CHROM. 5341

Rapid and sensitive measurement of 7-methylguanosine and N^6-isopentenyl derivatives of adenosine by cation-exchange chromatography

Because pure nucleic acids are generally available only in relatively small quantities, the nondestructive analysis of nucleic acid derivatives by UV spectrophotometry of chromatographic effluents continues to offer several advantages. The sensitivity of the procedure is dependent largely upon the construction of the columns and monitoring units. For example, it is possible to analyze 0.3 μg of nucleosides or bases ($\sim$ 1 nmole) and to detect as little as 0.015 μg ($\sim$ 0.05 nmole) by cation-exchange chromatography[1,2]. This analytical sensitivity compares favorably with both gas chromatography[3–6], radioactive measurements[7], and biological assays. One can monitor the effluent at a number of wavelengths so that spectral data as well as peak position are obtained. In addition, when volatile solvents are used the isolated material is readily recoverable in an unmodified form for further chemical, mass spectrographic, or biological assay.

One disadvantage of cation-exchange chromatography of the minor nucleosides and bases of RNA has been that strongly positively charged and highly hydrophobic substances are not readily eluted with totally aqueous solvents. For example, in the system described by UZIEL *et al.*[1], the compound 2-methylthio-N^6-isopentenyladenosine is eluted at about 64 h elution time, compared to about 3 h for most of the known nucleosides. The band is broad and not readily detectable. By incorporating ethanol into the solvent, we can now separate within 2.5 h a group of nucleosides characterized by high positive charge and/or a strong lyophobic substituent*.

Materials and methods

The instrumentation and chemical standards have been described previously[1]. 2-Methylthio-N^6-isopentenyladenosine was a gift of Dr. NELSON LEONARD. N^6-Isopentenyladenosine was a gift from Dr. R. H. HALL. *E coli* $tRNA_1^{Tyr}$ was prepared in our laboratory by chromatography of crude tRNA on Sephadex A-50 (ref. 8) followed by chromatography with the RP-4 system of KELMERS *et al.*[9]. This preparation was more than 90% homogeneous as judged by tyrosine acceptance (1550 pmole per A_{260} unit)**. Hydrolysis of this tRNA prior to analysis was done by a modification of the procedure previously described[1]. The tRNA was initially treated with RNase T_1 (0.1 mg/ml final concentration in 0.02 *M* ammonium acetate, pH 7.2) for 30 min at 37°.

The sequential use of RNase T_1 and a combination of venom diesterase and alkaline phosphatase[1] totally degrades tRNA to nucleosides. Although the combination of venom diesterase and phosphatase will, in theory, hydrolyze any tRNA, we find some samples to be refractory to only this treatment. The prior RNase T_1 step has always led to total hydrolysis. Any unhydrolyzed or cyclic phosphates appear at the breakthrough position.

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* Similar decreased retention volumes and peak widths have been observed by R. P. SINGHAL and W. E. COHN on anion-exchange chromatography.

** One A_{260} unit is the amount of RNA that gives an absorbance of 1.0 when it is dissolved in 1 ml of water and read in a path length of 1 cm at 260 nm.

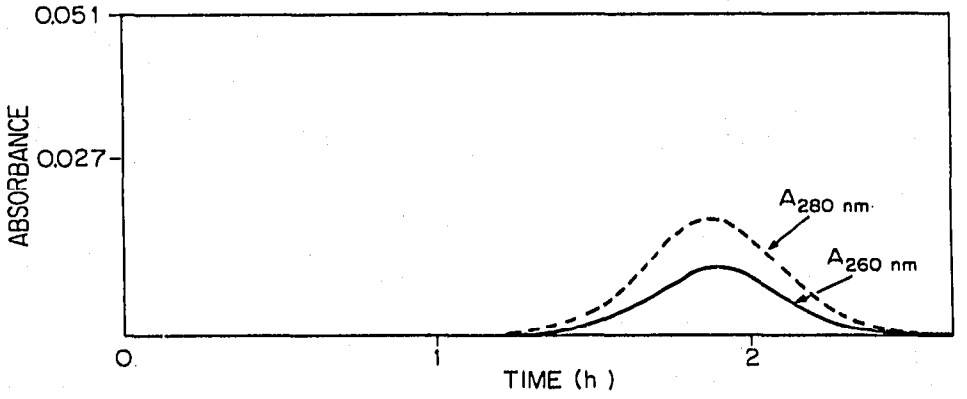

Fig. 1. Chromatography of 2-methylthio-N^6-isopentenyladenosine on a column of cation-exchange resin (Bio Rad A-6) with the dimensions 13 × 0.5 cm. The solvent had a final composition of 0.85 *M* ammonium acetate (pH 5.7) and 15% ethanol, and the temperature was maintained at 49°. A sample of 2.7 μg of compound was passed through the column at a flow rate of 0.29 ml/min.

