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Note

Improved method of resolving nucleotides by reversed-phase high-performance liquid chromatography

M. W. TAYLOR*, H. V. HERSHEY, R. A. LEVINE, K. COY and S. OLIVELLE
Department of Biology, Indiana University, Bloomington, IN 47405 (U.S.A.)
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Recent work in our laboratory has been directed to the relationship between the purine salvage enzymes and *de novo* purine biosynthesis in both eukaryotic and prokaryotic cells. We have recently demonstrated that in *E. coli* there is a selection for purR$^-$ (repressor negative) mutants in an hpt$^-$ gpt$^-$ background[1]. It has also been demonstrated that hgprt$^-$ cells may have altered regulation of the *de novo* purine pathway[2], and in man, individuals suffering from hgprt deficiency (Lesch-Nyhan syndrome) have elevated levels of uric acid[3]. Many other human diseases have been described that involve defects in purine salvage enzymes[4–6].

We have attempted to develop a rapid and sensitive method of quantitating changes that might occur in nucleotide pools in cells grown under different conditions using high-performance liquid chromatography (HPLC). Our aim has been to avoid the use of complex gradients that might require a programmer. Using simple isocratic elution and a C_{18} reversed-phase column we have obtained excellent and reproducible separation of nucleotides and bases in 25 min. Since we are using an isocratic elution system the column need not be equilibrated between runs of similar materials, and the baseline absorbance remains constant. The effect of varying ionic strength and pH on the elution profile of nucleotides was examined. Our results demonstrate that by choosing a specific combination of pH and ionic strength various types of separations can be achieved.

Thus depending on the interests of the investigator different conditions may be used for different types of separation.

MATERIALS AND METHODS

Apparatus

For HPLC, a Waters Assoc. (Milford, MA, U.S.A.) M45 solvent delivery system, Model U6K sample injector, and Model 440 wavelength detector were used. The column was a pre-packed reversed-phase column (30 × 0.4 cm I.D.), utilizing an octadecyl (C_{18}) chemically bonded stationary phase (Waters Assoc.). A pre-column consisting of a short stainless-steel column (5 cm × 4.6 mm I.D.) packed with C_{18} reversed-phase material was used to protect the main column.

Chemicals

Methanol (spectral quality) was from Fisher Scientific. All liquids were pre-

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filtered through a 0.2-μm Millipore filter. Nucleotide standards were obtained from Sigma (St. Louis, MO, U.S.A.), and were prepared as 1 mM stock solutions in double-distilled water, and diluted before use. Stock solutions were maintained at -20°C.

Preparation of eluents

A stock solution (1 M) of ammonium dihydrogen phosphate (Baker) was prepared, filtered, degassed, and diluted to the required ionic strength with double-distilled water. The pH was adjusted with NH_4OH or H_3PO_4. All eluents were pre-filtered through a 0.2-μm Millipore filter before use.

Chromatographic conditions

A Bondapak C_{18} column was used in all studies. Between use, the column was stored in methanol–water (30:70). The column was cleaned at the end of the day with the methanol–water mixture. The column was routinely run at 1–2 ml/min (1000–2000 p.s.i.).

