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Preparation of isotopically labeled ribonucleotides for multidimensional NMR spectroscopy of RNA

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ABSTRACT

A general method for large scale preparation of uniformly isotopically labeled ribonucleotides and RNAs is described. Bacteria are grown on isotopic growth medium, and their nucleic acids are harvested and degraded to mononucleotides. These are enzymatically converted into ribonucleoside triphosphates, which are used in transcription reactions *in vitro* to prepare RNAs for NMR studies. For ^{15}N-labeling, *E.coli* is grown on ^{15}N-ammonium sulfate, whereas for ^{13}C-labeling, *Methylophilus methylotrophus* is grown on ^{13}C-methanol, which is more economical than ^{13}C-glucose. To demonstrate the feasibility and utility of this method, uniformly ^{13}C-labeled ribonucleotides were used to synthesize a 31 nucleotide HIV TAR RNA that was analyzed by 3D-NMR. This method should find widespread use in the structural analysis of RNA by NMR.

INTRODUCTION

Recent advances in structure determination using NMR spectroscopy have relied heavily on uniform isotopic labeling to permit application of higher dimensional heteronuclear NMR techniques (1, 2, 3). Use of 3D- and 4D-NMR methodology in conjunction with ^{15}N- and ^{13}C-labeling has made a significant impact on protein solution structure determination. Proteins are usually prepared by overexpression in bacteria, and isotopic labeling only requires growth in isotopically labeled media. These techniques have only recently been applied to nucleic acids (4, 5, 6). DNA and RNA are routinely prepared either by chemical or enzymatic synthesis, and thus, isotopic labeling of nucleic acids requires the preparation of isotopically labeled monomers in a suitable form.

We describe here a procedure for preparation of uniformly isotopically labeled ribonucleoside triphosphates and their utilization in large scale *in vitro* transcription reactions to prepare isotopically labeled RNAs for NMR study. A similar method has recently been used to prepare uniformly ^{13}C-labeled ATP for mass spectrometry studies (7). The scheme, shown in Figure 1, is quite general, and can be adapted to the preparation of ^{13}C- and/or ^{15}N-labeled RNAs. In Step 1, bacteria, typically *E.coli*, are grown on glucose-mineral salts medium containing a labeled carbon and/or nitrogen source. We introduce the use of *Methylophilus methylotrophus*, which can be grown on methanol, an economical source of ^{13}C. The cells are harvested and disrupted by a rapid detergent lysis in Step 2, followed by a phenol extraction to remove proteins, and precipitation of the cellular nucleic acids (both DNA and RNA) with isopropanol. The nucleic acids are hydrolyzed enzymatically in Step 3 to mononucleotides that are then separated into deoxy- and ribonucleotide pools in Step 4. The ribonucleotides are then converted enzymatically into nucleoside triphosphates in Step 5, and the nucleotides are purified in Step 6. Step 7 involves transcription reactions to prepare large RNA molecules of the desired sequence, and the final Step 8 involves recycling of unincorporated nucleotides.

To demonstrate the applicability of this method to structural determination of RNA, we have prepared uniformly ^{13}C-labeled TAR RNA (u-^{13}C-TAR), and performed a 3D-HCCH-COSY experiment (8). TAR is a secondary structural element at the 5′ end of HIV mRNA that is the binding site for the transcriptional activator protein, Tat. We have characterized the structure of TAR alone and as a complex with arginine using two-dimensional, ^{1}H NMR methods (9). Thus, TAR will serve as a good test case to determine how much additional structural information for RNA will become available through the use of multidimensional heteronuclear NMR experiments.

MATERIALS AND METHODS

This procedure has been optimized to a great extent, and departures from the reported procedures should be avoided; in particular, the amounts and volumes reported should not be arbitrarily changed. It is strongly recommended that the procedures be rehearsed and reproduced using unlabeled materials before embarking upon a labeled preparation.

Materials

99% ^{13}C-methanol and 98% ^{13}C-glucose was purchased from Cambridge Isotope Laboratories. 98.7% ^{15}N-ammonium sulfate was obtained from the Los Alamos Stable Isotope Resource Program. Nuclease P1, phosphoglycerate mutase from rabbit

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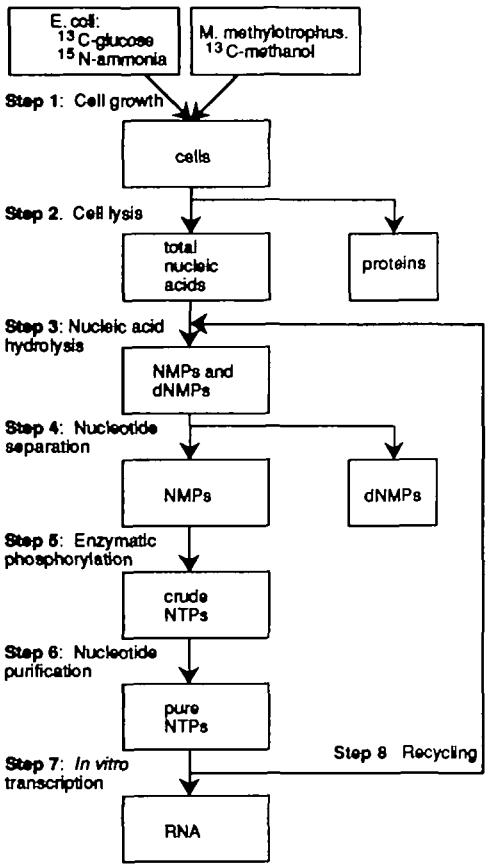

