HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY OF TRANSFER RNA BASES FOR ISOTOPIC ABUNDANCE

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SUMMARY

We present a quantitative analysis of the incorporation of stable isotopes into nucleic acids for verification of the site and determination of the abundance of the label. High-performance liquid chromatography and mass spectrometry of nucleic acid bases were used for quantitation of isotopic enrichment, with only μg amounts of available RNA. Conditions for acid hydrolysis of tRNA were optimized for quantitative yield of bases without destruction, and optimum conditions for the reversed-phase high-performance liquid chromatographic separation of the bases were determined. Three tRNA preparations, ^{13}C-enriched *in vivo* by incorporation of [^{13}C$_2$]adenine, [^{13}C$_2$]uracil and [^{13}C]methyl groups from methionine, were subjected to these procedures, followed by mass spectrometry of the bases. In natural abundance, ^{13}C is 1.08 atom %; in these tRNA preparations we found: 27% of all adenine was labeled at position 2 with ^{13}C atoms; 43 atom % ^{13}C at position 2 of uracil and 45 atom % ^{13}C at position 2 of cytosine; and 56.9 atom % ^{13}C at the methyl of thymine, respectively. The techniques described are important to the study of nucleic acid biosynthesis, modification and structure by nuclear magnetic resonance spectroscopy.

INTRODUCTION

Analysis of RNA nucleoside composition and sequences can now be accomplished with μg quantities of RNA when radiolabeling and gel sequencing techniques are utilized. Recently, we developed techniques for enzymatic hydrolysis and high-performance liquid chromatographic (HPLC) analysis of RNA nucleoside composition at less than μg level[1–3]. This sensitive method of analysis is especially suited to the identification of previously uncharacterized modified nucleosides. Modified nucleosides obtained in ng amounts from purified species of tRNA could be identified by mass spectrometry (MS)[4].

However, there was a need for related methodology applicable to investi-

gations of biosynthesis, biochemistry and biophysics of nucleic acids and their modified nucleosides. Information beyond that of identification and quantification of nucleosides could be obtained if the RNA is from cells grown in media containing a labeled precursor of nucleic acids, or the labeled presumed precursor of a nucleic acid modification. This labeling could be semi-specific, such as one particular carbon within a specific base, or extremely specific, such as the methyl carbon of ribothymidine, which occurs only once in a tRNA molecule. Verification of the site and abundance of the label would be applicable to the study of nucleic acid biosynthesis, modification and structural analyses. The methods presented here demonstrate that the incorporation of the stable isotope, ^{13}C, from specific nucleic acid precursors into RNA could be successfully identified and quantitated with only μg amounts of available RNA.

MATERIALS AND METHODS

Carbon-13-enriched tRNA

Enrichment of tRNA was accomplished at specific locations by addition of particular ^{13}C-enriched nucleic acid precursors to stringently defined media in which a selected strain of *Escherichia coli* was grown. The selection of *E. coli* C6 cys⁻met⁻rel⁻strain M1 and details of the media components, culture growth characteristics and tRNA extraction procedures have been published[5]. Briefly, cells were grown in media (15-l cultures) containing the nucleic acid bases, adenine, guanine, uracil and cytosine and the donor of all methyl group modifications, methionine. In order to achieve specific enrichment, only one of these added precursors contained a ^{13}C-enriched carbon. For the experiments reported here, [$^{13}C_2$]adenine, [$^{13}C_2$]uracil, and [^{13}C-methyl]methionine were obtained from Merck (Quebec, Canada), with enrichments of at least 90 atom % at the specified location[5,6]. The media also contained 6-mercaptopurine, an inhibitor of both purine biosynthesis *de novo* and adenosine–guanosine interconversion. This drug forces the cells to grow by utilizing the exogenously provided precursors for nucleoside synthesis via the "salvage pathways". The strain M1 was selected for its ability to rapidly express and efficiently utilize the "salvage pathways" for nucleoside synthesis. The tRNA was extracted by a method employing phenol denaturation of proteins, ethanol precipitation of nucleic acids, DEAE-cellulose column chromatographic separation of tRNA, and urea–polyacrylamide gel electrophoretic analysis of tRNA purity[5–7]. Yeast tRNA (Sigma, St. Louis, MO, U.S.A.) was used for comparison of ^{13}C abundance in bases.