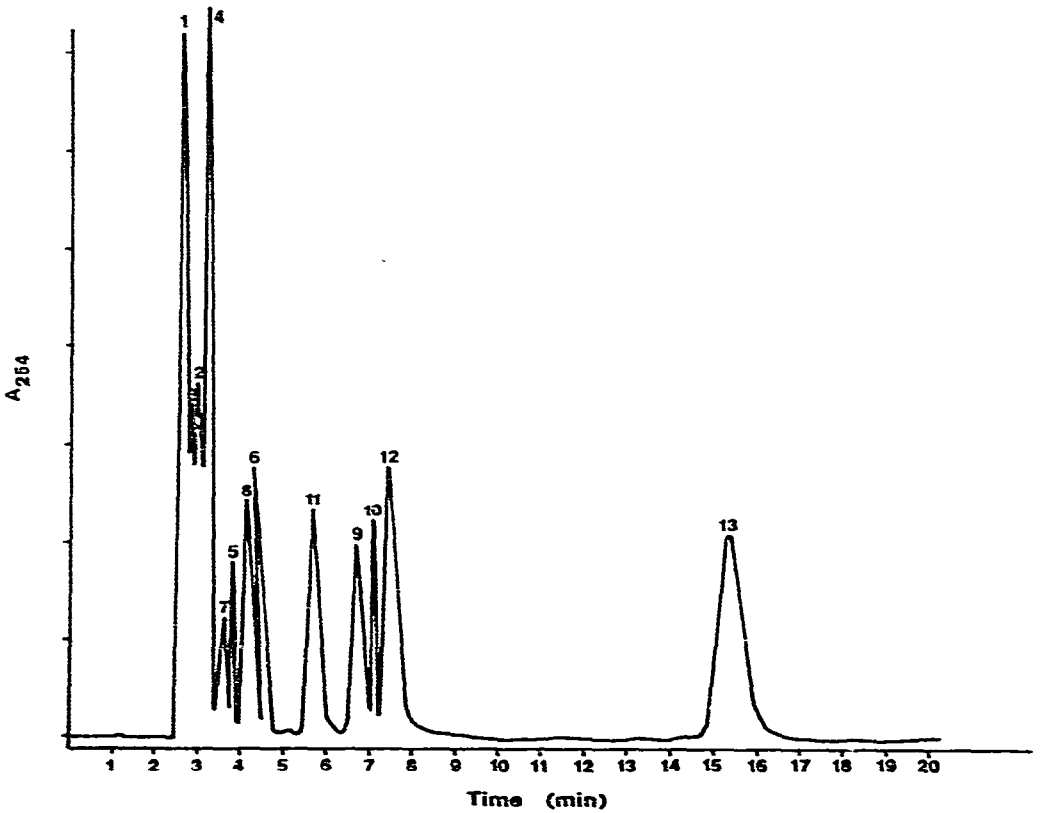

Fig. 1. Chromatogram of nucleotide standards on a Bondapak C_{18} column, using UV (254 nm) detection. Eluent 0.02 M $(NH_4)H_2PO_4$, pH 5.1. Flow-rate 1 ml/min. All peaks identified individually: 1 = CTP; 2 = CDP; 3 = UTP; 4 = UDP; 5 = CMP; 6 = UMP; 7 = GTP; 8 = GDP; 9 = GMP; 10 = IMP; 11 = ATP; 12 = ADP; 13 = AMP.

Cell extracts

Acid-soluble nucleotides and bases were extracted from tissue culture cells by a modification of the method of Jensen *et al.*[7]. Tissue culture cells were removed from flasks by trypsinization with 0.25% trypsin, washed in phosphate-buffered saline in the cold to remove the trypsin, and resuspended in 0.33 *M* formic acid. After 30 min on ice, the extract was centrifuged and the supernatant applied directly to the column. Under these conditions there is little breakdown of triphosphates to mono- or diphosphates. Soluble nucleotides were extracted from *E. coli* in a similar fashion.

RESULTS

Effects of ion concentration

Standard nucleotides (AMP, CMP, GMP, UMP, ADP, CDP, UDP, GDP, GTP, CTP, UTP, ATP) were eluted from the C_{18} column at 1.0 ml/min with $(NH_4)H_2PO_4$ (pH 5.1, 0.01 *M*) containing 6% methanol according to the method of Davis *et al.*[8] for the separation of nucleotides. However, in our hands this method did not give good resolution of nucleotides, all of them eluting very early. Resolution of the different nucleotides could be improved, by removing methanol and chromatographing with 0.02 *M* $(NH_4)H_2PO_4$ (pH 5.1) (Fig. 1). By increasing the ionic strength, better resolution could be achieved. The retention times of mono-, di- and triphosphates at pH 5.1 with 0.02, 0.05, 0.1, 0.2, and 0.5 *M* $(NH_4)H_2PO_4$ are compared in Fig. 2. At higher salt concentrations there was a greater retention time of adenine

