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Quantitative Measurement of Isoprenoid Nucleosides in Transfer Ribonucleic Acid*

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ABSTRACT: A method is described for the quantitative estimation of submicrogram quantities of isoprenoid nucleosides from hydrolysates of tRNA. It has the advantage of being more sensitive than ultraviolet absorbance assays and more rapid, specific, and precise than bioassays. Analysis involves degradation of tRNA to its nucleosides by base hydrolysis and enzymatic digestion; separation of the isoprenoid nucleosides from the bulk of the hydrolysis products by column partition chromatography; and resolution of their trimethylsilyl derivatives by gas chromatography. The derivatives are all well separated, with retention times of 5–20 min, when applied to a 4-ft glass column of 10% DC-11 on Gas Chrom Q at 255°.

The procedure was used to determine N^6-(Δ^2-isopentenyl)-adenosine, 2-methythio-N^6-(Δ^2-isopentenyl)adenosine, and N^6-(*cis*-hydroxy-3-methylbut-2-enyl)adenosine in tRNA from *Escherichia coli*, peas, and yeast. It should be especially useful for investigation of systems yielding milligram quantities of tRNA and for the examination of tRNA subspecies for isoprenoid nucleosides.

The demonstration of a relationship between the minor nucleotide content and the biological activity of certain tRNA species (Capra and Peterkofsky, 1968; Gefter and Russell, 1969) has prompted considerable speculation concerning the role of minor nucleosides in control of protein synthesis. Of particular interest are those nucleosides shown to have cytokinin activity (Hall *et al.*, 1967a; Burrows *et al.*, 1968): IPA,[1] *cis*-ZR (the cis isomer of zeatin riboside), and msIPA. In the past, assays for these substances have relied either upon rather insensitive ultraviolet absorbance measurements following chromatographic isolation or upon time-consuming bioassays of rather low precision. Hall and his coworkers (1967a) were able to detect as little as 10 μg of IPA or *cis*-ZR in hydrolysates of plant tRNA by measuring the absorbance of spots eluted from chromatograms. However this probably represents the lower limit

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[1] Abbreviations used are: IPA, N^6-(Δ^2-isopentenyl)adenosine; ms-IPA, 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine; *cis*-ZR, N^6-(*cis*-hydroxy-3-methylbut-2-enyl)adenosine; Me_3Si, trimethylsilyl.

of the method. The most sensitive bioassays can detect cytokinin concentrations of 1 μg/l. (Letham, 1967). Unfortunately, up to 30 days is required for maximum response, and generally the response is a nonlinear function of the cytokinin concentration. In addition, bioassays can give no information as to the chemical nature of the cytokinin present since several unrelated substances have been shown to possess activity (Chen and Hall, 1969; Giannattasio *et al.*, 1969; Leonard *et al.*, 1969).

Quantitative gas chromatographic analysis of the Me_3Si derivatives of both major and minor nucleosides has recently been shown to be feasible (Jacobsen *et al.*, 1968; Most *et al.*, 1968; Hancock, 1969). The extremely low level of isoprenoid nucleosides present in bulk tRNA hydrolysates however necessitates a partial purification prior to formation of the Me_3Si derivatives and gas chromatography. We describe here the application of partition and gas chromatography to the determination of isoprenoid nucleosides in tRNAs from several sources. In addition, we show how mass spectrometry in conjunction with gas chromatography may be useful for identification of minor nucleosides.

Experimental Section

Reagents. Calbiochem, Los Angeles, Calif., supplied zeatin riboside. Authentic IPA was the generous gift of Dr. Ross H. Hall, McMaster University. Baker's yeast, *Escherichia coli* W, and *E. coli* B tRNAs were obtained from Schwarz Bio-Research Inc., Orangeburg, N. Y. Bacterial alkaline phosphatase was purchased from Sigma Chemical Co., St. Louis, Mo.; *N,O*-bis(trimethylsilyl)trifluoracetamide from the Regis Chemical Co., Chicago, Ill. Celite 545 and Gas Chrom Q were obtained from Johns-Mannville Co. and Applied Science Laboratories, respectively. Teflon-lined aluminum tubing ($^1/_8$-in. o.d.) was purchased from Chemical Research Service Inc., Addison, Ill. Solvents were spectroscopic or reagent grade.

An authentic sample of ms-IPA was isolated from an alkaline hydrolysate of *E. coli* W tRNA by ethyl acetate extraction followed by chromatography upon Sephadex LH-20. Examination of the appropriate LH-20 fractions by gas chromatography showed them to contain a component which had ultraviolet and mass spectra identical with those reported by Burrows *et al.* (1969) for ms-IPA.

