An Analytical System for Rapid Separation of Tissue Nucleotides at Low Pressures on Conventional Anion Exchangers

Joseph X. Khym

An analytical anion-exchange procedure has been developed for the rapid separation of acid-soluble nucleotides (the so-called “free” or tissue nucleotides). It permits assay at low pressures (275–415 kPa; 40–60 psi) in less than 1 h on 10-cm columns of Aminex resins (conventional styrene-type anion exchangers) with alkaline citrate solutions as the eluent. Separation variables have been investigated, to determine optimum conditions for the routine analysis of samples containing tissue nucleotides. Also described here is a simple solvent-extraction procedure for removing HClO₄ or CCl₃CO₂H quantitatively from cell extracts that contain acid-soluble nucleotides: they are removed from aqueous acid solutions with a water-insoluble amine dissolved in a water-immiscible solvent.

Additional Keyphrases: quantitative separation of acid-soluble nucleotides • rapid chromatographic separation of tissue nucleotides at low pressures • analysis of “free” nucleotides • removal of trichloroacetic or perchloric acids by simple extraction

Various liquid-chromatographic procedures are available for isolating and assaying acid-soluble nucleotides (1). In some of the newer procedures, the nucleotides are separated on ion-exchange celluloses (2) and dextrans (3) or by reversed-phase systems (4, 5). However, because of the recent trend toward both speed and resolving power in the development of chromatographic systems (6–8), and because of the particular demands for speed, sensitivity, and quantitation in the routine determinations of cellular nucleotide pools, pellicular ion-exchange (9, 10) systems have been used more often than other chromatographic procedures. Thus, since 1967 when pellicular exchangers were first introduced (9) conventional resin anion exchangers have been used less and less in chromatographic separations of tissue nucleotides (1, 9, 10). However, in a recent preliminary communication, Khym (11) demonstrated that on the citrate form of Aminex A-27 (a conventional styrene-type anion exchanger, particle size 12–15 μm) with alkaline sodium citrate as the eluent, tissue nucleotides may be separated in about 1 h on columns only 10 cm in height. The citrate system compared very favorably with similar analyses carried out with pellicular anion exchangers, which have the disadvantage of lower capacity than conventional resins. Furthermore, because the A-27 columns are short, they can be operated at lower pressures (275–415 kPa; 40–60 psi) than are necessary for the pellicular ion-exchange columns (up to 34.5 MPa, or 5000 psi). This gives the added advantage of being able to operate with low-cost chromatographic equipment (e.g., glass columns).

The present work details the influence of various separation variables associated with the low-pressure liquid chromatography of tissue nucleotides on the citrate form of Aminex exchangers. A rapid, simple solvent-extraction procedure is also described for the quantitative removal of residual precipitating acid from cell extracts containing acid-soluble nucleotides.

Materials and Methods

Reagents

Sodium citrate (Na₃C₆H₅O₇·2H₂O), citric acid, and CCl₃CO₂H were purchased from the Mallinkrodt Chemical Works; sodium azide and sodium hydroxide solution (1 mol/liter) from Fisher Scientific Co.; 71% HClO₄ solution from J. T. Baker Chemical Co.; and Alamine 336 (tricaprylyl tertiary amine), a General Mills product, from McKerson Corp., Minneapolis, Minn. 55408. Freon-TF¹ (CCl₂FCClF₂, bp 47 °C) was obtained from E. I. du Pont de Nemours and Co., Inc. All reagents were of analytical grade. “A-grade” ribo- and deoxyribonucleoside 5’-mono-, -di-, and -triphosphates as well as ribo- and deoxyribonucleosides and purine and pyrimidine bases were obtained from Calbiochem.

Aminex A-27 (bead diameter, ~13 μm) and Aminex A-28 (bead diameter, ~9 μm) styrene/divinylbenzene anion exchangers were bought from Bio-Rad Laboratories.

¹ Nonstandard abbreviations used: Freon-TF, trichlorotrifluoroethane; and ADP-Rib, adenosine 5’-diphosphoribose.

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Equipment

Chromatographic equipment. The following accessories—originally sold by Chromatotronix, Inc. but now obtainable from Laboratory Data Control, Riviera Beach, Fla. 33404—were used throughout these investigations: jacketed glass columns (0.63 × 33 cm), interchangeable top and bottom adjustable internal bed support plunegs, off-column septum-type injectors (Model 164A11), and various lengths of 1/8-in. (o.d.) Teflon tubing with Cheminert connector fittings. The off-column injectors were modified by blocking the original inlet port and boring a new port about 2.5 cm up toward the septum holder. New septum holders were fabricated with needle guides of smaller diameter, to better accommodate Hamilton injection syringes.

