Rapid Preparation of Nucleotides from Acid-Soluble Pools by Chromatography on Silica, as Exemplified with Acid Extracts of Cultured Cells

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A rapid technique (5–10 min) has been developed for fractionating nucleotides from base and nucleoside contaminants in acid extracts of cells, by adsorption to silica gels. Silica gels (1-mL bed volume) were washed with 5 mL of water then with 5 mL of acetonitrile/water (90/10 by vol). After applying 3-mL samples, adjusted to 900 mL/L acetonitrile content, we washed the gel with an additional 10 mL of the acetonitrile/water solvent. More than 95% of the amounts of bases and nucleosides present, except for cytidine (92%), did not adsorb to silica under these conditions. Nucleotides were then quantitatively eluted with 9 mL of water. The retention volumes for positive, negative, and neutral nucleic acid components have been determined, to investigate the discriminatory properties of nucleic acid components on silica. Compounds (bases, nucleosides) that are not ionized at pH 7 do not adsorb to silica. However, negative, positive, and zwitterionic compounds are tightly adsorbed to the silica gels. This procedure has been used to purify nucleotides from several normal and transformed cell lines.

Additional Keyphrases: sample purification • nucleic acids • nucleotide pools from normal and transformed fibroblasts and epithelial cells • metabolic disease

Numerous low relative molecular mass compounds, including the nucleic acid components, nucleotides, bases, and nucleosides, are present in acid extracts of cells, serum, or urine. Adequate supply and regulation of these nucleotide pools are essential for normal nucleic acid metabolism (1). Cells can synthesize nucleotides de novo, or salvage purines and pyrimidines for nucleotide synthesis. Several clinical disorders have been associated with abnormal nucleic acid metabolism. Deficiency of the purine salvage enzyme hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) leads to an accumulation of uric acid in body fluids, and is associated with gouty arthritis and in some cases a severe neurological disorder, Lesch–Nyhan syndrome (2). In other patients a feedback-resistant enzyme in the purine biosynthetic pathway (phosphoribosyl pyrophosphate synthetase, EC 2.7.6.1) is associated with gouty arthritis (2, 3). Several other metabolic disorders have been associated with the absence of purine and pyrimidine biosynthetic enzymes (2). Recently the molecular basis for several severe immunological disorders has been identified (4, 5). The absence of adenosine deaminase (EC 3.5.4.17) or purine-nucleoside phosphorylase (EC 2.4.2.1), two successive enzymes in the purine salvage pathway, or of 5′-nucleotidase (EC 3.1.3.5), a general catabolic enzyme, has been associated with severe impairment of the immune system (4–6).

In these metabolic diseases, shifts in the composition of the acid-soluble pool occur, reflecting the enzymic lesion. Detection of the disease state is dependent on accurate and quantitative measurements of enzyme activity and of the acid-soluble pool from serum, urine, or cells. Khym et al. (7) have shown the importance of removing contaminants to make quantitative measurements of nucleotides from acid extracts; we have confirmed these observations.

We have also developed new techniques for rapid preparation of nucleotides and their constituent bases and nucleosides from acid extracts of cells. In addition to facilitating the preparation of acid-soluble pools, these procedures are readily applicable to enzymic measurements, simplifying and accelerating separation of substrate and product. The new procedures reduce the time and number of manipulations required to prepare several nucleotide groups before ion-exchange analysis of the nucleotide pools.

Earlier approaches to preparation of nucleotides from acid extracts have involved ligand exchange (8), gel filtration (9), borate complexing (10–12), and ion-exclusion chromatography (13). The most efficient separations were obtained on a partly hydrolyzed polycrylamide gel where nucleotides are excluded: borate-complexed nucleosides are only slightly retained, while the bases are retained sufficiently to be separated from nucleotides (13). This procedure, however, requires prior preparation of the gel and the routine performance of a chromatographic run and fraction collecting. We have recently described a technique for rapid group separations of nucleic acid components on 1-mL silica cartridges (14).

In this paper we describe an alternative procedure for preparation of nucleotides from acid extracts, also by adsorption to silica. To illustrate the usefulness of this procedure we have purified nucleotides from acid extracts of normal and transformed cell lines on the silica cartridges. We tested silica gels from several sources to determine whether the source of silica is a variable in this procedure. Retention volumes for neutral, positive, negative, and zwitterionic compounds were determined, to investigate the basis for retention on silica adsorbents. Charged nucleosides and nucleotides bind to the silica, but compounds without a charge at neutral pH are not retained.

