphragm pump, 3, through damping devices, 4, and through a modi-
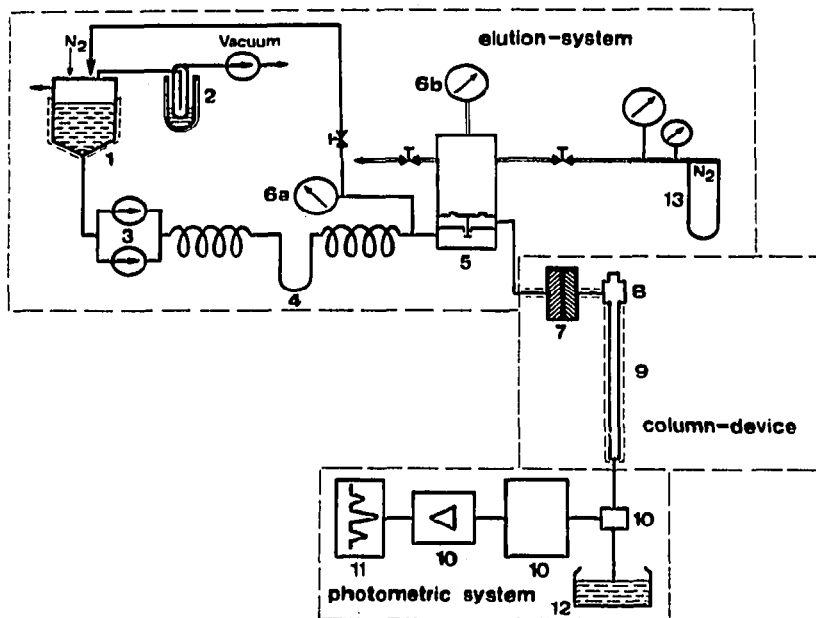
Fig. 1. Flow diagram of the apparatus. 1 = Thermostatted tank of mobile phase; 2 = cooling trap; 3 = DMP-1515 Orlita pump; 4 = resistance tube filled with quartz powder; 5 = modified Milla-flow pressure regulator; 6 = pressure gauges (a for liquid; b for gas); 7 = filter; 8 = injection port; 9 = separating column; 10 = Zeiss PM4 photometer; 11 = Philips recorder; 12 = waste; 13 = nitrogen cylinder for pressure regulator.

fied Millaflow pressure regulator, 5, to the separating column, 8, and the eluent is continuously monitored with a Zeiss PM4 photometer, 10. The resistance of the column packing is kept constant by precise temperature control of the reservoir and of the micro-column, which has previously been packed at a pressure 50% above the pressure used during the analysis. In packing the column, a modified "slurry package" procedure was used, first described by Kirkland[12]. In order to prevent changes in the resistance, gradient elution was not used.

In order to obtain maximum resolution, conventional, narrowly fractioned ion-exchange material of a particle diameter between 5 and 20 μm (Macherey, Nagel, & Co., G.F.R.) was used.

The separating column (Fig. 2) was designed in our workshop with an internal diameter of 2.3 mm and a total length of 300 mm (refs. 13 and 14). By maintaining the outer wall of the glass capillary, 16, at the column inlet pressure by means of the small channel Vz_2 (connecting the feeding line, 9, with the interspace V), the column can be run at pressures of more than 200 atm.

The analytical conditions for the following analysis were kept constant. The flow-rate of the mobile phase was 24 ml/h, the inlet pressure was 123 atm and the temperature was 34°.