Procedure. tRNA was prepared from the roots of 48-hr-old Alaska pea seedlings (*Pisum sativum* var. Alaska) by a modification of von Ehrenstein's procedure (1967) and was further purified and separated from 5S RNA by preparative scale polyacrylamide gel electrophoresis (Loening, 1967). Degradation to the constituent nucleosides was achieved by incubation in 0.3 M KOH at 37° for 24 hr followed by digestion of the neutralized hydrolysate with bacterial alkaline phosphatase (Fittler *et al.*, 1968). Following the procedure of Robins *et al.* (1967), the hydrolysate was lyophilized, applied to a Celite 545 column, and eluted with their solvent system E (ethyl acetate–1-propanol–water, 4:1:2). Early fractions from the column, which contained the isoprenoid nucleosides, were evaporated under dry nitrogen and trimethylsilylated by the addition of 10 μl of dry pyridine and 40 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide. Reaction was complete in 5 min at 60°. Two to five microliters was taken for injection.

Retention times and all quantitative data were obtained on silanized Pyrex gas chromatography columns (0.2 × 120 cm) coiled and packed with 10% DC-11 (Dow Corning silicone grease) on Gas Chrom Q (60–80 mesh) in a Varian 1520 gas chromatograph equipped with a hydrogen flame detector. Column operating parameters were: nitrogen carrier gas, 20 cc/min; hydrogen, 40 cc/min; air flow 200 cc/min. Injector and detector were 20–50° above column temperature. Columns were preconditioned for 48 hr before use and retention times were measured from the time of injection.

Mass spectra of isoprenoid nucleosides were determined on a Varian MATS CH 7 spectrometer using the direct inlet at 250–275° and ionization at 70 eV. Before measurement, samples from the partition column were dried and extracted with cold ether to remove impurities. To obtain the mass spectrum of the free nucleoside from a gas chromatographic peak a specially constructed glass trap was butted to the outlet of a 0.2 × 160 cm helical Teflon-lined aluminum column packed with 10% DC-11 on 100–120 mesh Gas Chrom Q. Carbon dioxide carrier gas and immersion of the trap in liquid nitrogen were necessary to minimize aerosol formation. The trapped Me_3Si derivative was hydrolyzed with water (15 min at room temperature) and mass spectra were obtained as above.

Results

Table I summarizes data collected on retention times as a function of temperature for the Me_3Si derivatives of authentic samples of major nucleosides and isoprenoid nucleosides known to occur in tRNA. In all cases, the isoprenoid nucleosides were separated from each other, from the major nucleosides, and from free bases. Presumably the greater retention times of the isoprenoid nucleosides are due to interaction of the isoprenoid side chains with the stationary phase. Although retention times were found to be very sensitive to column temperature and carrier gas flow, careful regulation of these parameters enables one to reproduce these values to within ±5%.

All nucleosides, with two exceptions, gave single peaks when examined by this technique. The exceptions were guanosine, which contained beside the major peak a minor slow-moving component (Jacobsen *et al.*, 1968), and commercial zeatin riboside, which gave two major peaks whose relative abundance was independent of the conditions and time of derivatization. The nature of these peaks will be discussed below.

The sensitivity of the technique to authentic samples of isoprenoid nucleosides was high. Calibration curves were obtained by injecting known amounts of IPA, ms-IPA, and ZR and were linear in the range 0.01–2.0 μg. Relative detector response to each compound was IPA 100, ms-IPA 75, and ZR 60. Neither peak height nor retention time was sensitive to the injection volume within the range 2–5 μl.

Figure 1 illustrates how partition chromatography separates the isoprenoid nucleosides from the bulk of the nucleosides present in a tRNA hydrolysate. Yeast tRNA (15 mg) was hydrolyzed, lyophilized, and mixed with 20 μg of authentic IPA and 20 μg of commercial zeatin riboside. The mixture was fractionated upon a partition column as described by Robins *et al.* (1967) and the early fractions were examined

TABLE I: Retention Times of Trimethylsilyl Nucleosides.

Compound	Retention Time (min) at Temp (°C) 235	245	255	270
N^6-(Δ^2-Isopentenyl)-adenosine	14.0	9.8	6.4	4.0
2-Methythio-N^6-(Δ^2-isopentenyl)adenosine	30.5		13.8	7.8
Zeatin riboside				
Peak A	29.5	19.7	12.5	7.2
Peak B	34.5	22.7	14.2	8.1
Adenosine	6.2	4.4	2.4	1.8
Cytidine	8.9	6.1	4.1	2.7
Guanosine	8.9	6.0	4.0	2.6
	9.9	6.6	4.3	
Uridine	3.8	2.6	1.8	1.2
N^6-(Δ^2-Isopentenyl)-adenine	1.3	1.1	0.75	0.55

by gas chromatography. The IPA (and, in experiments with *E. coli* tRNA, the ms-IPA) eluted with the void volume of the column (fractions 1–5). The two peaks of zeatin riboside eluted between fractions 5 and 20. Adenosine eluted beyond fraction 8. Routinely, fractions 1–5 were pooled and used for the determination of IPA or ms-IPA and fractions 5–20 for the determination of ZR.

