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Note

Rapid purification of radiolabeled trinucleoside diphosphates by reversed-phase high-performance liquid chromatography

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Trinucleoside diphosphates are valuable probes of protein biosynthesis. They have been used to decipher the genetic code[1], to probe the secondary structures of tRNA[2–6], and of 5S rRNA[7] and to probe the specificity of oligonucleotide binding to translational initiation factor IF3 (ref. 8). They have been synthesized enzymatically[9,10] and traditionally isolated by anion-exchange chromatography[11].

The isolation procedure usually requires an overnight elution with a salt concentration gradient, followed by another day of manipulations to remove the salt.

We have chosen to simplify and shorten the amount of time required in the isolation procedure by the application of deproteinized reaction mixtures to an octadecylsilyl porous silica microsphere column[12,13], and elution with a gradient of increasing methanol concentration[13].

Application of this technique allows separation of all 16 dinucleoside monophosphates from each other, and from trinucleoside diphosphates and synthesis byproducts that have been examined.

EXPERIMENTAL

The dinucleoside monophosphates were purchased from Sigma (St. Louis, Mo., U.S.A.) and used in reaction concentrations of 1 m*M*. [^{3}H]Guanosine diphosphate, ammonium salt, 12.7 kCi/mol and [^{3}H]uridine diphosphate, ammonium salt, 22 kCi/mol, were purchased from Amersham/Searle (Arlington Heights, Ill., U.S.A.) and used at 79 and 30 μM reaction concentrations, respectively. Primer-dependent polynucleotide phosphorylase was purchased from P-L Biochemicals (Milwaukee, Wisc., U.S.A.). Ribonuclease T1, pancreatic ribonuclease, and bacterial alkaline phosphatase were purchased from Worthington Biochemicals (Freehold, N.J., U.S.A.).

^{3}H-Labeled trinucleoside diphosphates were prepared similarly to ref. 3. After alkaline phosphatase treatment had been completed and the reaction stopped by boiling at 94° for 2 min, each reaction mixture was brought to 50% ethanol and allowed to sit one hour at −20°. The mixtures were then centrifuged at 12,000 *g* for 10 min at 4°. The supernatants were withdrawn and dried under nitrogen gas, then redissolved in 70 μl of water. Each solution was then injected onto a 250 × 3 mm stainless-steel column (Laboratory Data Control, Riviera Beach, Fla., U.S.A.)

slurry-packed with Spherisorb ODS 5 (Phase Separations, Queensferry, Great Britain) that had been equilibrated with 50 m*M* KH_2PO_4 pH 5.5. Oligonucleotides were separated into mono-, di- and trinucleotide reaction products by a gradient from solvent B (50 m*M* KH_2PO_4, pH 5.5) to solvent A (80% methanol). The gradient was delivered by a Perkin-Elmer 2/2 liquid chromatograph flowing at 1 ml/min, starting at 100% solvent B, and changing to solvent A at 1%/min for 30 min, or 5%/min for 12 min. Collection of the effluent in 0.5-min fractions was done with an ISCO 328 fraction collector equipped with a flow interrupter. The effluent was monitored by a Perkin-Elmer LC-55 variable-wavelength detector at 260 nm; the retention times and areas under the peaks were quantitated by a Columbia Scientific CSI-38 integrator. The molar amounts of oligonucleotides were obtained by converting the integrator counts, in μV·sec, for each oligonucleotide peak, onto moles, as in ref. 13. The extinction coefficients were obtained or calculated from ref. 14.

The oligonucleotide peaks, along with synthesis byproducts, were identified by retention times, as compared with unlabeled standards (Sigma), and by peaks in cpm that were detected by diluting 10-μl aliquots of collected fractions in 3 ml of scintillation fluid (67% toluene, 33% Triton X-100, 0.3 g/l 1,4-bis[2-(5-phenyloxazoyl)]-benzene, 5 g/l 2,5-diphenyloxazole) and counting in a Beckman LS-233 scintillation counter. The desired fractions were then lyophilized, redissolved in water and stored at −20°.

RESULTS AND DISCUSSION

Separation of all four nucleoside-5′-monophosphates and their constituent nucleosides (Table I) from all 16 dinucleoside monophosphates (Table II), and eight synthesized [^{3}H]trinucleoside diphosphate and reaction byproducts (Table III) was accomplished by gradient elution at a solvent change rate of 1%/min. Nucleosides, nucleotides, dinucleoside monophosphates and trinucleoside diphosphates could be separated at a solvent change rate of 5%/min, but resolution was significantly better at 1%/min. The longest chromatographic run required was 2000 sec, for the separation of the ApAp[^{3}H]G reaction mixture, shown in Fig. 1.