Results and discussion

The simple inclusion of ethanol in the solvent described in our earlier paper caused the 2-methylthio-N^6-isopentenyladenosine to elute at about 16 h rather than the original 64 h[1]. We then increased the ionic strength and pH from 0.4 *M* and pH 4.65 to the values given in Fig. 1. The higher pH reduces the positive charge on groups ionizing near pH 4.7, the increased ionic strength reduces the retention volume due to ion exchange, and the ethanol increases the solubility of the compound(s) in the moving phase, again causing smaller retention volumes and narrower peak widths.

Fig. 1 illustrates the chromatography of 2-methylthio-N^6-isopentenyladenosine

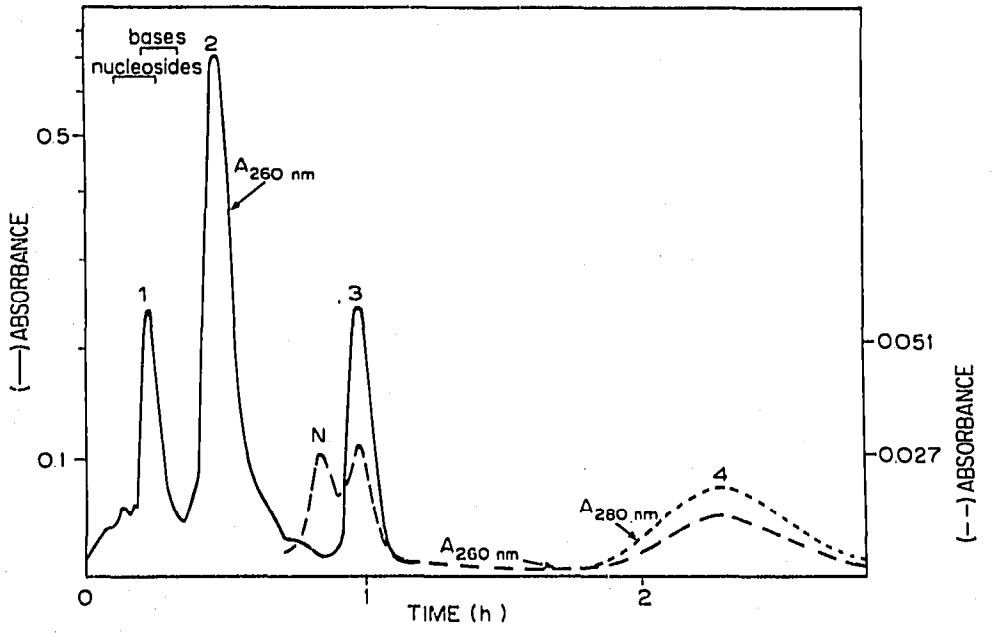

Fig. 2. The column and solvent described in Fig. 1 was used to obtain the positions of the usual four nucleosides, four bases (brackets), adenosine (1), N^6-isopentenyladenosine (2), 7-methylguanosine (3), and an enzymatic hydrolysate of *E. coli* $tRNA_1^{Tyr}$ (--- — — —) which contained an unknown compound N as well as 7-methylguanosine and 2.6 μg 2-methylthio-N^6-isopentenyladenosine (4). The flow rate was 0.25 ml/min.

