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Preparation of carbon-13 labeled ribonucleotides using acetate as an isotope source

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NMR spectroscopy is an experimental method capable of providing high-resolution structural and dynamic information for RNA molecules in solution. The power of NMR as a structural–analytical tool is greatly enhanced when heteronuclear methods can be applied to molecules enriched with ^{13}C. These heteronuclear NMR methods have revolutionized the field of protein structure determination in the past several years. However, heteronuclear NMR has not yet been as widely exploited in the analysis of RNA, in large part due to the difficulty, inefficiency and expense of current methods of preparing isotopically enriched ribonucleotides. This currently presents a significant barrier to the application of NMR to the analysis of RNA.

Here we describe an alternative method for the preparation of ^{13}C enriched ribonucleotides, the essential precursors for either chemical or enzymatic *in vitro* synthesis of ^{13}C enriched RNA. This method combines the advantage of using an inexpensive isotope source (sodium acetate) with the advantages of using *Escherichia coli* as the source of crude nucleotides. In addition, this method permits the distribution of ^{13}C within the ribonucleotide products to be controlled by mixing various combinations of enriched and unenriched sodium acetate in the cell growth medium. This work is of particular significance to investigators using NMR methods to characterize the structure and dynamics of RNAs.

Protocols have been previously reported for the preparation of ^{13}C enriched ribonucleotides (1–4) and deoxyribonucleotides (5). These protocols call for using either glucose or methanol as the source of isotope. Procedures based on glucose involve growing *E.coli* on a defined medium containing ^{13}C enriched glucose and isolating the cellular RNA, which is then digested to nucleotide monophosphates (NMPs). The NMPs are then converted to nucleotide triphosphates, the precursors for *in vitro* RNA synthesis. The main disadvantage of this procedure is that it requires an isotope source (glucose, ^{13}C enriched at all six positions) that can be prohibitively expensive for large-scale preparations. Procedures based on methanol produce labeled ribonucleotides using raw material from the organism *Methylophilus methylotrophus* (1) or *Methylobacterium extorquens* (4). Although methanol is a relatively inexpensive source of ^{13}C, the use of methylophiles have several significant disadvantages when compared to *E.coli*: they have a significantly lower ribonucleotide content than *E.coli* per gram of cells (1); the growth of *E.coli* is easier and more familiar to most researchers; and since *E.coli* is used by many investigators in preparing ^{13}C-labeled proteins for NMR analysis (6), ribonucleotides can be isolated from *E.coli* as useful side products of protein preparations.

In the work presented here, ^{13}C enriched ribonucleotides were prepared from material isolated from *E.coli* grown using ^{13}C sodium acetate as the source of carbon. The media contained the following components (per liter): 7 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 240 mg $MgSO_4$, 10 mg $CaCl_2$, 0.2 mg $FeCl_3$, 0.15 mg $ZnCl_2$, 25 µg $CoCl_2$, 25 µg $CuSO_4$, 25 µg $MnCl_2$, 0.5 mg thiamine and 1.5 g of sodium acetate. The media was inoculated using cells from a culture growing in the same media but with ^{12}C-glucose rather than sodium acetate as the carbon source. Cells grown using acetate as the carbon source had significantly slower growth rates than cells grown on an equal mass of glucose, with typically 3 h doubling time versus 45 min. However, the cells ultimately reached the same density and total mass. Cells were harvested when the culture reached an absorbance of between 1.0 and 1.4 at 600 nm. The ^{13}C-enriched ribonucleotides were isolated from the *E.coli* as described in excellent detail by Batey *et al.* (1). The harvested cells were resuspended in STE buffer (0.1 M NaCl, 10 mM Tris, 1 mM EDTA, pH 8) and frozen. After thawing, SDS was added up to a concentration of 1% to the cell lysate, which was then vigorously mixed. Cellular proteins were removed by phenol extraction. After centrifugation the RNA-containing aqueous layer was removed from above the phenol and white protein-containing interface. Additonal STE buffer was added to the phenol–protein mixture, which was again mixed and centrifuged to extract additional RNA. The ribonucleotide-containing aqueous layers were combined and extracted with 24:1 chloroform–isoamyl alcohol. The cellular RNA was precipitated with ethanol, and dissolved in acetate buffer at pH 5.2. The cellular RNA was digested to mononucleotides by adding 0.1 mM $ZnCl_2$ and 30 U RNAse P1 and incubating at 37°C overnight. The crude cell lysate contained 4100 A_{260} U/l cells. After phenol and chloroform extraction and ethanol precipitation, typically 1400 A_{260} U of nucleotides were obtained/l of cells. In our hands, there was no significant difference in nucleotide yield from cells grown on glucose or either doubly or singly labeled acetate as an isotope source. With 99% ^{13}C-enriched sodium acetate as the only significant source of carbon in the cell growth media, isotope enrichment of the ribonucleotides appeared to be nearly complete as judged by carbon–carbon coupling in ^{13}C NMR spectra. This media was also used for preparing doubly