As shown in Figure 1, peaks A and B (designated on the basis of retention times; see Table I) of commercial zeatin riboside were differentially distributed across fractions 5–20, indicating that the commercial product contains two compounds. Partial separation of the two components occurred when 500 μg of commercial ZR was fractionated on a partition column. An early fraction from the column (corresponding to fraction 7, Figure 1) gave on gas chromatographic analysis 56% peak A and 44% peak B. A late fraction (corresponding to fraction 14, Figure 1) gave 15% peak A and 85% peak B. Mass spectra of the two fractions were almost identical. Hall *et al.* (1967a) reported the mass spectra of *cis*- and *trans*-ZR to differ most notably in the relative intensities of peaks at *m/e* 192, 228, and 331. Comparison of our spectra with the published spectra and the relative mobilities of the two compounds in solvent system E identified A as *cis*-ZR and B as *trans*-ZR. Additional support for this interpretation was obtained from a partial nuclear magnetic resonance spectrum of the commercial mixture in D_2O. The methyl peak was split by 4 ppm, indicating the presence of cis and trans isomers within the mixture. We therefore conclude that the commercial ZR is a mixture of 30% *cis*-ZR and 70% *trans*-ZR.

In order to investigate the stability of the isoprenoid nucleosides during hydrolysis and subsequent conversion into the Me_3Si derivatives, 30 mg of salt-extracted commercial yeast RNA containing less than 0.2 μg of IPA was mixed with 20 μg each of IPA and ZR, hydrolyzed, and subjected to partition chromatography. The appropriate fractions were trimethylsilylated and examined by gas chromatography.

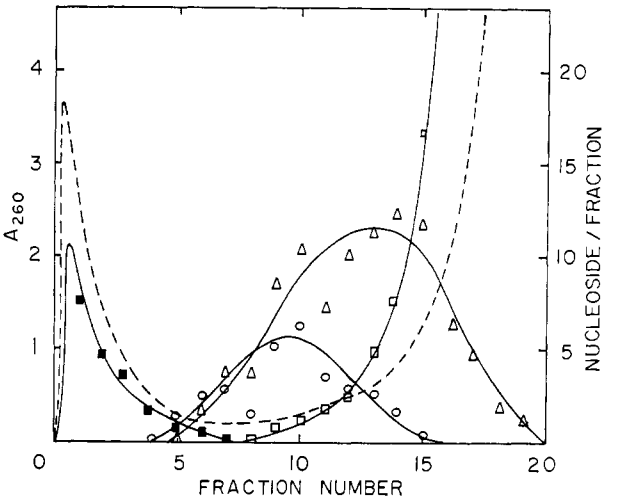

FIGURE 1: Partition chromatography of fortified tRNA hydrolysate. Celite 545 (13 g) was equilibrated with 6 ml of the lower phase of solvent system E and packed into a 0.9 × 60 cm column. The lyophilized hydrolysate and added nucleosides were dissolved in 1 ml of lower phase E, mixed with 2.3 g of Celite, and packed onto the column. Elution was with upper phase E at 1 ml/min. Fractions of 1 ml were collected, absorbance at 260 nm was read, and nucleoside content of each fraction was determined as described in the text. One unit of nucleoside represents 1 μg of adenosine (□), 2 μg of IPA (■), and 0.1 μg of ZR peak A (○) or ZR peak B (△). (----) Denotes A_{260}.

A phosphate buffer blank was treated in a like manner. No extra peaks indicative of degradation products were obtained from the fortified hydrolysates, and recoveries were near quantitative.

Recovery data were obtained by fortifying *E. coli* B tRNA hydrolysates with 1–20 μg of isoprenoid nucleoside. Samples were lyophilized, fractionated on 0.3 × 75 cm partition columns, converted into the Me_3Si derivatives, and subjected to gas chromatography. Table II summarizes the data, which indicate recovery is quantitative to at least the 1-μg level. The ms-IPA contents of the unfortified samples were also determined as discussed below. These values are included in Table III.