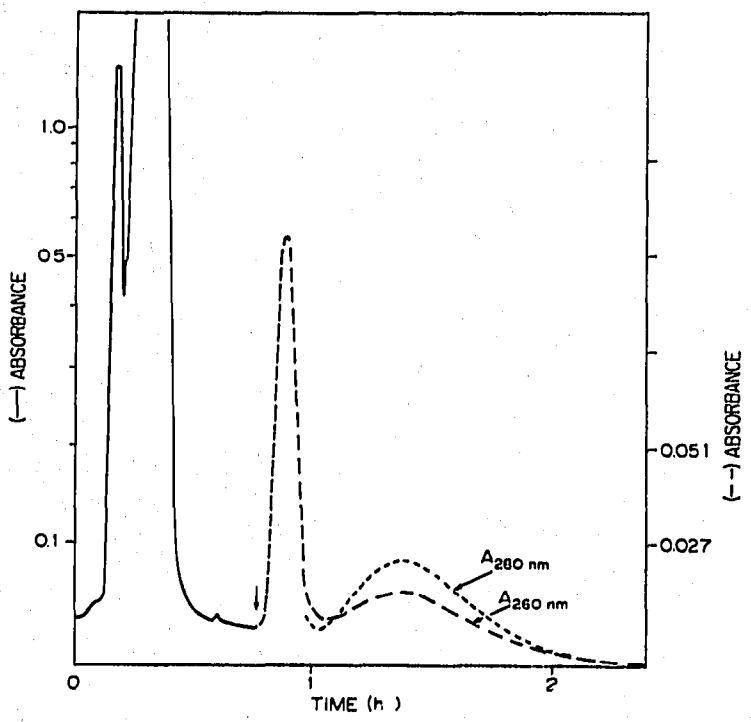

Fig. 3. Increasing the ethanol concentration resulted in an earlier elution of 2-methylthio-N^6-isopentenyladenosine. The column described in Fig. 1 was used and the solvent composition was 0.8 *M* sodium acetate and 20% ethanol. The assay was complete in about 2 h.

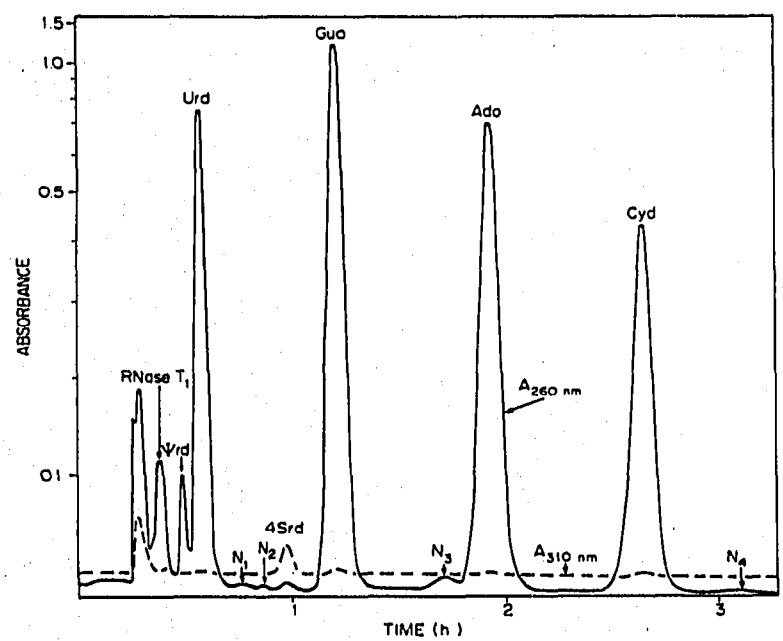

Fig. 4. A column, 23 × 0.6 cm, of cation exchanger[1] was used to separate the nucleoside obtained from mixed *E. coli* B tRNA. About 3 nmoles of tRNA were analyzed. Ψrd = pseudo-uredine; Urd = uridine; 4 Srd = 4-thiouridine; Guo = guanosine; Ado = adenosine; Cyd = cytidine.

in 0.85 *M* ammonium acetate, pH 5.7 (15% ethanol v/v). The sample contained 2.7 μg of material, based upon the spectral data of BURROUGHS *et al.*[10]. One-fifth of this amount would be readily detected in this system. The sensitivity of this assay is comparable to the biological assay of N^6-substituted adenines using the chlorophyll preservation test[11] described in Table I. A reduction of the column diameter (× 1/2) and the volume in the flow cell can further increase the sensitivity fourfold.

Fig. 2, which is a composite of two chromatograms, shows the separation of adenosine, N^6-isopentenyladenosine, 7-methylguanosine, 2-methylthio-N^6-isopentenyladenosine, and an unknown compound N. The 7-methylguanosine was a synthetic standard. The dashed lines represent a hydrolysate of *E. coli* B $tRNA_I^{Tyr}$. We routinely scan at least two wavelengths to give supporting evidence for the identity of the compound. The yield of 2-methylthio-N^6-isopentenyladenosine was 1450 pmole/A_{260} indicating 0.94 moles/mole tRNA. The brackets at the front of the chromatogram show the elution positions for the usual nucleosides and bases.

Fig. 3 illustrates the effect of increasing the ethanol concentration to 20% and reducing the salt concentration to 0.8 *M* ammonium acetate. The sample was a hydrolysate of ~ 16 nmole of *E. coli* B $tRNA^{Phe}$ (65% pure prepared by the Oak Ridge National Laboratory). The yield of 7-methylguanosine is low (see later paragraphs). For comparison, Fig. 4 shows a typical pattern of nucleosides obtained from mixed *E. coli* tRNA chromatographed in the usual way[1].