Figure 1. Flowchart outlining the procedure for generating isotopically labeled nucleoside triphosphates.

muscle, nucleoside monophosphate kinase from beef liver, and calf intestinal alkaline phosphatase were purchased from Boehringer Mannheim. Enolase from baker's yeast, myokinase from chicken muscle, pyruvate kinase from rabbit muscle, guanylate kinase from porcine brain, and 3-phosphoglycerate (barium salt) were obtained from Sigma. Affi-gel 601 boronate-derivatized polyacrylamide gel and AG 50W-X8 ion exchange resin (H^+ form, 200–400 mesh) were purchased from Bio-Rad. Centrifugal microconcentrators with a 10,000 molecular weight cutoff were purchased from Amicon. Reverse phase HPLC was performed with a 250×4.6 mm 5 micron Econosphere C8 column purchased from Alltech. The Vydac Nucleotide Analysis HPLC column was obtained from Rainin. Toyopearl DEAE 650 M Tsk-Gel, 40–90 micron, was obtained from Supelco. A Jenco pH/ORP Controller, Model 3671, was purchased from Markson. Milli-Q 18 MΩ H_2O was used in all procedures.

Cell growth

Culture of Methylophilus methylotrophus. Methylophilus methylotrophus (ATCC 53528) was grown in a liquid medium containing 10 (per liter): K_2HPO_4, 0.95 g; NaH_2PO_4, 0.78 g; $(NH_4)_2SO_4$, 1.8 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 50 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $MnSO_4 \cdot 5H_2O$, 50 μg; $ZnSO_4 \cdot 7H_2O$, 50 μg; $CaCl_2 \cdot 2H_2O$, 1.3 mg; $CoCl_2$, 10 μg; H_3BO_3, 7 μg; $NaMoO_4$, 10 μg. A solution containing only the phosphate salts was made separately, the pH adjusted to 6.8, and autoclaved. After this solution had cooled, it was supplemented with a filter sterilized solution of the other nutrients and 1 ml 99% ^{13}C-methanol per liter. *M.methylotrophus* was maintained in this same medium, either on 15% agar plates where the (unlabeled) methanol concentration was increased to 5 ml per liter media, or in stock cultures containing 30% added glycerol at −20°C (11). A 5 ml culture was inoculated with a single colony and grown with agitation for 12 h at 37°C. The entire 5 ml culture was used to inoculate a 50 ml culture that was subsequently grown for 12 hours at 37°C with agitation. Alternatively, a 50 ml culture was inoculated with 1 ml of glycerol stock culture, and 1 ml of this revived overnight culture was used to inoculate a fresh 50 ml culture. The 50 ml culture from either procedure was used to inoculate 1 liter cultures. Cells were grown aerobically in 4 liter shake flasks at 37°C for 48 hours with agitation. A 1 ml test aliquot of the culture was harvested by centrifugation in a microfuge for 5 min. Cultures that formed pink, tightly packed cell pellets were harvested immediately. Cultures that contained large flocculent white pellets were allowed to age at 25°C for 24 hours without agitation prior to harvest. The culture was harvested by centrifugation at 6000 rpm in a JA-10 rotor for 20 minutes. Each liter of culture yields approximately 2 g of wet packed cells, which have a distinct pink color. Eight liters of culture will yield enough NTPs for one NMR-scale transcription.

Culture of E.coli. To obtain ^{13}C and ^{15}N-labeled nucleic acids, *E.coli* (ATCC 15224) was grown on a minimal media containing (per liter): KH_2PO_4, 13.6 g; $(^{15}NH_4)_2SO_4$, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 15 mg; Na_2EDTA, 30 mg; $FeCl_3 \cdot 6H_2O$, 25 mg; $CuSO_4 \cdot 5H_2O$, 240 μg; $MnSO_4 \cdot 5H_2O$, 180 μg; $ZnSO_4 \cdot 7H_2O$, 27 μg; $CoCl_2$, 27 μg. A solution containing only KH_2PO_4 was made separately, the pH adjusted to 7.0 with KOH, and autoclaved. The other components were then added as filter sterilized solutions, along with 5 ml of a filter sterilized 20% (w/v) solution 98% ^{13}C-glucose. For ^{15}N-labeled nucleic acids, *E.coli* (ATCC 15224) was grown on a minimal media containing (12) (per liter): glycerol, 21.5 g; KH_2PO_4, 1.6 g; NaH_2PO_4, 5.3 g; $(^{15}NH_4)_2SO_4$, 0.7 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; Na-citrate, 0.5 g; $CaCl_2 \cdot 2H_2O$, 750 μg; Na_2EDTA, 30 mg; $FeCl_3 \cdot 6H_2O$, 25 mg; $CuSO_4 \cdot 5H_2O$, 240 μg; $MnSO_4 \cdot 5H_2O$, 180 μg; $ZnSO_4 \cdot 7H_2O$, 27 μg; $CoCl_2$, 270 μg. A solution containing glycerol and the phosphate salts was made separately, the pH adjusted to 7.0, and autoclaved. The other nutrients were added as a filter sterilized solution after the autoclaved media had cooled. Cultures were inoculated with 5 ml of an overnight culture of *E.coli*. Cells were grown aerobically for 15 hours in 4 liter shake flasks at 37°C with agitation, and harvested by centrifugation at 6000 g in a JA-10 rotor for 20 minutes. Each liter of culture yielded approximately 4 g of wet packed cells.