Purity control of AdN modified in the purine moiety

The activity of ATP-N^1-oxide in the translocation process across the inner
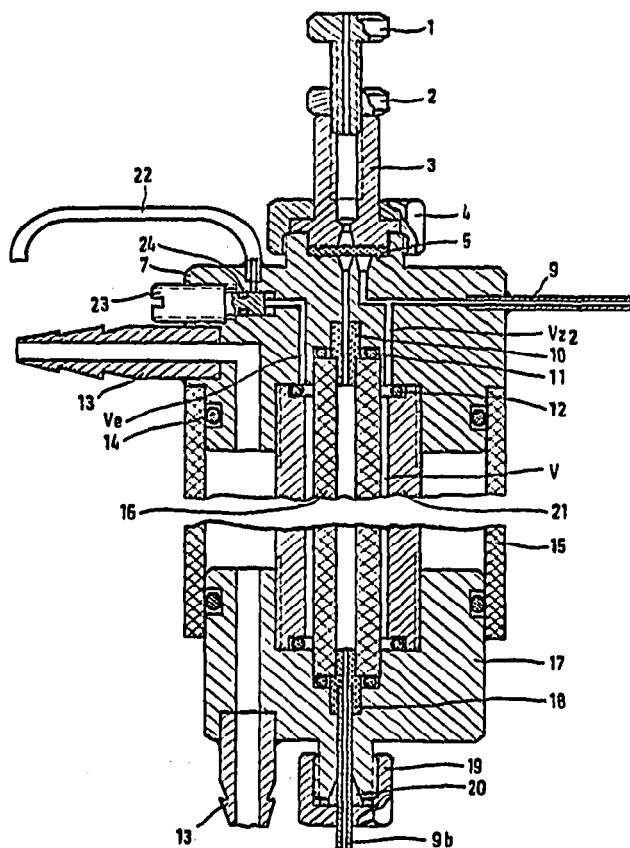
Fig. 2. Glass-walled separating column for HPLC. 1 = Connector for injection needle; 2 = lock-nut; 3 = needle guide and diaphragm plate; 4 = cap-nut; 5 = diaphragm; 7 = injection port; 9 = feeding line (capillary of S.S.); 9b = effluent capillary (of S.S.); 10 = Kel-F spacer; 11 = O-ring (Viton); 12 = O-ring (chloroprene); 13 = hose connectors; 14 = O-ring (chloroprene); 15 = glass jacket; 16 = glass capillary; 17 = column end fitting; 18 = Kel-F spacer; 19 = cap-nut; 20 = sealing taper; 21 = jacketing cylinder (of S.S.); 22 = interspace(V) vent; 23 = vent (22) valve; 24 = O-ring (chloroprene); V = interspace between 16 and 21; Vz_2 = connection between 9 and interspace V.

mitochondrial membrane[4] as well as in biological oxidation was tested in our laboratory. ^{14}C-labelled ATP-N[1]-oxide was synthesized[15] for this purpose. It could be demonstrated by HPLC analysis (see chromatogram, top of Fig. 3) that an oxygen atom had been introduced at the 1-N position only and that the reaction was complete. However, the amount of mono- and diphosphate produced was relatively high. Purity control of the second preparation (see chromatogram, bottom of Fig. 3) showed that the reaction was incomplete and that the major product had a longer retention time and was therefore not ATP-N[1]-oxide.

   The N-alkylated monophosphates used were commercially available products (Terra-Marine Bioresearch, La Jolla, Calif., U.S.A.) and were tested for their purity. N[1]-CH$_3$-AMP (see chromatogram, left-hand side of Fig. 3) showed three impurities: AMP, N[6]-CH$_3$-AMP and N[6]-(CH$_3$)$_2$-AMP. Their presence was confirmed by cation-
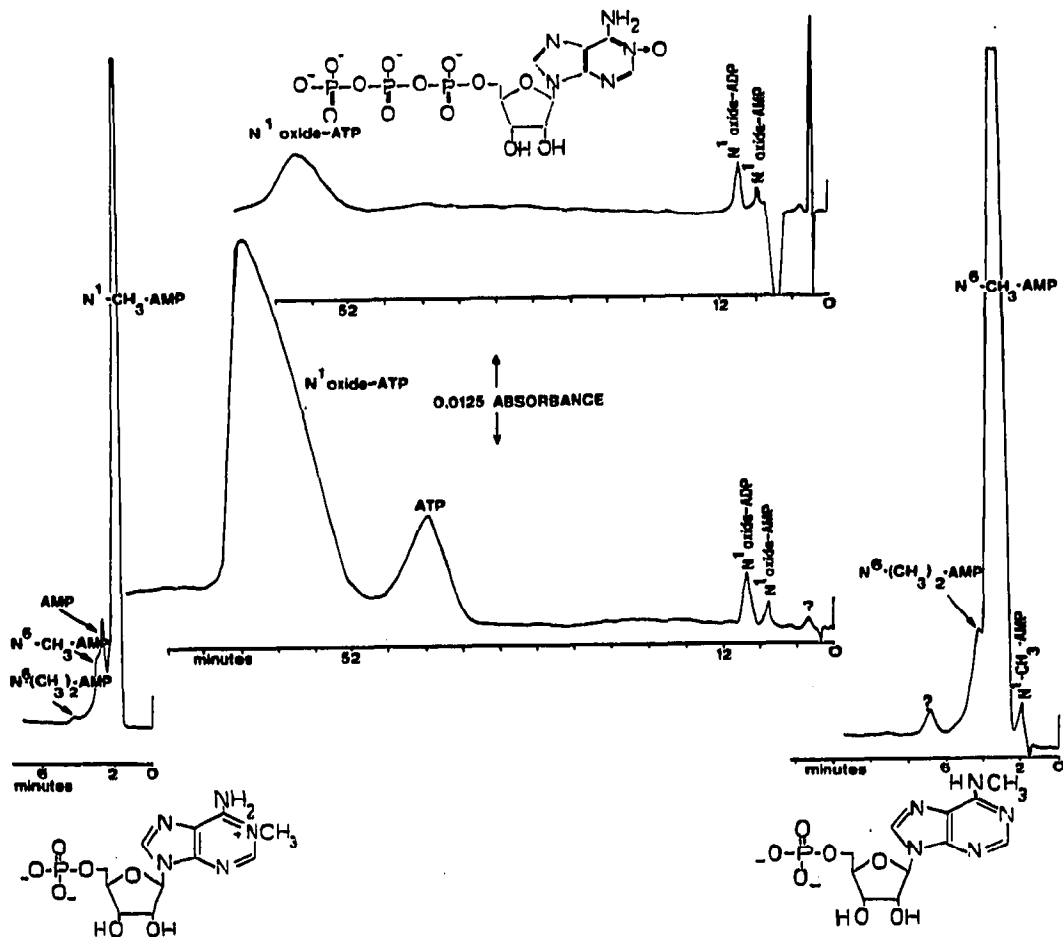
Fig. 3. Chromatograms showing purity controls of purine-base modified AdNs.