Acid hydrolysis of nucleosides

Stock standards[1,2] were weighed on a microgram balance to afford working solutions of fine accuracy: adenosine, 1.02 m*M*; cytosine, 1.00 m*M*; guanosine, 0.42 m*M*; and uridine, 1.00 m*M*. The hydrolysis procedure reported earlier[3] entailed placing 100 μl of the stock nucleoside solution into a 100 × 16 mm screw-cap culture tube. The solvent was removed with a stream of pure nitrogen while the tube was kept at 45°C in a heating block. When dryness was confirmed, 1 ml of trifluoroacetic acid (TFA)–formic acid (1/1, v/v) was added, the tube was capped, and the solution was mixed and placed in a 150°C heating block. Times for the hydrolysis were varied between 0.5 and 8 h. After removing the tube from the heating block, it was placed

in a freezer at $-20°C$ for *ca.* 10 min to allow condensation of the TFA and formic acid. The acids from the hydrolysate were removed with a stream of pure nitrogen gas in a heating block maintained at 45°C. Exactly 1 ml of double-distilled water was added to the dry hydrolysate followed by sonication before HPLC of 0.1-ml aliquots.

Acid hydrolysis of tRNA

The aqueous tRNA sample of *ca.* 15 μg was quantitatively transferred into a 100×16 mm Pyrex culture tube. The water was removed by placing the tube in a heating block at 50°C under a gentle stream of purified nitrogen gas. Then 1 ml of the TFA–formic acid mixture was added to the tube, and a PTFE-lined screw cap was firmly attached. This solution was mixed and placed in a heating block at 150°C for 4 h, no more than one-third of the tube being placed in the block. When hydrolysis was complete, the tube was carefully placed in the freezer for at least 15 min before opening the screw cap. The acids were then evaporated to dryness on the 50°C heating block under a gentle stream of nitrogen gas. Care was taken not to bake the dry hydrolysate. Redistilled nanopure water (0.4 ml) was added and sonicated to dissolve all of the residue. An aliquot (0.2 ml) of this solution was injected on to the HPLC column for separation of major and modified bases.

HPLC of bases

Major and modified bases from acid hydrolysates of tRNA were separated by reversed-phase HPLC. A μBondapak C_{18} column (600×4 mm) maintained at 35°C was used for the separation. Bases were eluted with a linear gradient of methanol (0 to 10%) in 0.10 M $NH_4H_2PO_4$ (pH 5.1). The flow-rate was 1.0 ml/min, with the methanol concentration increasing at a rate of 0.5%/min. The ultraviolet absorption of the bases was measured at 254 nm. An analytical run was made for the bases in each of the samples, then a preparative run was made, and *ca.* 60% of the central portion of the peak of interest was collected. This fraction (1.5 ml) was further purified by re-injection into the same chromatographic system. However, the elution solution was changed to 10% methanol in water, in order to remove the $NH_4H_2PO_4$. Use of a higher concentration of methanol not only gave a more concentrated base fraction, but also changed the retention characteristics of the bases. Thus, the purity of the final material was further ensured. Again, only 60% of the central portion of the peak was collected. An aliquot of this collection was used for the MS measurement.

Mass spectrometry

The MS equipment consisted of a modified CEC 21-110B high-resolution mass spectrometer, operating in conjunction with a Japan Electron Optics Laboratory JEC-6 spectrum-computer and a JMA-IC-O automatic data analyzer for photoplate detection. The instrument has a demonstrated resolution in excess of 1/30,000. Before MS analysis, detection thresholds were established for the RNA bases. Enough of each base was isolated from the tRNA for a complete mass spectrum and single-ion monitoring of the M^+ and $M^+ + i$ peak height ratios.

The electron multiplier on our mass spectrometer is of the 16-stage Allen type with beryllium–copper dynodes and is inherently noisy. Therefore, after a period of testing by which we were assured we could routinely achieve $\pm$ 10% accuracy when

the ratio of $M^+ + 1$ to M^+ was $<10\%$, and $\pm 5\%$ accuracy at ratios $>10\%$, measurements were made in the following manner. Half of each sample, purified by and collected from the HPLC, was introduced by direct probe into the mass spectrometer source, and a complete low-resolution spectrum was made to determine if any impurities were present. After the low-resolution runs, the instrument was tuned to a resolution of 1/10,000 to further ensure that there would be no contribution from ions other than M^+ and $M^+ + 1$ (the 1/10,000 resolution still allowed ample sensitivity for our purpose). Channel "A" of the high-resolution mass measurement section was then focused on the pertinent M^+ ion, while channel "B" was focused on the corresponding $M^+ + 1$ ion. The sample was then introduced into the instrument, and, as it began to volatilize, the two channels were scanned alternately at 1-sec intervals. The signals were recorded on a CEC-5-124 recording oscillograph. The three "sets" (channels "A" and "B") of signals just prior to and just after the maximum signal strength were then averaged, and the enrichment was calculated by the following formula.

$$\text{Mole \% of labeled species} = \frac{[(M^+ + 1) - A^*(M^+)]}{M^+ + [(M^+ + 1) - A^*(M^+)]} \times 100$$

where A^* is the normal isotopic abundance of $M^+ + 1$ calculated separately for each non-enriched sample.