Materials and Methods

Materials

Commercially prepared silica cartridges (no. 51900) were obtained from Waters Associates, Milford, MA 01757. Other silica gels were silicic acid (no. 23847) from Mallinckrodt Chemical Works, St. Louis, MO 63160; Silica Gel HA (cat. no. 131-1180) from Bio-Rad Lab., Richmond, CA 94804; Unisil from Clarkson Chemical Co., Williamsport, PA 17701; and Silica Gel (no. SX144-06) from Matheson Coleman and Bell, Norwood, OH 45212. Nucleotides and nucleosides were purchased from Calbiochem, La Jolla, CA 92112; Sigma Chemical Co., St. Louis, MO 63178; and P-L Biochemicals, Milwaukee, WI 53205. Acetonitrile was purchased from Fisher Scientific Co., Fair Lawn, NJ 07410. The anion-exchange resin (Aminex A-28) and fritted columns for the silica gels (cat. no. 731-1110)
The eluted of Samples Silica wash) Anion-Exchange tested at ciates) Apparatus were delay bacteriostatic eluted were applied to the silicas and nucleosides elute in the wash. Nucleotides were then eluted with 9 mL of water.

Apparatus

The commercially packed silica gels (Waters Associates) were inserted in the end of a 20-mL syringe (Becton-Dickinson Co., Rutherford, NJ 07070). Buffers and samples were applied to the gels with a Pasteur pipette; a flow rate of about 4 mL/min was maintained. After solutions were passed through the gel, the cartridge was removed from the end of the syringe and the plunger was withdrawn to refill the syringe. The silica cartridge were prepared for reuse by drying for 1 h at 130 °C.

Approximately 1 mL (dry volume) of each of the other tested silicas were placed in a fritted column, washed, and eluted as described.

Anion-Exchange Chromatography

The anion-exchange resin was prepared and used as suggested by Khyrn (15), except that chloroform was used as the bacteriostatic agent instead of sodium azide and the gradient delay was 8 min, not 6 min. Samples of 100 μL or less were applied to the resin with an in-line injection device (model no. 7120; Rheodyne Co., Berkeley, CA 94710). Column dimensions were 300 × 3.9 mm, and the flow rate was 0.7 mL/min. The effluent was monitored at 254 and 280 nm (Duol Monitor no. 1222; LDC, Riviera Beach, FL 33404). Quantitations were made by converting peak areas into nanomoles of substance (16). Individual values for molar absorptivities at 254 nm were those determined by Khyrn (15).

Preparation of Acid-Soluble Pools from Cultured Cells

Acid-soluble pools were extracted with 5 mL of ice-cold 0.5 mol/L trichloroacetic acid for 10 min at 3 °C. Cells and supernatant liquid were removed from the dishes, and each dish was washed with an additional 2 mL of the acid. The entire supernat was centrifuged for 10 min at 16 000 × g. The supernat was neutralized and the acid extracted with a slight excess of a water-insoluble tertiary amine (Alamine 336; General Mills Chemicals, Kankakee, IL 55435) in a water-immiscible solvent (Freon TF; DuPont Co., Wilmington, DE 19898), as described by Khyrn (15). The aqueous phase was concentrated by evaporation (Rotary Evapo-Mix; Buchler Instruments, Fort Lee, NJ 07024). Acid-soluble pools were then processed on the silica cartridges, and the nucleotide fractions were concentrated and chromatographed on the anion-exchange resin.

Tissue Culture

Normal (IARC-20) and transformed (IARC-28) rat liver epithelial cells were cultured in William's media supplemented with 100 mL of fetal calf serum per liter, as previously described (17). Normal hamster embry fibroblasts, prepared from pregnant hamsters (10-12 days' gestation), and transformed hamster embryo fibroblasts [B(a)P-2] were cultured in Dulbecco's medium supplemented with 100 mL of fetal calf serum per liter (18). Nucleotides were extracted from normal fibroblasts on the third passage in subculture.

Results

Optimum Conditions for Preparation of Nucleotides

To determine optimum conditions for separating nucleotides from bases and nucleosides, we chromatographed mixtures of each of several different acetonitrile concentrations on the silica cartridges. The results from these experiments are recorded in Tables 1, 2, and 3 for bases, nucleosides, and nucleotides, respectively. The results indicate that greater than 90% of the quantities of bases and nucleosides do not bind to the silica gel when applied in a solution that is 900 mL/L or less acetonitrile. When a sample is adjusted to 95/5 (by vol) acetonitrile/water and chromatographed on a silica gel, a significant amount of the more polar nucleosides (cytidine and guanosine) is bound to the gel. Less polar nucleosides (adenosine, uridine, and ribothymidine) and bases are not significantly bound to the gel under these conditions. Greater than 90% of the amount of nucleotides present bind to the silica gel, when samples are adjusted to 900 or 950 mL/L of acetonitrile per liter and chromatographed on cartridges equilibrated with these solutions. However, nucleotides are not bound quantitatively to a gel equilibrated with 850 mL of acetonitrile per liter.