TABLE II: Recovery of Isoprenoid Nucleosides from a tRNA Hydrolysate.

tRNA (mg)	Added IPA (μg)	IPA Content by GC[a] Anal. (μg)	% Recov of Added IPA
5		0.37	
5		0.39	
5		0.34	
10		0.85	
30		2.64	
5	1.00	1.22	85
5	5.00	5.55	103
5	10.0	10.5	101
5	20.0	19.6	96

[a] GC = gas chromatographic.

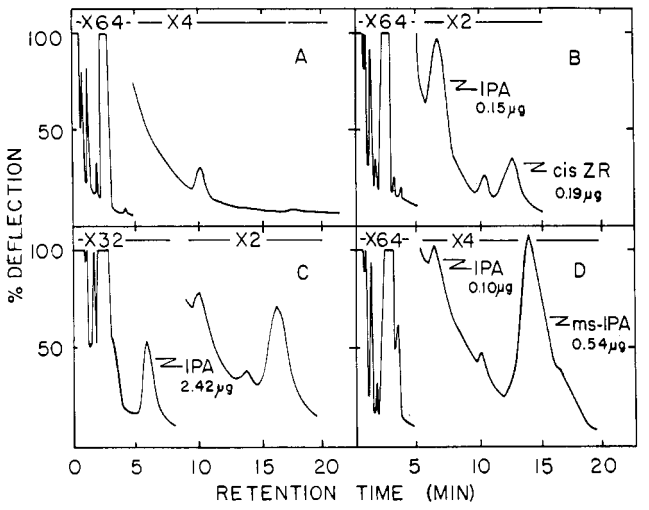

FIGURE 2: Gas chromatographic analysis of fractionated tRNA hydrolysates. (A) Phosphate buffer carried through analytical procedure; (B) 11.2 mg of pea root tRNA (first partition column fraction, containing 100% of IPA and 50% of *cis*-ZR present in hydrolysate); (C) 30 mg of baker's yeast tRNA; (D) 30 mg of *E. coli* W tRNA. Attenuation factors are noted on the figure. Temperature, 255°.

Figure 2 illustrates profiles obtained when the method was applied to purified pea root, *E. coli* W, and yeast tRNAs. Early peaks (with retention times less than 5 min) were due to solvent, solvent impurities, adenosine, and possibly methylated adenosines. An unidentified peak at 10 min was seen in all samples including the phosphate buffer blank. Coinjection with standards indicated each sample contained IPA. *E. coli* tRNA also contained ms-IPA, and pea root tRNA contained *cis*-ZR.

Because the presence of IPA in tRNA from many species is well established it was felt that identity of mobility with an authentic sample in both gaseous and liquid systems was sufficient to establish its presence. In the case of *cis*-ZR, however, further confirmation was sought by trapping the appropriate gas chromatographic peak and subjecting it, after hydrolysis, to mass spectrometry. The mass spectrum, shown in Figure 3, proved to be identical with that obtained for authentic *cis*-ZR.

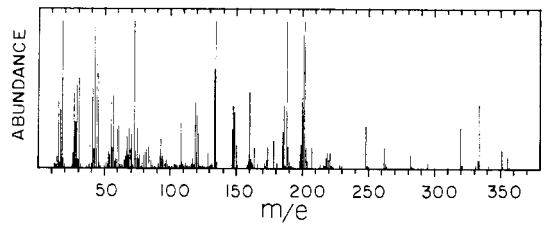

FIGURE 3: Mass spectrum of *cis*-ZR isolated from pea root tRNA. The gas chromatographic peak of Me_3Si-*cis*-ZR from a hydrolysate of 20 mg of pea root tRNA was trapped and hydrolyzed, and the resulting free nucleoside (10 μg) was examined in the MATS-7 direct inlet, 250°, 70 eV. Major peak assignments *m/e:* 351, molecular ion; 334, loss of OH; 320, loss of CH_2OH; 262 and 248, loss of sugar fragments; 219, free base; 202, free base less OH; 188, free base less CH_3 and OH; 135, adenine. Peak at 281 is found in background scans at 50°. Peaks at 73 and 147 are characteristic of polysilyated compounds.

TABLE III: Isoprenoid Nucleoside Content of Unfractionated tRNAs.

Source	Nucleoside Content (% w/w)		
	IPA	ms-IPA	*cis*-ZR
E. coli W	0.010	0.048	nd[a]
E. coli B	0.009	0.085	nd
Pea root	0.031	nd	0.075
Yeast	0.17	nd	nd

[a] nd = not detectable.

Figure 2 also illustrates the sensitivity of the gas chromatographic assay. The peaks of Figure 2B correspond to 0.15 μg of IPA and 0.19 μg of *cis*-ZR respectively, based upon calibration curves determined with authentic samples. From such data, the isoprenoid nucleoside content of each tRNA was calculated (Table III).