Pumps and gauges. The flow rate of eluent through a column and its component parts was controlled with a Milton-Roy Minipump (0–1000 ml/h maximum flow rate). Gauges (Durogauge, 0–200 psi) with an AISI 316L tube and socket were obtained from Dresser Industrial Valve, Stratford, Conn. 06497.

Ultraviolet detector. In the initial work (11), the column was monitored with a UA-2 ISCO ultraviolet analyzer (single cell, 10-mm path length, 50-μl capacity) (Instrumentation Specialties Company, Lincoln, Neb. 68505). In the present work, monitoring was done with a more sensitive UA-5 ISCO instrument (dual cell, 10-mm path length, 19-μl capacity). External recorders (e.g., Beckman 10-in. recorder) were attached to the ISCO's when peak areas were measured quantitatively.

Each ultraviolet detector was calibrated independently by determining extinction coefficients as follows: Single authentic compounds, dissolved at a known concentration in citrate buffer of pH 8.2, were allowed to flow directly through the measuring cell of the ultraviolet analyzer. Because the optical cells had a 10-mm pathlength, the extinction coefficients were calculated from the equation:

\[ \varepsilon_{254} = \frac{\text{absorbance at 254 nm}}{\text{concentration of authentic compound (μmol/ml)}} \]

The results of one such calibration are shown in Table 1. Measurements on the UA-5 ISCO were made in the absorbance range 0.02–1.0; deviation from linearity was not noted.

To prevent bubble formation in the measuring cells of the optical units, about 3 m of small-bore (0.3 mm) Teflon tubing (Chromatotronix, part No. T063012) was affixed to the exit side of the cell. Such tubing adds about 100 kPa (15–20 psi) to the operating pressure of the chromatographic system.

Elution

Preparation of sodium citrate eluents. All citrate eluent solutions contained 3 × 10⁻⁴ mol of sodium azide per liter, to prevent bacterial growth in both the citrate eluents and on the citrate form of the Aminex resins. Both the dilute (0.025 mol/liter) and the concentrated (0.5 mol/liter) eluents were prepared from 1 mol/liter sodium citrate solution, which was filtered through a 0.45-μm Millipore membrane after its preparation. pH was adjusted by adding small amounts of either citric acid (1 mol/liter) or NaOH (1 mol/liter), or both, to prepared citrate eluents. If not protected from atmospheric CO₂, the pH of citrate solutions will decrease markedly.

Gradient apparatus. The eluent, generated as a convex gradient (25 to 500 mmol of citrate per liter, pH 8.2), was delivered to the column by the apparatus shown in Figure 1. The advantage of this design is that, at the completion of a run, the column is easily equilibrated with dilute buffer in anticipation of the next run by means of a three-way stopcock. Furthermore, as seen in Figure 1, this design allows the apparatus to be detached and cleaned during the 15- to 20-min equilibration.

The gradient employed does not impair the reproducibility of the analyses or cause the resin bed to swell or shrink noticeably. Depending on the amount and nature of sample impurities, about 50 consecutive determinations can be performed on a single column, after which usually only the first few millimeters have to be replaced.

Column Preparation

Before packing in a column, 3–5 ml of Aminex A-27 or A-28 was consecutively treated with the following solutions: one part of concentrated HCl plus two parts of ethanol (20 min); 1 mol/liter HCl (4 h); 1 mol/liter NaOH (1 h); 1 mol/liter citric acid (until the filtrate was acid); 0.5 mol/liter sodium citrate, pH 8.3 (until the filtrate was free of Cl⁻). Slurries of the resins and the various wash solutions were filtered through 15-ml sintered-glass funnels (ASTM, "medium" porosity Pyrex No. 36060). After the last treatment, a concentrated slurry of resin and sodium citrate (pH 8.2, 25 mmol/liter), consisting of 1 part resin and about 0.5 part citrate eluent, was prepared.