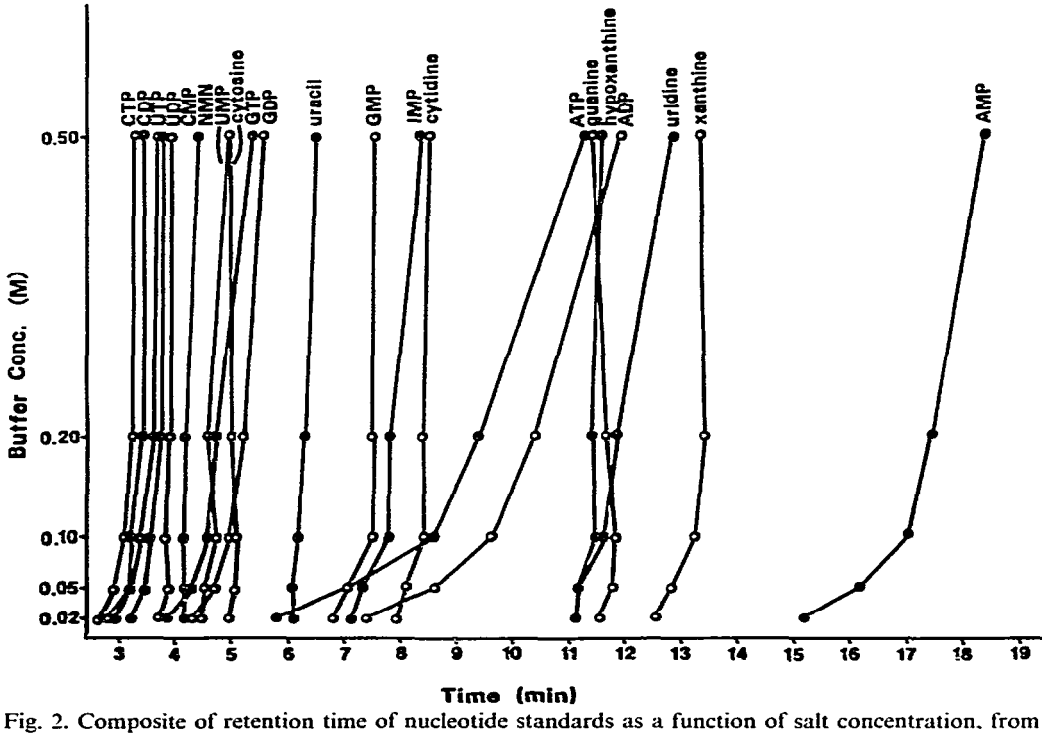

Fig. 2. Composite of retention time of nucleotide standards as a function of salt concentration, from a Bondapak C_{18} column (30 × 0.4 cm I.D.) using UV (254 nm) detection. Isocratic elution with 0.02 *M* $(NH_4)H_2PO_4$–0.5 *M* $(NH_4)H_2PO_4$, pH 5.1. Flow-rate 1 ml/min.

nucleotides, with ATP, ADP and AMP eluting later at higher salt concentration. Increasing salt concentration had very little effect on time of elution of other nucleotides.

Effect of pH

Nucleotides were eluted from the C_{18} column using 0.2 *M* $(NH_4)H_2PO_4$, and 0.5 *M* $(NH_4)H_2PO_4$ at pH 3.4–6.6 in 0.5 pH unit differences. As expected[9,10], pH had a much greater effect on separation than salt concentration. The elution of cytidine and uridine nucleotides was not influenced by changes in pH as markedly as the purine nucleotides. In particular the retention time of ATP, ADP and AMP was greatly altered at different pH values. The monophosphates were most affected and AMP retention was particularly sensitive to pH alteration (13 min at pH 6.6, 20 min at pH 5.5, and 11.5 min at pH 3.65). These data are summarized in Fig. 3. The pK_a of the primary phosphate group (*ca.* 6 for all the nucleotides) probably accounts for these results, with the polarity of the base and the ratio of base to phosphate groups accounting for the greater shifts in monophosphates and adenine nucleotides. The pK_a values of the bases appear to have only a minor effect.