The percentage enrichment of ^{13}C in each of the tRNA bases was calculated according to the following example for thymine. The thymine molecular ion (m/e 126) peak height (designated M^+) and the molecular ion plus one (m/e 127) peak height ($M^+ + 1$) were used to determine the enrichment. In a natural-abundance sample of thymine, $M^+ + 1$ should be 6.3% of the peak height, M^+. Thymine has the molecular formula $C_5H_6N_2O_2$, and the percentages of natural abundance for ^{13}C, ^{15}N and ^{17}O are 1.08, 0.37 and 0.037, respectively. Hence, the percent natural abundance of the $M^+ + 1$ peak from non-labelled thymine is calculated as percent isotopes at $M^+ + 1$: $(5 \times 1.1\%) + (2 \times 0.37\%) + (2 \times 0.037\%) = 6.314\%$. The percent enrichment would be calculated for thymine as follows:

$$\text{Mole \% of labeled species} = \frac{[(M^+ + 1) - 6.3(M^+)]}{M^+ + [(M^+ + 1) - 6.3(M^+)]} \times 100$$

RESULTS AND DISCUSSION

The ability to quantify isotopic enrichment within specific bases of tRNA is very much dependent on complete hydrolysis of the nucleosides to bases[5], quantitative separation and purification of the bases and MS. The combined chromatographic–MS procedures must be made applicable to small amounts, (μg quantities) of tRNA, because only limited amounts of sample are available. Therefore, verification of complete hydrolytic release of bases is important.

Release of bases from μg quantities of the four major ribonucleosides, ribothymidine (5-methyluridine) and 2-methylguanosine was investigated. Bases were separated and quantitated by reverse-phase HPLC and UV absorbance measurements under conditions specified in Materials and methods. Fig. 1A is a chromato-

gram of the base separation we have achieved. The release of bases from nucleosides by acid hydrolysis was quantitated over an 8-h period. Yields of the bases are shown in Fig. 2A, B. The major purine nucleosides were quantitatively hydrolysed to bases in less than 1 h; whereas pyrimidine nucleosides required at least 4 h of hydrolysis. This difference between purine and pyrimidine nucleoside hydrolysis was also evident for the two modified nucleosides. The 2-methylguanosine hydrolysis was complete in less than 1 h; ribothymidine hydrolysis required 4 h.
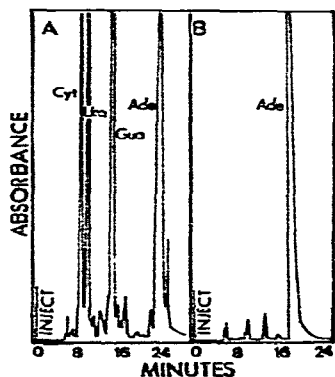

Fig. 1. Reversed-phase HPLC of bases. A: tRNA that had been acid hydrolysed to bases (see Materials and methods) was injected on to a μBondapak C$_{18}$ column (600 × 4 mm) and bases were eluted with a 0–10% methanol gradient in phosphate buffer. Absorption at 254 nm was measured with a full-scale deflection of 0.5 absorbance units. B: The central 60% of the adenine fraction from an initial chromatography, such as that shown in A, was subjected to re-chromatography on the same column but with elution by 10% methanol in water. Ade = adenine; Cyt = cytosine; Gua = guanine; Ura = uracil.
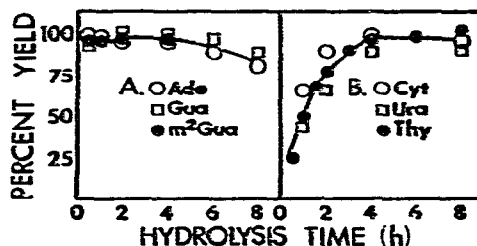
Fig. 2. Release of bases from acid hydrolysis of nucleosides. Major and modified nucleosides [adenosine, cytosine, guanosine, uridine, 2-methylguanosine (m^{2}G) and ribothymidine] were acid hydrolysed. Aliquots taken at various time intervals were assessed for the amount of base released. Release of bases was quantitated by HPLC and UV absorption and is plotted as percent yield.