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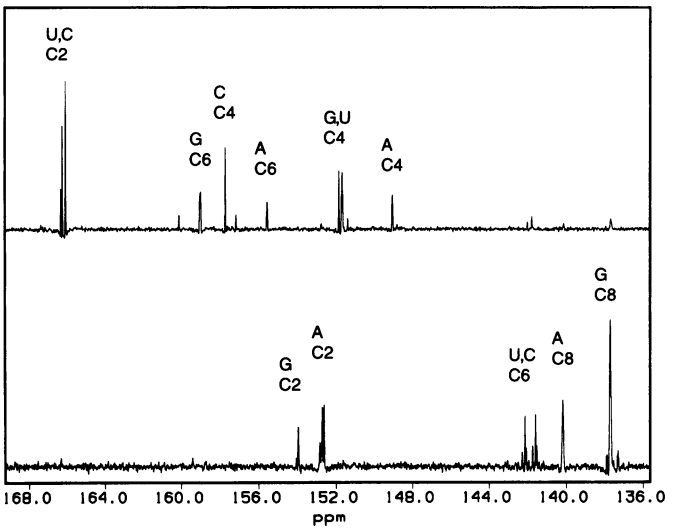

Figure 1. A section of the proton-decoupled ^{13}C NMR spectra at 125.7 MHz of ribonucleotides isolated from *E.coli* grown using singly labeled acetates $^{12}CH_3{}^{13}COONa$ (upper spectrum) or $^{13}CH_3{}^{12}COONa$ (lower spectrum) as the source of carbon. The dramatic differences between the upper and lower spectra are indicative of the non-unformity of isotope distribution within the ribonucleotides. The spectra were obtained with 1024 scans of 32K points, a sweep width of 26 318 Hz. The spectra contain skewed sine-bell resolution enhancement, and are referenced to external (trimethylsilyl)propionic acid.

labeled ribonucleotides (^{13}C and ^{15}N), where $^{15}NH_4Cl$ was used as the nitrogen source.

We also investigated the distribution of ^{13}C within ribonucleotides prepared using singly labeled acetate ($CH_3{}^{13}COONa$ or $^{13}CH_3COONa$) as the carbon source. ^{13}C-enrichment at the various nucleotide positions was determined by analyzing the multiplets arising from ^{13}C coupling and peak intensities in one- and two-dimensional ^1H and ^{13}C NMR spectra (Table 1). The non-uniformity of the ^{13}C distribution within the purified ribonucleotides is striking, as can be seen in Figure 1. With the exceptions of C1′, C2′ and C3′, the carbons within the nucleotides appear to be essentially completely labeled or unlabeled, depending upon which singly labeled acetate was used as the isotope source. This non-uniformity of isotope distribution is potentially useful in NMR applications. For example, it is particularly interesting that a sample prepared using $^{13}CH_3COONa$ is highly ^{13}C-enriched at all of the positions useful for proton-detected heteronuclear NMR experiments, except for the ribose C3′. Proton-detected heteronuclear methods are now universally employed as the most powerful NMR methods for analyzing RNA structure (1–4,7,8). A highly useful double-labeled RNA sample could be prepared using singly labeled $^{13}CH_3COONa$ and $^{15}NH_4Cl$ as the only isotope sources. The differential labeling of C3′ versus C2′ is also potentially useful, since the C3′ and C2′ carbons cannot be distinguished based on unique ^{13}C chemical shifts (8). Using acetate as an isotope source, it is also possible to prepare ribonucleotides with a wide range of isotope enrichment using media containing mixtures of $CH_3{}^{13}COONa$, $^{13}CH_3COONa$ and CH_3COONa, and $^{13}CH_3{}^{13}COONa$.

Table 1. The distribution of ^{13}C in ribonucleotides prepared using singly labeled acetates $^{12}CH_3{}^{13}COONa$ or $^{13}CH_3{}^{12}COONa$ as the source of carbon.

Position labeled	Carbon-13 source	
	$^{12}CH_3{}^{13}COONa$	$^{13}CH_3{}^{12}COONa$
Purines		
C2	<5	>95
C4	>90	<5
C5	<5	>95
C6	>90	<5
C8	<5	>95
Pyrimidines		
C2	>95	<5
C4	>95	<5
C5	<5	>90
C6	<5	>90
Ribose		
C1′	10 ± 5	90 ± 5
C2′	20 ± 10	80 ± 10
C3′	75 ± 10	25 ± 10
C4′	<5	>90
C5′	<5	>90

Values are % ^{13}C enrichment. Nucleotides prepared from cells grown with 99% enriched $^{13}CH_3{}^{13}COONa$ as the carbon source have a ^{13}C content of ~99%

In summary, we have described an efficient, inexpensive and generally useful method for preparing ribonucleotides containing a wide variety of distributions of ^{13}C enrichment. This method has significant advantages in terms of cost and flexibility over previously reported procedures, and will be beneficial to investigators using heteronuclear NMR spectroscopy in the study of RNA, currently an important and active area of research.

REFERENCES

1 Batey, R.T., Inada, M., Kujawinski, E., Puglisi, J.D. and Williamson, J.R. (1992) *Nucleic Acids Res.* **20**, 4515.
2 Nikonowicz, E.P., Sirr, A., Legault, P., Jucker, F.M., Baer, L.M. and Pardi, A. *Nucleic Acids Res.* **20**, 4507.
3 Michnicka, M.J., Harper, J.W. and King, G.C. (1993) *Biochemistry* **32**, 395.
4 Hines, J.V., Landry, S.M., Varani, G. and Tinoco, I. (1994) *J. Am. Chem. Soc.* **116**, 5823–5831.
5 Zimmer, D.P. and Crothers, D.M. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 3091–3095.
6 Venters, R.A., Calderone, T.L., Spicer, L.D. and Fierke, C.A. (1991) *Biochemistry* **30**, 4491.
7 Farmer, B.T., Müller, L., Nikonowicz, E.P. and Pardi, A. (1993) *J. Am. Chem. Soc.* **115**, 11040.
8 Varani, G. and Tinoco, I. (1991) *J. Am. Chem. Soc.* **113**, 9349.