exchange chromatography of the enzymatically produced ribosides, where three additional unidentified small peaks were seen[8]. N[6]-CH₃-AMP (see chromatogram, right-hand side of Fig. 3) showed three impurities, two of which were identified as N[1]-CH₃-AMP and as N[6]-(CH₃)₂-AMP. Here again, cation-exchange HPLC revealed additional peaks, one of which was identified as adenosine.

Ring opening of the ribose moiety of the ATP strongly affects the binding properties of the molecule with the AdN carrier in the mitochondrial membrane[16]. The ribose ring was opened between 2-Cpr and 3-Cpr by oxidation, followed by reduction to the diethylene glycol derivative[17]. This leads to a distortion[17] with twisting of the phosphate moiety around the axis between 4-Cpr and the (former) furan oxygen atom. The purine base is twisted in an antiparallel manner. As shown in Fig. 4 and later in Fig. 9, this change of conformation markedly affects the chromatographic behaviour of the modified ATP. The amount of unmodified material can be determined by HPLC analysis, making it possible to be taken into consideration in the results of the biochemical tests.
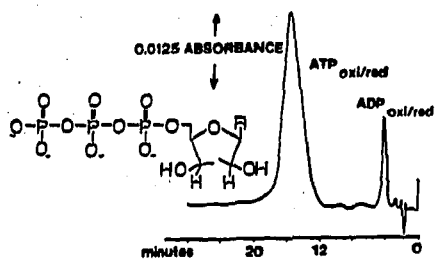
Fig. 4. Chromatogram showing purity control of ATP modified in the purine moiety.
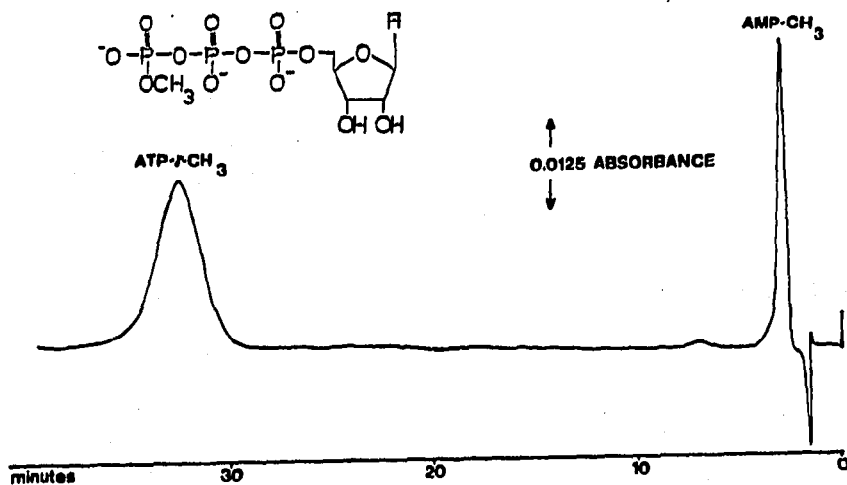