Table 1. Values for Molar Absorptivities, Determined at 254 nm for Nucleoside Phosphates in Citrate Buffer (pH 8.2)⁴

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \varepsilon_{254} )</th>
<th>Compound</th>
<th>( \varepsilon_{254} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMP</td>
<td>0.34</td>
<td>AMP</td>
<td>13.0</td>
</tr>
<tr>
<td>CDP</td>
<td>6.0</td>
<td>ADP</td>
<td>13.1</td>
</tr>
<tr>
<td>CTP</td>
<td>12.8</td>
<td>ATP</td>
<td>12.4</td>
</tr>
<tr>
<td>UMP</td>
<td>12.7</td>
<td>GDP</td>
<td></td>
</tr>
<tr>
<td>UDP</td>
<td>7.9</td>
<td>GTP</td>
<td></td>
</tr>
<tr>
<td>UTP</td>
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</table>

⁴Identical values for \( \varepsilon_{254} \) were obtained with either the 19- or 50-μl cells when placed in a ISCO type 6 optical unit. Determinations were made on 0.02, 0.1, and 1.0 absorbance range scales.
To operate at lower pressures, the middle disk (0.006 in. mean pore diameter) contained in the adjustable bed support plungers is removed, and the two outer disks (0.034 in.) are put back in place. With the column at room temperature and the bottom support plunger in place, the concentrated resin slurry is added to a column with a disposable pipet. After inserting the top plunger, dilute citrate eluent is pumped through the column until most of the resin has settled. The pumping is stopped, and more resin is added to the column until the bed height remains stationary at 10 cm. At this point the column temperature is brought to 70 °C by means of a Haake Model F constant-temperature circulating-water bath, and 0.5 mol/liter citrate eluent is pumped through the column for about 2 h. (The pressure decreases noticeably when the column is operated at 70 °C). After equilibrating, with dilute citrate eluent, the column is ready for use.

After packing, a new column often shows improved resolution after a few column runs have been made. In some instances, adjacent pairs such as UTP and GDP will not separate at all initially, but will do so in subsequent determinations.

Removal of Perchloric or Trichloroacetic Acids

Acid-soluble nucleotides are usually extracted from tissues with either CCl₃CO₂H (about 0.3 mol/liter) or HClO₄ (0.3 to 0.5 mol/liter); because these acids interfere with most subsequent chromatographic assays, they customarily are removed from samples before analysis.

The procedure used in these studies involves neutralizing the acid solutions with a water-insoluble amine (Alamine 336, mol wt 392) contained in a water-insoluble organic solvent (Freon-TF). The extractions are done as follows. Equal volumes of tissue-extract samples and amine/Freon solutions (about 0.5 mol of Alamine 336 per liter) are mixed gently for 3–4 min in a screw-cap container. The phases may be separated in a few minutes by low-speed centrifugation. Extraction of aqueous HClO₄ solutions yields two clear organic phases, both cleanly separated from a single aqueous layer; extraction of aqueous CCl₃CO₂H solutions yields clear phases for both the organic and aqueous layers. In either case nucleotides, nucleosides, and purine and pyrimidine bases remain quantitatively in the aqueous (top) phase, which now has a pH of 4–5. Aliquots of the aqueous phase may be placed directly on the Aminex columns from the aqueous phase or first be concentrated by vacuum distillation. Citrate eluent at pH 8.2 is usually added to extracted samples so that the final citrate concentration is 25 mmol/liter.

Fresh preparations of Alamine/Freon solutions are clear and colorless and impart negligible amounts of ultraviolet-absorbing material to aqueous phases during extractions. Upon standing for several days, Alamine may crystallize from the Freon solvent and the preparations may turn yellow.

Column Operation

The columns are routinely operated at a flow rate of 0.6 ml/min and at a temperature of 70 °C. Under these conditions, Aminex A-27 columns develop a pressure drop across the resin bed of 275–345 kPa (40–50 psi) when dilute citrate eluent is being pumped through the system. An increase of about 140 kPa (20 psi) occurs as the concentration of citrate eluent approaches the limit concentration of 0.5 mol/liter. Under the same conditions of flow rate and temperature, Aminex A-28 columns operate at pressures 140–170 kPa (20–25 psi) higher than those of equivalent columns of A-27. As noted in the legend information, operating pressures vary considerably with either flow rate or temperature.

Small sample volumes (10–200 μl) were injected with Hamilton syringes without interrupting the flow of eluent. Larger amounts (up to 1.0 ml) were applied to the column with the pumps momentarily off.