Since release of pyrimidines from nucleosides required the longer time, release of thymine from μg quantities of tRNA was studied in detail. *E. coli* unfractionated tRNA (13.7 μg) was subjected to acid hydrolysis as described in Materials and methods. Release of thymine over an 8-h period is depicted in Fig. 3. At 4 h, the release of thymine was complete and continued at the same level for the remaining 4 h of hydrolysis. An assessment of recovery was determined by addition of 121.6 ng of thymine to 13.7 μg of tRNA. This mixture, hydrolysed and chromatographed, resulted in 99.6% recovery of the added thymine.

With the appropriate conditions successfully achieved for the tRNA hydrolysis and quantification of bases, analysis of isotopically enriched tRNA was accomplished. Unfractionated tRNA was obtained from a strain of *E. coli* grown in stringently defined media that contained either [^{13}C$_2$]adenine, [^{13}C$_2$]uracil or [^{13}C-methyl]-methionine[5]. The ^{13}C-enrichment of these nucleic acid precursors was ascertained by nuclear magnetic resonance (NMR) spectroscopy and MS to be site-specific and greater than 90 atom%[5,6,9]. Unlabeled yeast tRNA was extensively dialyzed against glass-distilled water and used for comparison of the ^{13}C content of bases. Unlabeled
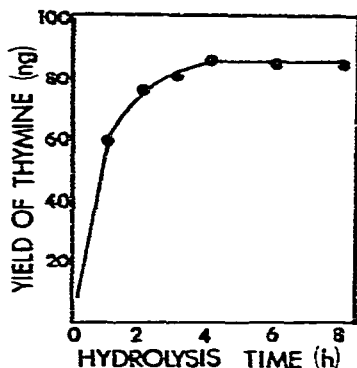
Fig. 3. Release of thymine from tRNA. tRNA was acid hydrolysed, and aliquots of the hydrolysate were taken at various time intervals and subjected to HPLC. The release of thymine from the tRNA over 8 h is plotted in nanograms.

and ^{13}C-enriched tRNAs were acid hydrolysed, and the bases were separated by HPLC. The base of interest was subjected to chromatography a second time and eluted with 10% methanol in water. This procedure further purified the base and removed all salts from the first chromatography before MS analysis; Fig. 1B is the elution profile of adenine during the second chromatography. The retention time for each base was shorter during the second chromatography under conditions of 10% methanol, as seen by comparison of adenine in Figs. 1A and 1B.

The purified bases were subjected to MS analysis. Due to the non-volatility of guanine in the mass spectrometer ion source, a trimethylsilyl (TMS) derivative was made by reacting the purified base with 0.5 ml of bis(trimethylsilyl)trifluoro-acetamide (BSTFA) in 0.5 ml of acetonitrile for 15 min at 150°C. Both a complete mass spectrum and single-ion monitoring of the molecular ion (M^+) and the molecular ion plus one mass unit ($M^+ + 1$) were accomplished for each base. For the mass spectrum of TMS-guanine, m/e 208 and 209 were used to find the ^{13}C atom% enrichment, because this pair contained the least spectral background when compared to other measurable fragments. The MS analyses of M^+ and $M^+ + 1$ of bases from unlabeled and ^{13}C-enriched tRNA preparations are shown in Fig. 4. Unlabeled tRNA contained bases with ^{13}C abundance comparable to the theoretical values based on 1.1 atom% natural abundance. The tRNA obtained from cultures grown with ^{13}C$_2$-enriched adenine was found to contain enrichment only within adenine, and to the extent of 27 atom%. tRNA obtained from cultures grown with [^{13}C$_2$]uracil was found to contain enrichment within both uracil and cytosine and to the extents of 43 and 45 atom%, respectively. E. coli was cultured in the presence of 6-mercaptopurine, which blocks de novo purine biosynthesis and adenosine–guanosine interconversions. This drug is incapable of blocking the interconversion of pyrimidines. Thus, both uracil and cytosine were labeled in the tRNA of cells grown on [^{13}C$_2$]uracil. By NMR spectroscopy of the adenine and uracil/cytosine tRNA preparations, the base location of the labels was verified, and it was shown that the labels were present only at the C_2-positions (Fig. 5).