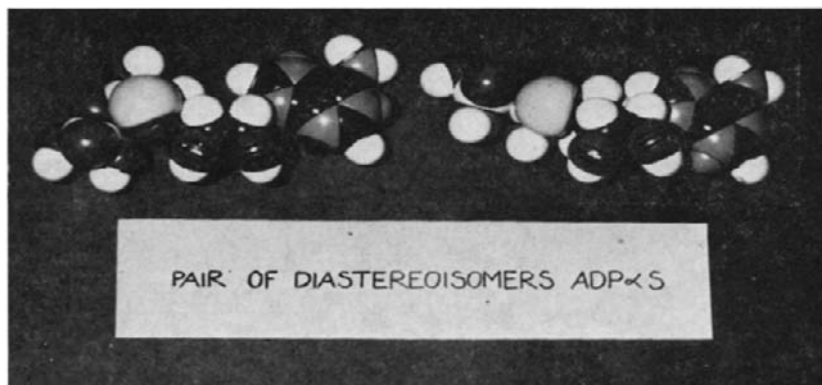Fig. 5. Chromatogram showing the separation of two alkylphosphate esters of AdN.

Fig. 6. Molecular model of the diastereoisomers of ADP-α-S analogue (A, B)

Modifications of the phosphate moiety strongly affect oxidative phosphorylation[5-7]. They may also influence kinase reactions. Replacing an oxygen atom in the α-position of the phosphate moiety of ATP by sulphur leads to two diastereoisomers[2], as shown in the molecular model (Fig. 5). The two isomeric forms of the diphosphate could be separated by HPLC anion-exchange chromatography with a resolution value of approximately 0.8 (see left-hand chromatogram of Fig. 6). Using Eckstein's nomenclature, the first peak corresponds to the so-called A form.

REACTION MONITORING BY HPLC

The advantage of HPLC in reaction monitoring could be clearly demonstrated by the following experiment[18]. ATP and its α-S-analogue were incubated separately and simultaneously in equimolar amounts with hexokinase and glucose. Samples of 5 μl were taken from the two incubations at three different intervals (after 15 min, 16 h and 40 h) and were directly injected into the separating column successively at approximately 1-min intervals. As shown in the chromatograms in Fig. 6, the A form of the diphosphate-α-S analogue was already present in a considerable amount after 15 min. Under these conditions (see Note Fig. 7) this analogue proved to be an equal or even better substrate than ATP, whereas the B form was the least active substrate. Hydrolysis of the α-S-ATP was excluded by an enzyme-free control incubation.