Identification and Quantitation

In these studies identifications were made solely on the basis of the retention time (or volume to peak) of a given compound. Elution positions were established with authentic compounds (either ribo- or deoxyribonucleoside 5'-mono-, -di-, or -triphosphates or related compounds) under a specific set of column operating conditions. The reference compounds (1 to 50 nmol), dissolved in citrate eluent (25 mmol/liter), were applied to the column singly or in various simple mixtures until elution positions were established beyond any doubt. For example, once the elution

Fig. 1. Gradient apparatus for 10-cm Aminex column

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order of, say, the nucleoside monophosphates was established, two nucleoside diphosphates were added to a mixture of the monophosphates and the analyses were repeated. If there was any doubt about elution position, concentrations of the compounds in question were changed in the mixtures under study.

Peaks were quantitated by peak-height and peak-width measurement \((8, 12)\) according to the equation:

\[
\text{nmol} = \frac{H_A \cdot W_{\text{ml}} \cdot 1064}{\varepsilon_{254}}
\]

where \(H_A\) is the net peak height (in absorbancy units) measured at 254 nm, \(W_{\text{ml}}\) is the peak width (in volume units) measured at half peak height, \(\varepsilon_{254}\) is the extinction coefficient of a particular compound as previously defined, and 1064 is an equation constant.

Results and Discussion

Removal of Acid from Tissue Extracts

Even though \(\text{CCl}_3\text{CO}_2\text{H}\) and \(\text{HClO}_4\) are widely used to extract nucleotides from tissues, there has been no agreement as to the best method for quantitative extraction \((13-15)\). Not only cold acids, but other solvents \((13-15)\) are often used for extractions, the choice depending upon the origin of the kind of tissue being analyzed \((16-18)\). Nonetheless, by the Alamine/Freon procedure given here, acid-soluble nucleotide material, once extracted from tissue fragments, quantitatively remains in the aqueous phase, while both \(\text{CCl}_3\text{CO}_2\text{H}\) and \(\text{HClO}_4\) (and, presumably, any other strong acid) are partitioned quantitatively to the organic phase. This was observed by two different sets of experiments. In one, separate mixtures of either ribonucleoside 5'-mono-, or -di-, or -triphosphates were dissolved in cold \(\text{CCl}_3\text{CO}_2\text{H} (0.3 \text{ mol/liter})\) or \(\text{HClO}_4 (0.4 \text{ mol/liter})\) and shaken with an equal volume of 0.5 mol/liter Alamine in Freon solvent. In each case, upon separation of the phases, the aqueous layer had a pH of 4-5 and contained all of the added nucleotides, as ascertained spectrophotometrically. In the other experiment, it was observed that losses were slight, if they occurred at all, when mixtures of nucleoside phosphates in either acid were extracted with Alamine/Freon solvent, concentrated to dryness in a micro rotary evaporator, and then dissolved in 15 mmol/liter citrate eluent and separated on the citrate form of Aminex A-27. A typical experiment of this type is described in Figure 2.

Removal of acids with amines dissolved in water-immiscible solvents is not new; the system was first used by Smith and Page \((19)\) to remove mineral acids from protein hydrolysates. Although simple and convenient (as compared with removal of the \(\text{ClO}_4^-\) anion as its potassium salt or of \(\text{CCl}_3\text{CO}_2\text{H}\) by repeated extractions with ether), this technique has not been used routinely to remove acid extractants from tissue preparations. The choice of amine and (or) solvent is arbitrary (e.g., see references \(17, 19, 20)\) as long as the chosen solvent system carries out the intended task. The choices of reagents here were based on the facts that Alamine 336 is obtainable in a very pure state, is very insoluble in water, and does not yield ultraviolet-absorbing impurities, and that Freon-TF and water are virtually insoluble in each other \((21)\).

Analytical Variables Affecting Nucleotide Separations on the Column

Effect of temperature. The optimum temperature for separating nucleoside phosphates on the citrate form of A-27 (or A-28) at pH 8.2 is 70 °C (Figure 3). At temperatures in the range of 50-60 °C AMP/CDP, GDP/UDP, and ATP/GTP pairs do not separate, while at 75 °C AMP/CDP separate cleanly but UTP tends to overlap GDP. AMP and CDP may be completely separated at 70 °C by having a gradient delay of 2-3 min—that is, after sample injection, dilute citrate eluent is pumped momentarily a few minutes longer before the gradient is started (or, alternatively, the exit tube from the gradient device to the column is made correspondingly longer).

Because 70 °C is the temperature used, it is essential to determine the stability of the nucleoside phosphates at this temperature. The results of this study are shown in Figure 4. Sodium azide was not added to stock preparations of nucleoside phosphates, nor was it present during the 2-h heating period as described in Figure 4. Sodium azide solution, even at a concentration of 0.3 mmol/liter, degrades nucleoside phosphates slowly over a period of several days. Thus, even though it was present in all citrate eluents, it...
was not added to nucleoside phosphates dissolved in citrate buffers.