Chromatography of cell extracts

In order to ascertain that similar results were obtained with biological samples, total nucleotide pools of Chinese hamster cells (CHO), L-cells, and *E. coli* were

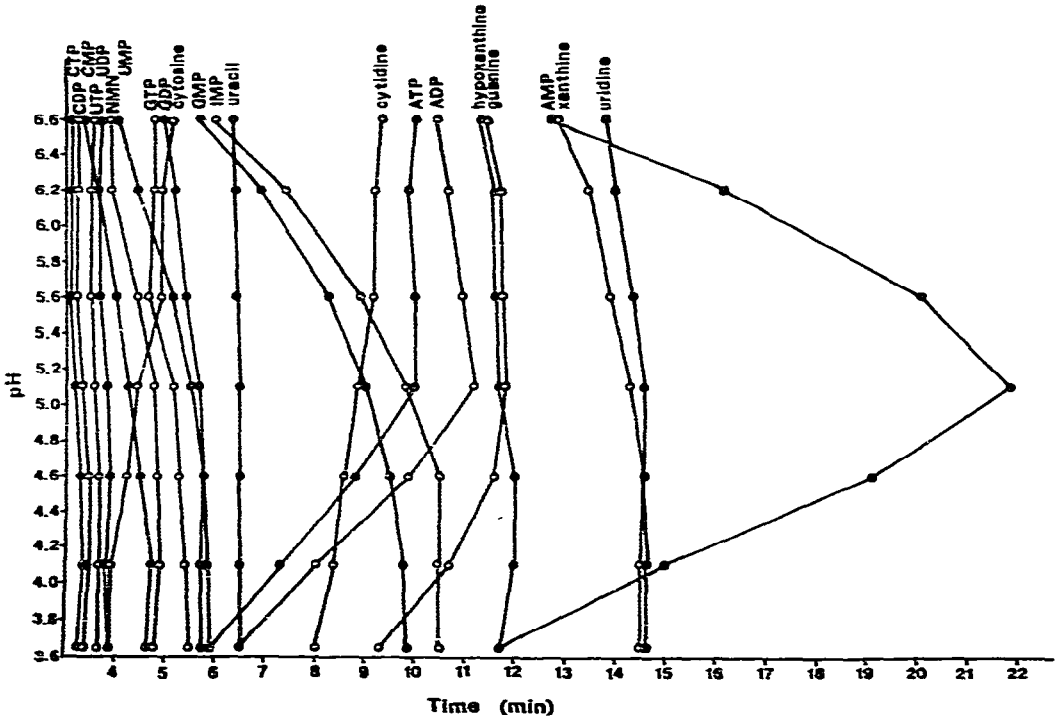

Fig. 3. Composite of retention time of nucleotide standards as a function of pH. Conditions as in Fig. 1, except 0.2 *M* $(NH_4)H_2PO_4$ used at different pH values.