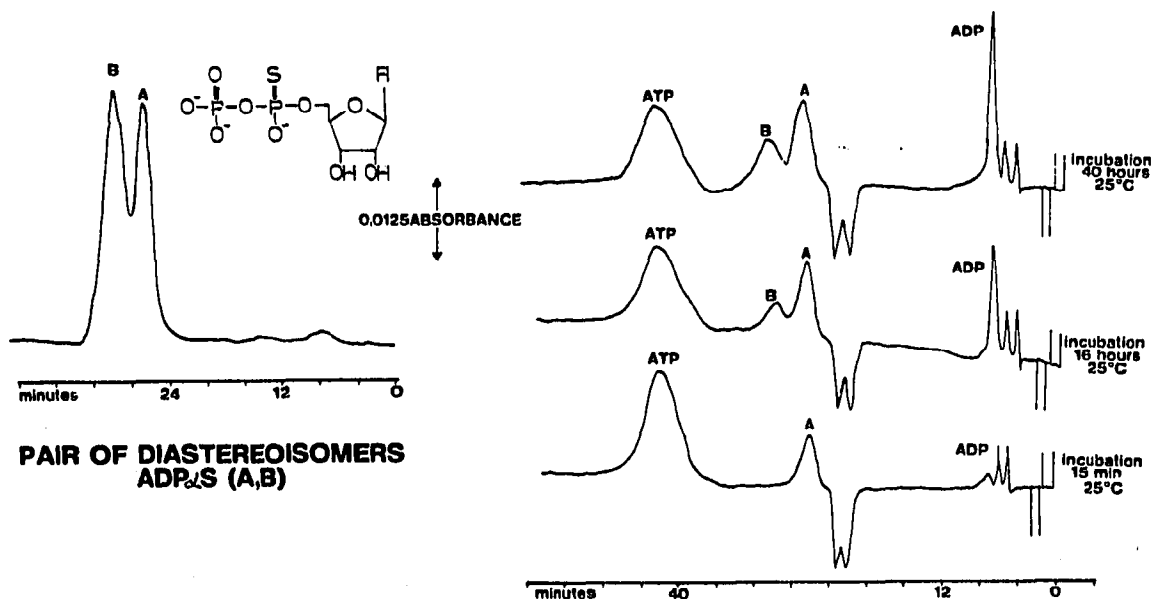**SPECIFICITY OF ATP AND ATPαS IN HEXOKINASE REACTION**

Fig. 7. Chromatograms monitoring hexokinase reactions with ATP and its α-thio analogue. Note: the incubation conditions for the enzyme were kept non-optimum, thus reducing the reaction velocity.

Besides N-alkylation in the purine moiety, esterification of the phosphate moiety leads to a pronounced chromatographic forward shift of the analogue with regard to the unmodified adenine nucleotide (see chromatogram, Fig. 7). This knowledge was of great help in monitoring the ATP.alkylation experiments with the carcinogen ENU, as follows.

ATP (10 mM) was incubated at pH 6.4 for 4 h at 37° with the carcinogen N-ethyl[1-^{14}C]nitroso urea in 3 ml of twice-distilled water. The reaction was initiated by the addition of Cu^{2+} (5 mM) bound to a small amount of weakly acidic cation-exchange material. The pH was kept constant with an automatic titration apparatus (Radiometer, Copenhagen) by addition of 0.5 M NaOH. Samples of 5 μl were taken from the incubation medium at different intervals and were injected into the separating column. After incubation for 4 h, chromatogram B (Fig. 8) showed only a minor
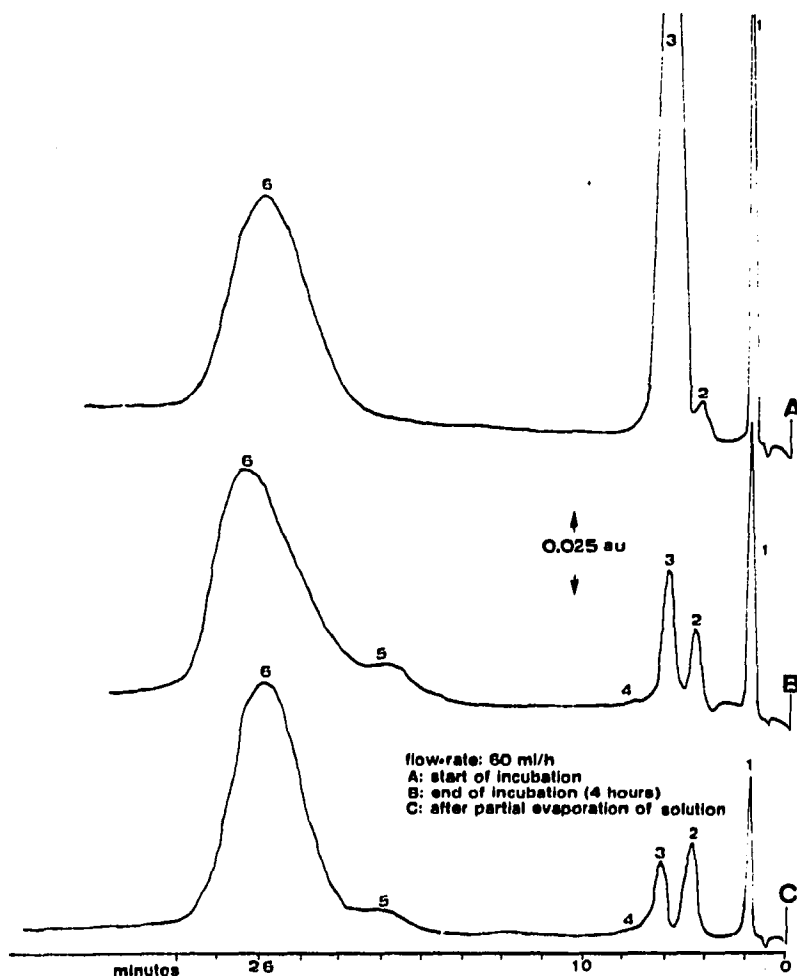