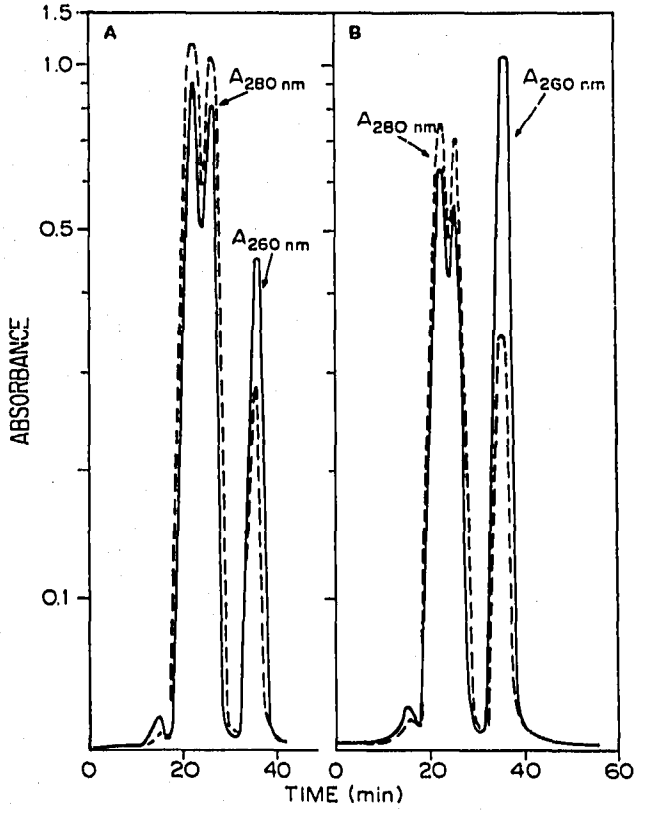

Fig. 5. Analyses done on the column described in Fig. 4. Seven nmoles of 7-methylguanosine were hydrolyzed at 80° in 0.3 *N* NaOH for 15 min (A) and 40 min (B). The A_{280}/A_{260} ratio was constant over the double peak near the breakthrough, and the loss of A_{260} from this double peak on prolonged treatment with alkali was recovered entirely in the increase of absorbance in peak 3. This suggests that all three compounds have the same extinction coefficient.

TABLE I

ASSAY FOR CHLOROPHYLL PRESERVATION BY ADDED ADENOSINE DERIVATIVES

Additions[a]	*M*[b]	*μg chlorophyll remaining*[c]	*Net chlorophyll remaining in leaf tips*[d]
Four days' incubation		19 ± 2	0
No incubation		40 ± 7	21
A-A-Ap	1×10^{-6}	20 ± 3	0
A-A-Gp	1.2×10^{-6}	20 ± 1	0
Kinetin	1×10^{-6}	36 ± 2	17
2-Methylthio-N^6-isopentenyladenine	0.5×10^{-6}	34 ± 6	15

[a] The assay consists of incubation of eight wheat leaf tips for four days in 0.05 *M* potassium phosphate buffer, pH 6.5, in the absence (lines 1 and 2) or presence of various nucleotide derivatives. All additions were hydrolyzed for 1 h at 100° in 1 *N* HCl and neutralized prior to addition to the assay medium (final volume 5 ml).

[b] Molarity of the substance in the assay medium (2.5 to 5 nmoles were used in the various experiments).

[c] Measured residual chlorophyll normalized to one wheat leaf tip.

[d] This value is obtained by subtracting the amount of chlorophyll remaining in the wheat leaf tips after four days' incubation in just the buffer.

The procedures we have described here give quantitative recovery of 2-methylthio-N^6-isopentenyladenosine, N^6-isopentenyladenosine, or 7-methylguanosine from the column chromatography. The overall low yields of 7-methylguanosine we have observed are due mainly to the conditions for preparation of the nucleosides. For example, alkaline hydrolysis followed by phosphatase yields three new components from the original 7-methylguanosine on cation-exchange chromatography[1] (Fig. 5).

Examination of the hydrolysis products indicates that two chemically related compounds with similar spectral data are formed rapidly and subsequently degraded to peak 3. The increase in absorbance of peak 3 equals exactly the loss of absorbance from peaks 1 and 2. This indicates there is no change in extinction coefficient, which leads us to suggest that alkali yields a symmetrical cleavage of the imidazolium ring, giving the 5-formyl and 6-formyl isomers, and that peak 3 presumably arises from the further hydrolysis of the formyl group to give N^6-ribosyl-5,6-diaminoisocytosine. The increased retention of peak 3 due to the liberated amino group is consistent with this hypothesis.

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