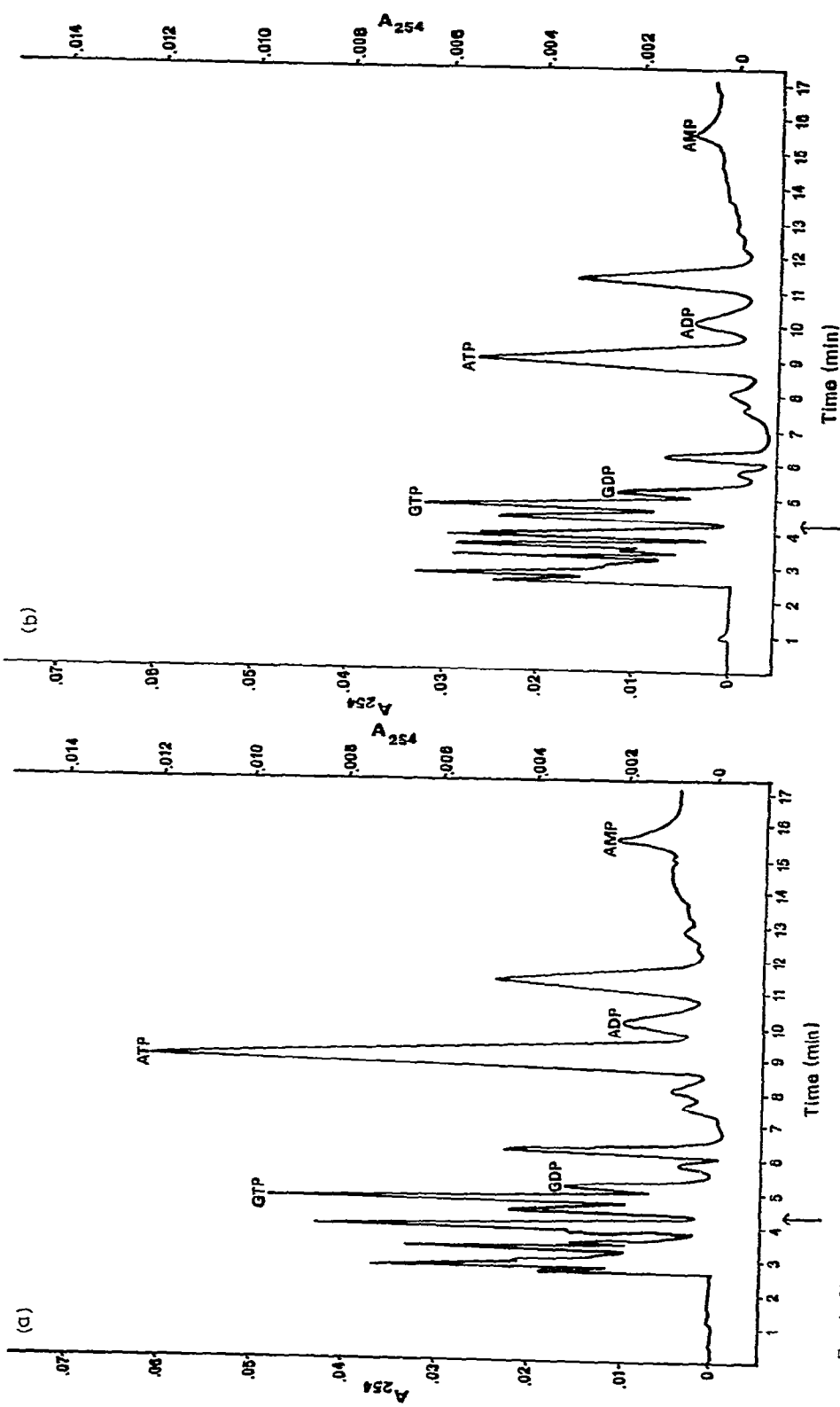

Fig. 4. Chromatogram of total acid-soluble pool of *E. coli* grown in the presence (a) and absence (b) of azaserine ($1 \cdot 10^{-4}$ *M*). Chromatographic conditions: 25 µl injected; flow-rate 1 ml/min; 0.2 *M* $(NH_4)H_2PO_4$, pH 5.1.

measured. To test whether nucleotide pool changes could be detected using our analytical method, an exponential culture (5 ml) of *E. coli* strain TL525 (ara, Δleu, V169, StrA purE::lac) was treated with azaserine ($1 \cdot 10^{-4}$ M) for 15 min. The glutamine analogue azaserine has been shown to inhibit *de novo* purine biosynthesis[11]. After this period samples were removed and the nucleotide pools extracted with 0.33 *M* formic acid. Cultures that had not been exposed to azaserine were processed identically and served as controls.

As can be seen in Fig. 4a and b, addition of azaserine specifically affects the pools of purine nucleotides. There is approximately a two-fold difference in ATP and GTP concentrations. Thus the separation of nucleotides in a cell extract is identical to that received using standards.