Fig. 8. Chromatograms monitoring ATP *in vitro* alkylation by ENU.

remaining amount of ENU (peak 3). On the other hand, at the end of incubation, the ATP peak 6 showed a distinct asymmetry, with new peaks 4 and 5 appearing. By comparing chromatograms B and C (before and after partial evaporation of the solution), a volatile character is indicated for peaks 1, 4 and 5 because of their decrease in size. This was proved by re-chromatography of the evaporated solutions collected. This volatile character suggests esterification in the phosphate and/or ribose moieties. The greatest amount of radioactivity was found in peaks 1 and 2, suggesting the elution of polyalkylated AdN. Further characterizations of these products by other chromatographic and spectrometric methods are in progress.

HPLC DATA AND MONONUCLEOTIDE CONFORMATION

From the correlation between the peak variances and the retention times, one can calculate the average number of theoretical plates, which is normally used to express the efficiency of the column. As shown in Fig. 9, the average number of theoretical plates for the analogues γ-CH$_3$-ATP ester and ATP-N^1-oxide is nearly twice the value for the thiophosphate analogues and the normal AdNs. A similar finding is noted in Fig. 10, where the peak variances and retention times of common and modified nucleosides in cation-exchange HPLC are correlated by an approximated curve.
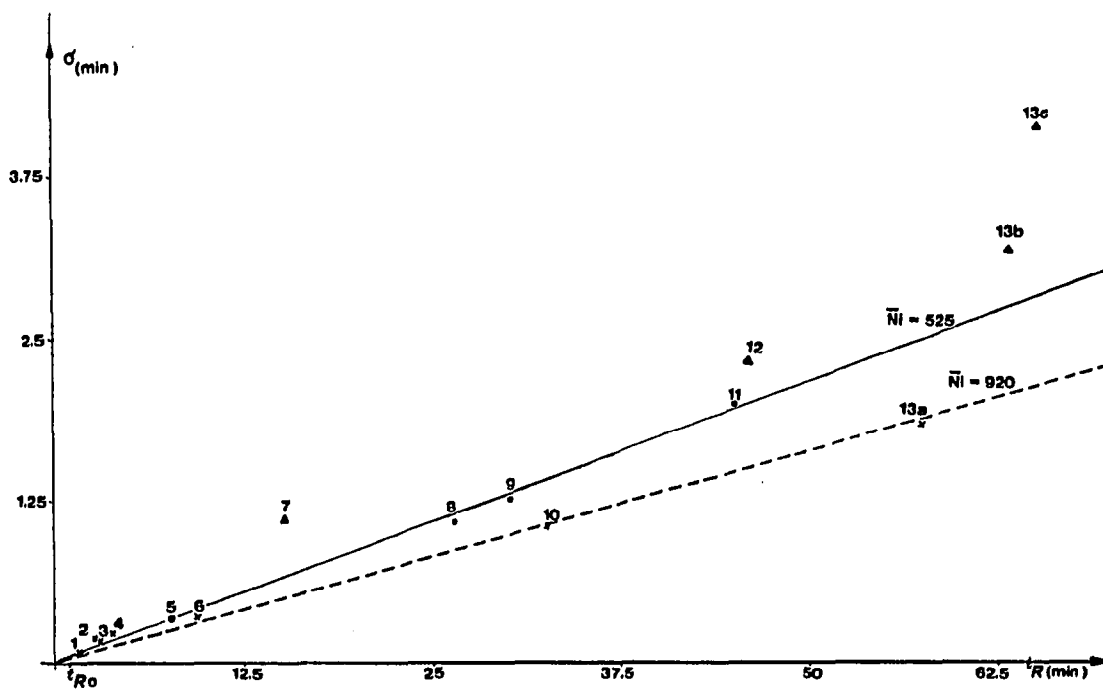