Discussion

The procedure discussed here enables isoprenoid nucleosides to be determined in very small samples of RNA. The least amount of IPA giving a reproducible response using the hydrogen flame detector was 0.01 μg. Injecting one-tenth aliquots, one could detect IPA in a hydrolysate containing 0.1 μg of IPA. Data in Table II demonstrate that 5 mg of unfractionated tRNA is therefore sufficient for analysis when the isoprenoid nucleosides are present at a minimal level of 0.01%.

The resolution of the method is high and is comparable to that achieved by earlier workers. Hancock (1969) was able to separate the Me_3Si derivatives of the α and β anomers of pseudouridine. In our hands, the method was capable of the comparable resolution of *cis*- and *trans*-zeatin ribosides. The separation and retention times observed for the Me_3Si derivatives of IPA and ZR are comparable to those obtained by Most *et al.* (1968) upon SE-52 columns.

The IPA and ms-IPA contents of *E. coli* W and *E. coli* B tRNA (Table III) agree quite closely with those obtained on analysis of gram quantities of *E. coli* B tRNA (0.003 and 0.05%, respectively) by Burrows *et al.* (1969).

Robins *et al.* (1967) examined enzymatic hydrolysates of yeast tRNA, and reported yeast tRNA to contain 0.065% IPA (one-third our value). Fittler *et al.* (1968), however, observed incomplete release of IPA from tRNA hydrolysates using enzymatic hydrolysis, possibly due to decreased activity of diesterases to *N*- and *O*-methyl substitution of the adjacent nucleoside. Because of the possibility of incomplete hydrolysis and the difficulty of quantitative elution of isoprenoid nucleosides from paper chromatograms utilized in Robin's determination, we feel our values to be closer to the true IPA content of yeast tRNA. These larger values are perhaps more reasonable in view of the numerous tRNA subspecies

now known to contain isoprenoid nucleosides (Armstrong *et al.*, 1969).

We have confirmed Hall's (1967a) report of *cis*-ZR in tRNA from peas. The low yields of *cis*-ZR and IPA he obtained from (a presumably nonquantitative) isolation of these compounds from whole garden pea tRNA cannot, however, be directly compared to our analysis of pea root tRNA. It is interesting to note that a 1:2 ratio of IPA:*cis*-ZR is found in both tissues.

The values for isoprenoid nucleoside content determined by our method do, of course, depend on the purity of the tRNA. Commercial *E. coli* and yeast tRNA were found to give only 15–17 A_{260} units/mg in 0.005 M $MgCl_2$–0.01 M Tris-Cl (pH 7.4). In addition analytical gel electrophoresis showed these materials to be contaminated with at least 10% 5S RNA. For this reason, the actual isoprenoid nucleoside contents of these species are probably 20–30% higher than the values of Table III.

Routine preparation of plant tRNA by the conventional techniques of phenol extraction followed by DEAE chromatography yields products contaminated with 5S RNA, DNA fragments, and polysaccharides. We have found that a preparative electrophoretic step is sufficient to remove these contaminants. The *cis*-ZR content of pea root tRNA in Table II reflects therefore only experimental losses during analysis.

The nature of the peak with a retention time of 16.6 min present in the yeast tRNA (and sometimes pea root tRNA) hydrolysates is obscure. It may be related to the second isoprenoid nucleoside reported to be in yeast tRNA by Hall *et al.* (1967b) since its mobility on partition chromatography and its extended retention time are indicative of a nonpolar molecule. Hecht *et al.* (1969) have recently reported that wheat germ tRNA contains 2-methylthiozeatin riboside, which would be expected to have such chromatographic properties. We have preliminary data (R. O. Morris, D. F. Babcock, and G. B. Jacobsen, unpublished observations) indicating that wheat germ tRNA contains IPA, *cis*-ZR, the unknown component with a retention time of 16.6 min found in pea and yeast tRNA, and a fourth even more strongly retained species. Somewhat surprisingly, ms-IPA does not appear to be present. Mass spectroscopy in conjunction with gas chromatographic separation should shed light on the identity of these compounds.

In summary, separation and quantitation of trimethylsilyl derivatives of isoprenoid nucleosides are feasible and more advantageous than previous methods for analysis of these components. We are now using the technique to identify the distribution of isoprenoid nucleosides among isoaccepting subspecies of pea root tRNA.

Since this work was submitted, we learned that Upper and Helgeson (1970) have utilized a similar procedure to measure isoprenoid nucleosides. Their procedure has comparable sensitivity and resolution.

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