Chinese hamster ovary cells and L-cells, grown as monolayers, were extracted as described in materials and methods. A 100-μl volume of the cell extract (from 10^6 cells/ml) was sufficient to give a good profile of nucleotides. All the nucleotides and bases could be identified from standards. To identify peaks, radioactive and non-labelled standards were injected and co-eluted with the sample. Fig. 5 compares the total nucleotide pools of CHO cells and L-cells grown in culture. The differences in nucleotide pools, ATP/AMP and GTP/GMP ratios, as well as the amounts of nicotinamide mononucleotide (NMN) are quite striking. The resolution in this case has been enhanced (two-fold) by using two columns in tandem.

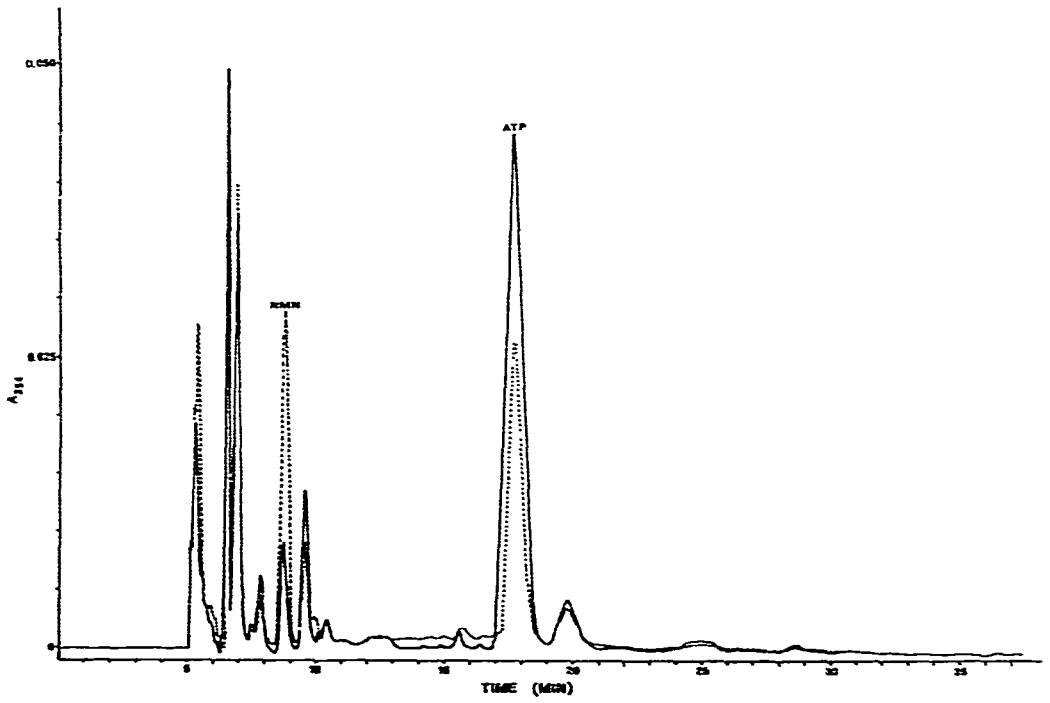

Fig. 5. Comparison of total nucleotide pools of CHO (———) and L (ATCC) (– – –) cells. Chromatographic conditions: 50 μl cell extract injected; 0.05 A_{254} total absorbance; flow-rate 1 ml/min; 0.2 *M* $(NH_4)H_2PO_4$, pH 5.1

The analysis of total nucleotides using reversed-phase column HPLC has numerous advantages over other methods. The method is rapid, highly reproducible, and does not require equilibration between samples. Cyclic nucleotides and nucleosides are only eluted if methanol is added to the salt solution, or after a lengthy period of time (over 60 min) without methanol (unpublished results).

The normal procedure used in examining nucleotide contents of biological samples relies on radioactive labelling procedures. However it is difficult in such procedures to calculate the specific activity of each nucleotide pool, or to measure the turn-over rate of various nucleotides. The method described in this paper does not rely on such measurements, and can also be used in conjunction with labelling to directly measure the fate of any specific radio-labelled compound added to the medium. The specific activity of each nucleotide can thus be calculated.

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