TABLE I

NUCLEOSIDE AND NUCLEOTIDE RETENTION TIMES

Sample	*Retention time (sec. ±1%)*	
	At 1%/min	*At 5%/min*
A	844	552
C	199	185
G	438	405
U	225	191
pA	219	155
pC	93	85
pG	125	111
pU	98	93

TABLE II

DINUCLEOSIDE MONOPHOSPHATE RETENTION TIMES

ND = Not done.

Sample	*Retention time (sec, ±1%)*	
	At 1%/min	*At 5%/min*
ApA	1186	641
ApC	ND	551
ApG	1149	587
ApU	1117	578
CpA	ND	521
CpC	382	341
CpG	ND	525
CpU	448	375
GpA	1009	554
GpC	536	444
GpG	927	469
GpU	ND	499
UpA	916	509
UpC	ND	417
UpG	ND	429
UpU	593	411

TABLE III

TRINUCLEOSIDE DIPHOSPHATE RETENTION TIMES

ND = Not done.

Sample	*Retention time (sec. ±1%)*	
	At 1%/min	*At 5%/min*
ApApG	1385	605
ApGpU	1324	ND
ApUpG	1089	550
CpUpG	717	ND
GpApU	1147	ND
CpGpU	987	ND
UpApG	1050	541
UpUpG	898	470

Retention times were reproducible over a six-month period to ±1%. Quantitation by integration, dependent only on Beer's law, rather than on internal standards, indicated recoveries of 102% ± 11%, for samples in the range of 0.25–5.0 nmol, for all samples run over a six-month period.

Earlier methods of separation of synthesized trinucleoside diphosphates involved an ion-exchange column and a molecular sizing column with lyophilization steps in between column runs. These steps result in a finite loss of material, and involve a 3-day process per trinucleoside diphosphate.

By use of reversed-phase high-performance liquid chromatography, sample manipulation and chromatography are reduced to 2 h. The amount of material loss

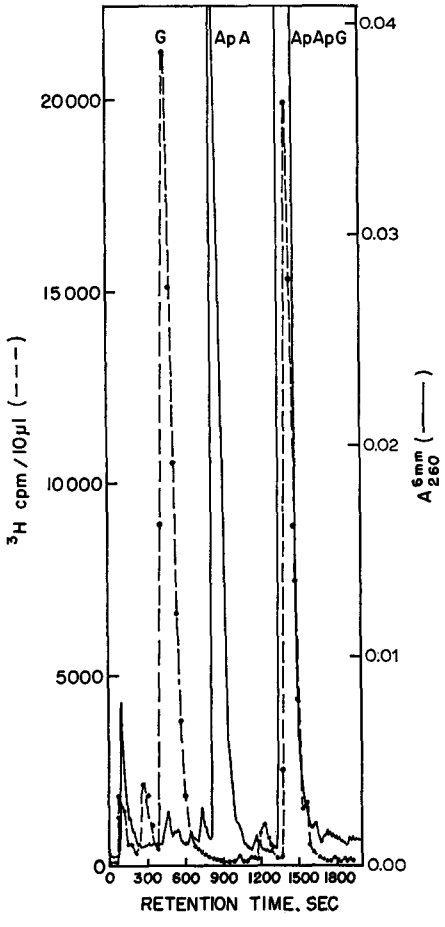

Fig. 1. Purification of ApAp[^{3}H]G. Deproteinized reaction mixture, 70 μl, was applied to a 250 × 3 mm Spherisorb ODS 5 column equilibrated with 50 mM KH_2PO_4, pH 5.5, flowing at 1.0 ml/min, at room temperature, and eluted with a 30-min linear gradient to 35 mM KH_2PO_4, pH 5.5, 24% methanol as described in the Experimental section. Solid line: A_{260} (6 mm); filled circles and dashed line: ^{3}H cpm per 10-μl aliquot of each 30-sec fraction.

is minimal, and fractions containing product are left unfrozen for a minimum amount of time, thus lowering the chance of product hydrolysis to mono- and dinucleotides.

Following submission of this manuscript, similar results have been reported for unlabeled nucleosides, nucleotides and dinucleoside monophosphates[15].

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