**Effect of pH.** The optimum pH range for separating nucleoside phosphates on the citrate form of either A-27 or A-28 is between 8.0 and 8.3 (these are room temperature values; the actual pH values of citrate eluent at 70 °C were not determined). Because of slight differences in different batches of resins, small adjustments in pH values within the generally optimal range sometimes improve the resolution of adjacent nucleoside phosphate peaks.

Between pH 8.0 and 8.3, the retention time for uridine and guanosine nucleoside phosphate increases markedly (and more noticeably at pH's higher than 8.3) as the pH is increased. This is because of increased ionization of the acidic groups in the base moieties of the uridine and guanosine phosphate derivatives. Thus, if certain adjacent pairs such as UDP/CTP or UTP/GDP are not completely resolved, an appropriate increase or decrease in pH should increase the resolution. Adenosine and cytidine nucleoside phosphates do not have acidic groups or their base moieties that ionize in the range between pH's 8.0 to 8.3, hence elution positions remain the same for these derivatives with small changes in pH of the citrate eluent. Thus the resolution between AMP and CDP cannot be improved by pH changes, but is remedied by controlling citrate concentrations as mentioned previously.

**Speed of analysis.** Since 1967, speed and efficiency have been the main incentives for using pellicular exchangers rather than other types of exchangers for tissue nucleotide assay (8-10, 22). However, as shown in Figure 5, with analytical resolution (8) such analyses can be completed in about 40 min on columns of conventional resin exchangers that are only 10 cm in height. Also, as seen in Figure 5, there is complete resolution of adjacent peaks when the flow rate is decreased from 1 to 0.3 ml/min; at this slower flow rate an analysis takes about 2.5 h to complete, but there is the advantage of low operating pressures (only 240-340 kPa, or 35-50 psi).

If greater speeds are important in chromatographic assays of tissue nucleotide samples, 5-cm columns of A-27 or A-28 may be used to carry out nucleotide

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**Fig. 3.** Effect of temperature on the resolution and retention time of nucleoside phosphate peaks

The same mixture of ribonucleoside phosphates, each at a concentration of about 0.1 μmol/ml, was assayed on a 10-cm A-27 column at a flow rate of 0.6 ml/min and at the temperatures shown. In each case a 50-μl aliquot was used.

**Fig. 4.** Stability of nucleoside phosphates at 70 °C in citrate buffer (pH 8.2)

A mixture of ribonucleoside phosphates, dissolved in 25 mmol/liter citrate eluent, was analyzed directly on a 10-cm A-27 column and again after heating the mixture at 71 °C for 2 h. Identical aliquots (75 μl) were injected into the column (operated at 70 °C and a flow rate of 0.6 ml/mm).

**Fig. 5.** The same mixture of ribonucleoside phosphates, each at a concentration of about 1.0 μmol/ml, was assayed on a 10-cm A-28 column at the flow rates and pressures shown.

A 30-μl aliquot was used in this case and the chart speed of the external recorder was 0.1 in./min and full scale deflection was 1.0 absorbancy units.
separations in about 25 min. On the smaller columns, resolutions are still sufficient, but there is considerable overlapping of some adjacent peaks.

Quantitative Measurements

Samples were examined routinely on A-27 or A-28 columns at a flow rate of 0.6 ml/min and a temperature of 70 °C. In these determinations, external recorders attached to the column-monitoring systems were operated at 12 in./h. To test for quantitative recovery under these conditions, AMP/ATP mixtures of known concentration were examined. The results of these assays (Table 2) were calculated by means of equation 2. Peak-height and peak-width were measured from chromatograms typified by the one shown in Figure 6, which also shows the retention times for all 12 common nucleoside phosphates run under the same conditions as the AMP/ATP mixtures.

Practical Applications

At present, this method of analyzing tissue nucleotides is being used to evaluate different methods for extracting "free" nucleotides from Escherichia coli preparations.3 Table 3 shows some preliminary results.

Another application of practical interest is testing reagent chemicals for purity. An example of this is the simultaneous analysis of four commercial ribonucleoside diphosphate preparations. Purposely, preparations several years old were chosen for this test. The diphosphates were dissolved in dilute citrate eluent, and the resulting mixture was assayed on an Aminex A-27 column. The chromatogram (Figure 7) showed that each diphosphate contained its corresponding monophosphate in a proportion ranging from 17 to 22%.