| Table 1. Effect of Acetonitrile on Adsorption of Bases on Silica |
|----------------|----------------|----------------|----------------|----------------|
| Solvent composition, acetonitrile/water | % of total bases recovered |
|                |                 |                 |                 |                 |
|                | Wash | Bound | Wash | Bound | Wash | Bound | Wash | Bound |
| 85/15 | 100 | ND | 100 | ND | 100 | ND | 100 | ND |
| 90/10 | 100 | ND | 100 | ND | 100 | ND | 100 | ND |
| 90/10 | 100 | ND | 100 | ND | 100 | ND | 100 | ND |
| 95/5 | 96 | 4 | 95 | 5 | 94 | 6 | 81 | 19 |

Procedure as in Table 1.
Table 3. Effect of Acetonitrile on Adsorption of Nucleotides on Silica

<table>
<thead>
<tr>
<th>Acetonitrile/water</th>
<th>CMP</th>
<th>UMP</th>
<th>AMP</th>
<th>GMP</th>
<th>CTP</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>85/15</td>
<td>6</td>
<td>94</td>
<td>70</td>
<td>30</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>90/10</td>
<td>ND</td>
<td>100</td>
<td>ND</td>
<td>100</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>90/10</td>
<td>ND</td>
<td>100</td>
<td>ND</td>
<td>100</td>
<td>11</td>
<td>89</td>
</tr>
<tr>
<td>95/5</td>
<td>ND</td>
<td>100</td>
<td>ND</td>
<td>100</td>
<td>6</td>
<td>94</td>
</tr>
</tbody>
</table>

% of total nucleotides recovered

Procedure as in Table 1.

Table 4. Acid-Soluble Nucleotides from Normal and Transformed Cells

<table>
<thead>
<tr>
<th>Nucleosides</th>
<th>Cytidylates</th>
<th>Uridylylates</th>
<th>Adenylates</th>
<th>Quantitites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>CMP</td>
<td>UMP</td>
<td>AMP</td>
<td>GMP</td>
</tr>
<tr>
<td>IARC-20</td>
<td>1.3</td>
<td>61.3</td>
<td>7.9</td>
<td>ND</td>
</tr>
<tr>
<td>IARC-28</td>
<td>3.3</td>
<td>24.5</td>
<td>9.8</td>
<td>2.1</td>
</tr>
<tr>
<td>3HEF</td>
<td>3.8</td>
<td>77.1</td>
<td>26.8</td>
<td>1.3</td>
</tr>
<tr>
<td>B(a)P-2</td>
<td>ND</td>
<td>109.1</td>
<td>54.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Nucleotides from Acid Extracts of Cultured Cells

Neutralized acid extracts of cultured cells were adjusted to 900 mL of acetonitrile per liter by 10-fold dilution with acetonitrile and then chromatographed on silica cartridges equilibrated with the same solvent. Bases and nucleotides were eluted with 10 mL of the 900 mL/L solvent. Nucleotides were then quantitatively eluted with 9 mL of water. Nucleotides were concentrated by evaporation and subsequently chromatographed on the anion-exchange resin (15). A typical chromatograph of acid-soluble nucleotides on the anion-exchange resin is illustrated in Figure 1. Acid-soluble components not bound to the silica gel were also chromatographed on the anion-exchange resin to illustrate the removal of contaminants (Figure 1). With this protocol we have prepared nucleotides from normal and transformed cell lines, and have quantitated (nmol/10^6 cells) the amount of each individual nucleotide (Table 4). The nucleoside triphosphates are the major constituents of the acid-soluble nucleotide pools, for both epithelial and the fibroblast cell lines. The purine nucleotides are more abundant than the pyrimidine nucleotides, and there are also significant quantities of nucleotide sugars and nicotineamide coenzymes (Figure 1). Although the quantities vary slightly for a given nucleotide, we see no significant differences in pool sizes, when comparing the normal cell with its transformed counterpart. However, the nucleotide pools are in general...
larger in the fibroblasts [HEF, B(a)P-2] than in the epithelial cell lines [IARC-20, IARC-28].