The most successful labeling of tRNA occurred with cells grown on [^{13}C-methyl]methionine. This may be due to the strain's methionine auxotrophy. Thymine
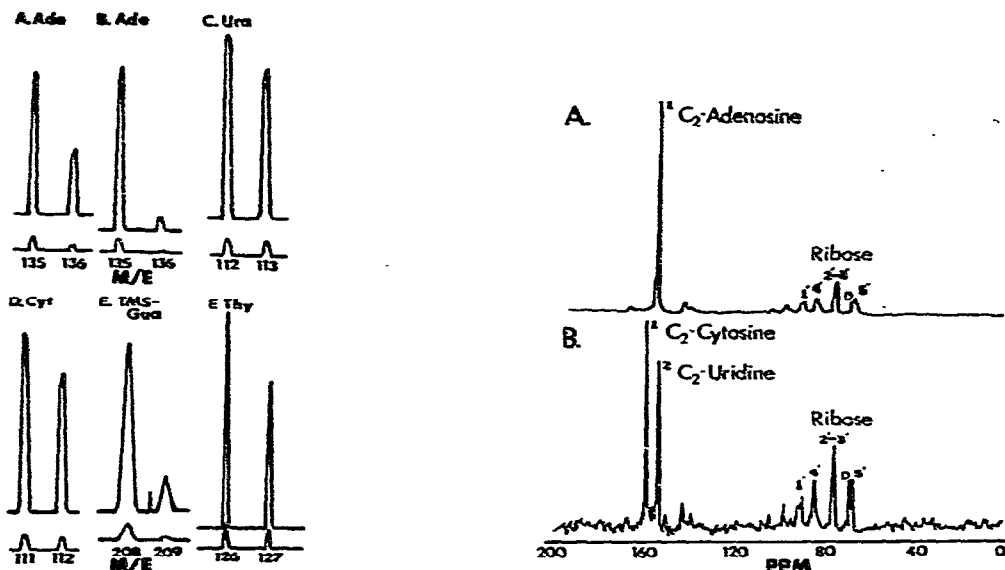
Fig. 4. Mass spectometry of bases from labeled and unlabeled tRNA samples. Bases obtained from unlabeled and ^{13}C-enriched tRNA samples were subjected to mass spectral analysis. The figures show the results of single-ion monitoring in which the molecular ion (M$^+$) and the molecular ion plus one mass unit (M$^+$ + 1) are detected. A, adenine from tRNA of cultures grown with ^{13}C$_2$-enriched adenine; B, adenine from unlabeled tRNA; C, uracil from tRNA of cultures grown with ^{13}C$_2$-enriched uracil; D, cytosine from tRNA of cultures grown with [^{13}C$_2$]uracil; E, TMS-guanine from tRNA of cultures grown with [^{13}C$_2$]adenine; F, thymine from tRNA of cultures grown with methionine, ^{13}C-enriched in the methyl group.

Fig. 5. Carbon-NMR spectroscopy of [^{13}C$_2$]adenine and [^{13}C$_2$]uracil/cytosine tRNA. Carbon-NMR spectra of ^{13}C-enriched tRNA were taken on a Varian XL100 FT NMR spectrometer equipped with a Nicolet 1180 computer and Varian temperature control. Samples (2 ml) of ca. 2 mM tRNA were placed in 12-mm tubes and for the spectra shown were kept at 76.5°C for the time-averaged acquisition of 8192 scans. Chemical shifts (ppm) are relative to tetramethylsilane with the internal standard dioxane (signal designated D in the figures) located at 67.4 ppm. Resonance signals 1', 2'–3', 4' and 5' emanate from the natural-abundance ^{13}C of the five ribose carbons. A, tRNA from cultures grown with ^{13}C$_2$-enriched adenine exhibited only one enriched signal (number 1 in the figure); this signal corresponds in chemical shift to that of C$_2$ of adenine, adenosine and polyA^6. B, tRNA from cultures grown with [^{13}C$_2$]uracil exhibited two enriched signals (numbered 1 and 2); signal 2 corresponds in chemical shift to that of the C$_2$ of uracil, uridine, UMP and polyU and signal 1 to the C$_2$ of cytosine, cytidine and CMP^6.

isolated from tRNA of this culture was labeled to the extent of 56.9 atom%; NMR spectroscopy of this tRNA verified that ^{13}C-enrichment occurred only at methyl groups and not in base-ring carbons[5,6].

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