Chromatography of Compounds Related to Ribonucleoside Phosphates

Under the column operating conditions described here, NAD and NMN elute before UMP and, if present in concentrations about equal to that of UMP, tend to overlap this peak. NADP elutes in the same position as AMP. When assayed singly, NADH gives a chromatogram with a small breakthrough peak and another small peak with a retention time greater than GTP. Other similar compounds such as ADP-Rib, FAD, etc., have not been investigated.

Mixtures of deoxyribonucleoside phosphates yield chromatograms that are similar to those obtained for the ribo-derivatives. However, as seen in Figure 8, dAMP and dCDP as well as dCTP and dTDP do not separate, and dADP and dTTP separate only partially. Other sets of conditions are being investigated for

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3 These experiments carried out with the collaboration of R. L. Schenley of the Biophysics and Cell Physiology Section at Oak Ridge National Laboratory.
Table 3. "Free" Nucleotides in Escherichia coli Cells* after Various Treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>UMP</th>
<th>AMP</th>
<th>CDP</th>
<th>GMP</th>
<th>UDP</th>
<th>ADP</th>
<th>GDP</th>
<th>ATP</th>
<th>GTP</th>
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<tbody>
<tr>
<td>Hot H₂O</td>
<td>3</td>
<td>16</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
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<td>T</td>
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<td>Sonicated</td>
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<td>5</td>
<td>4</td>
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<td>sonicated</td>
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<tr>
<td>Hot H₂O</td>
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<td>T</td>
<td>T</td>
</tr>
</tbody>
</table>

a About 10⁶ cells/ml of extractant.
b Cold CCl₃CO₂H was added to samples after treatment. None was added to the experiment described by the next-to-last row of data.
c CMP, CTP, UTP not measured.
d T = trace.

Efficiency of Separation and Comparisons to Pellicular Ion-Exchange Systems

Column efficiency is expressed quantitatively in terms of the number of theoretical plates (N) found for a column (see references 8, 9, 10, or 12). N may be calculated by (9, 23):

\[ N = 5.53 \left( \frac{V_{mi}}{W_{mi}} \right)^2 \]  

where \( V_{mi} \) is the retention time of a peak expressed in volume units and \( W_{mi} \) is the same quantity given for equation 2. Because \( N \) is proportional to column length (L), a more useful measure of column efficiency may be calculated from the height equivalent to a theoretical plate (HETP), which is defined by:

\[ \text{HETP} = \frac{L}{N} \]  

Another measure of efficiency is the plates per second (N/s) encountered by a peak as it passes through a column; this is obtained from (9, 24, 25):

\[ \frac{N}{s} = \frac{N}{t_R} \]  

where \( t_R \) is the retention time for a peak, in seconds. However, when gradient elution is used, calculations of \( N \) and HETP by equations 3 and 4, respectively, are not valid, because the mass distribution coefficient of a solute will not remain constant throughout a column when the eluate is changing concentration. Thus, to obtain some idea of efficiency values for nucleotide separations with the citrate/Aminex procedure, the four common 5'-ribonucleoside monophosphates were chromatographed at constant eluate concentration (Figure 9). Accordingly, efficiency values were calculated for the separation shown in Figure 9 by the use of equations 3 to 5; these values, given in Table 4, compare favorably, and in most instances are better than similar values found for pellicular ion-exchange systems (9, 10, 24, 25). Efficiency can be improved further by using A-28 rather than A-27 columns. This has been judged qualitatively by noting that, when compared under identical conditions, nucleoside phosphate peaks have narrower band widths on A-28 columns.

In some instances, pellicular exchangers have an advantage over conventional resins in tissue nucleotide work. One advantage is that separations can be

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performed at ambient temperatures with the newer pellicular ion-exchangers (22). As Figure 2 shows, this is not important for the common ribonucleoside phosphates, but for more labile compounds (e.g., NAD) hydrolytic decompositions would be of concern. Another advantage of the pellicular analytical system is that very sensitive detectors (e.g., less than 0.02 absorbancy units full scale) can be used without serious baseline noise and drift (9). In the present work, considerable baseline drift occurred on the 0.02 absorbance scale (presumably because of the steep gradient used), especially toward the very end of a chromatogram. Nevertheless, because the capacities of conventional resins are several hundred fold greater than those of the pellicular resins, larger sample volumes may be injected into the A-27 or A-28 columns used here. Thus, less-sensitive absorbancy ranges are not always needed.

References