Retention Volumes for Minor Components of RNA on Silica Gels

To investigate the basis for binding of nucleic acid components to silica, we have determined the retention volumes for 10 methylated nucleosides (Figure 2). By determining the retention volumes of minor components of RNA, some of which have a positive charge at neutral pH, we have been able to investigate whether the nature of the charge (positive vs. negative) is important for binding to silica. Minor nucleosides were loaded under conditions where (a) bases do not bind, (b) nucleosides bind and are then eluted by neutralizing the borate complex, and (c) nucleotides are eluted last with water (14). Nucleosides that are positively charged at neutral pH chromatograph like negatively charged nucleotides (14) on the silica gels. Nucleosides with no charge at neutral pH are eluted with acetoni/tri/tol (90/10 by vol). On this basis the cationic nucleosides 3-methyl adenosine, 7-methyl guanosine, and 3-methyl cytidine can be separated from neutral nucleosides such as cytidine, uridine, adenosine, or guanosine.

Chromatography on Different Commercial Preparations of Silica

To determine whether the source of silica is a variable in these procedures, we chromatographed nucleotide mixtures containing bases and nucleosides on silicas purchased from different manufacturers. For these experiments we used the three-step fractionation procedure (14). Nucleotide mixtures containing bases and nucleosides were chromatographed on approximately 1 mL of four different silica preparations and the prepacked silica cartridges. Pooled fractions were concentrated and then analyzed on the anion-exchange resin to determine the cross contamination of one group with another. Good separations of nucleosides from bases and nucleotides were obtained with all silica preparations. Partial separations of bases from nucleosides were observed with four commercial preparations (Bio-Rad; Mallinckrodt; Clarkson; and Matheson Coleman and Bell), in that a variable amount of nucleosides eluted with the bases in the borate wash. The variable binding of the nucleoside–borate complex is probably a reflection of increased dead space with the loosely packed silicas. Quantitative separations of bases from nucleosides were obtained with the prepacked silica cartridges (Waters Associates).

Discussion

We have described a new procedure for rapid isolation of nucleotides from acid extracts. The procedure is based on batch adsorption and elution and can be easily adapted to multiple, simultaneous extractions with considerable saving of time. Separations of nucleotides from other components of acid extracts can be done routinely in 5–10 min with standard equipment. The only equipment required is a syringe for loading samples, several test tubes to collect fractions, and the silica gel itself. We prefer this procedure to our previously described three-step fractionation method (14) for preparation of nucleotides. With the two-step fractionation, the concentrate contains no contaminating salts, which would ordinarily interfere with subsequent analysis of the acid-soluble pools. However, the three-step procedure is invaluable for studies in which bases and nucleosides must be resolved from each other as well as from the nucleotides.

Recently, Khym et al. (7) demonstrated the importance of removing the contaminants in the acid extracts to make quantitative measurements of acid-soluble nucleotides. We have confirmed their observations and, using our technique, have purified nucleotides from acid extracts of normal and transformed fibroblasts and epithelial cells in culture. After silica chromatography, the nucleotides were analyzed on an anion-exchange resin and quantitated as described above. Using our technique to compare the normal cell (epithelial or fibroblast) with its transformed counterpart, we observed no significant differences in the nucleotide concentrations in the two cell types. Also, the gentleness of this procedure is illustrated by the fact that nucleoside triphosphates are the major components of the acid-soluble pools. Under less than optimum extraction conditions, the nucleoside mono- and di-phosphates are often observed as the major components of the acid-soluble pool (19, 20).

To investigate the nature of the retention mechanism for nucleic acid components on silica gel, we have chromatographed charged, as well as neutral, compounds on the silica gels. Charged nucleosides and nucleotides—either positive, negative, or zwitterionic—are strongly bound to silica. Compounds with no charge at neutral pH are not retained. A negatively charged nucleoside–borate complex forms, separating the neutral nucleosides from bases, 2′-O-methyl nucleosides, and deoxynucleosides, none of which can complex with borate. Cationic nucleosides separate from nucleotides with no charge and chromatograph like negatively charged nucleotides on the silica gels.

The observation that charged compounds, irrespective of the nature of the charge, bind to silica gel suggests that ion-exchange is not the primary mode of retention. If ion-exchange were the primary mode of retention, either positive or negative compounds would bind to the gel, but not both. As discussed by Morris and Morris in a recent review (21), it is now generally agreed that hydrogen bonding to surface silanol groups is the basis for retention on silica adsorbents. Our observations are consistent with this proposal.

Several genetic diseases are a result of aberrant nucleotide metabolism and are marked by altered concentrations of nucleotides, bases, or nucleosides in cells, serum, and urine. Identification of the disease is dependent on quantitative measurements of contents of acid-soluble pools or accurate measurement of enzyme activity. In either case, preparation of acid extracts or separation of substrate and product should be rapid and precise to be of value in routine analysis. The procedures we have described for silica chromatography of nucleic acid components will facilitate routine diagnosis of these diseases by simplifying the assay and decreasing the assay time.

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References