Fig. 9. Graphic determination of the average number of theoretical plates ($\bar{N}_t$) for common and modified AdNs. 1 = N^1-CH$_3$-AMP; 2 = AMP-CH$_3$; 3 = N^6-CH$_3$-AMP and AMP; 4 = ADP$_{oxl/red}$; 5 = ADP; 6 = ATP-N^1-oxide; 7 = ATP$_{oxl/red}$; 8 = ADP-α-S (A); 9 = ADP-α-S (B); 10 = ATP-γ-CH$_3$; 11 = ATP; 12 = dATP (impure); 13 = ATP-N^1-oxide (a, pure; b and c, impure).
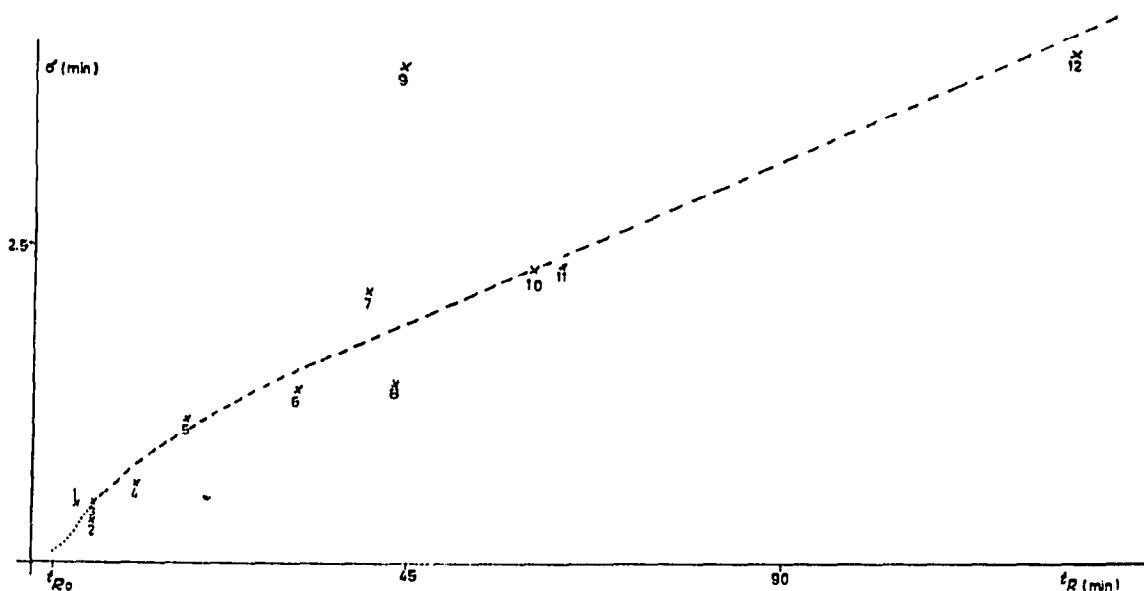
Fig. 10. $\sigma = f(t_R)$ for cation-exchange HPLC of common and modified nucleosides. 1 $=3',5'$-Cyclic-AMP; 2 = uridine; 3 = N^1-oxide-adenoside; 4 = inosine; 5 = guanosine; 6 = adenosine; 7 = N^6-CH$_3$-adenosine; 8 = cytidine; 9 = C^8-bromoadenosine; 10 = imidazoadenosine (3-β-D-ribofuranosylimidazo[2-1-i]purine); 11 = N^6-(CH$_3$)$_2$-adenosine; 12 = N^1-CH$_3$-adenosine.

The variance of the retention time ratio of C^8-bromoadenosine clearly differs from the values of all other substances, suggesting the elution of two peaks with a resolution value of less than 0.4. As the elementary analysis and the UV spectrum are consistent with a C^8-bromo derivative (mol. wt. 346.9. Found: Br 22.74; C 34.73; N 20.32; H 3.59. Calculated: Br 23.03; C 34.59; N 20.18; H 3.74), these two incompletely separated peaks might be the two conformational forms, *anti* and *syn*, in which the nucleosides can exist. In nucleotides, additional conformational changes can be induced by the phosphate moiety, giving rise to conformational forms with slightly different distribution coefficients. The above findings of the low variance values for ATP N^1-oxide and γ-CH$_3$-ATP may be explained by the so-called "gear effect"[19] of the substituents.

The explanation given above is still speculative. However, if it is true, it shows that high-performance HPLC, besides being a rapid method for qualitative and quantitative analysis, may furnish further information and thus lead to new promising biochemical experiments.

ACKNOWLEDGEMENTS

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