Cell lysis

Lysis procedure. Approximately 4 g of wet packed cells were resuspended in an equal volume of STE buffer (0.1 M NaCl, 10 mM Tris·Cl (pH 8.0), 1 mM EDTA (pH 8.0)). Resuspended cells were slowly added to a mixture of 90 ml of STE buffer and 5 ml 10% SDS at 37°C with rapid stirring. Phenol containing 0.1% 8-hydroxyquinoline (w/v) was equilibrated against STE buffer, a 24:1 chloroform:isoamyl alcohol solution was prepared, and a 1:1 solution of the phenol and chloroform solutions was preheated to 65°C. After the cellular lysate had been thoroughly homogenized for 10–15 minutes, the solution was added to 200 ml of preheated phenol:chloroform and incubated for 30 minutes

at 65°C with occasional vigorous mixing to keep the organic and aqueous phases emulsified. After centrifugation for 10 minutes at 6000 rpm in a JA-10 rotor, the aqueous layer was removed, taking care not to disrupt the white protein inclusion layer. The organic and inclusion layers were extracted twice with 100 ml of STE buffer in a Waring blender for 5 minutes, and the aqueous layer was removed after centrifugation. The aqueous layers from each extraction were pooled (~300 ml total volume) and extracted with an equal volume of 24:1 chloroform:isoamyl alcohol. Nucleic acids were precipitated by adding 30 ml 3 M sodium acetate, pH 5.2, and 330 ml isopropyl alcohol to the aqueous phase and incubating overnight at −20°C. Precipitation at low temperature for more than four hours is critical for ensuring complete precipitation of all cellular nucleic acids. The solution was centrifuged at 6000 rpm for 30 minutes in a JA-10 rotor, the liquid decanted, and the pellet was dried and resuspended in 25 ml of STE buffer. The solution should contain between 1100 and 1375 A_{260} units of nucleic acids if *M.methylotrophus* is used, or 3500−3750 A_{260} units if *E.coli* is used.

Nucleic acid hydrolysis

Nuclease P1 digest. The resuspended nucleic acids from a single lysis procedure (~1200 A_{260} units in 25 ml STE) were denatured in a boiling waterbath for 5−6 minutes. The solution was cooled in an ice bath before adjusting to 15 mM sodium acetate, 0.1 mM $ZnSO_4$, pH 5.2. Nucleic acids were hydrolyzed by adding 6 units nuclease P1 and incubating for 1 hour at 37°C (13). To ensure complete hydrolysis of the nucleic acids, another 6 units of nuclease was added after one hour, and incubation was continued for an additional hour. Complete hydrolysis of the nucleic acids to NMPs was verified by reverse phase HPLC using a C8 column.

Reverse phase HPLC. Reverse phase HPLC was used to monitor the hydrolysis of DNA and RNA during the P1 nuclease digestion. Separation of ribonucleotides and deoxyribonucleotides on this column is improved by dephosphorylation with calf intestinal alkaline phosphatase. A sample of 20−30 μg of nucleoside monophosphates was dephosphorylated by incubating with 2 units of calf intestinal alkaline phosphatase for 30 minutes at 37°C in phosphatase buffer (50 mM Tris·HCl, pH 8.5, 0.1 mM EDTA, pH 8.5), followed by another 2 units of phosphatase and continued incubation for 30 minutes. HPLC analysis of the reaction mixture was performed on a 250×4.6 mm Econosphere C8 column using an isocratic elution with methanol-83.3 mM triethylammonium phosphate, pH 6.0 (4:96 v/v) as the mobile phase at a flow rate of 1 ml/min (14). Sample volumes of 10−20 μl were injected and nucleosides were detected at 268 mn with a UV detector, as shown in Figure 2a.

Nucleotide separation

Boronate affinity chromatography. To prepare the affinity chromatography column, 5 g Affi-gel 601 was hydrated in TE buffer (10 mM Tris·HCl, pH 8.0, 1 mM EDTA, pH 8.0) and packed in a 20×2.5 cm glass column. A wide column was important for good flow rates because the resin volume changes with pH and ionic strength. 1 M TEABC, pH 9.5, was prepared by bubbling CO_2 through 141 ml triethylamine in 700 ml H_2O at 5°C until the pH drops to 9.5, and bringing to 1 liter volume with H_2O. It is crucial that all steps be performed at 5°C in order for the ribonucleotides to bind to the column. The column

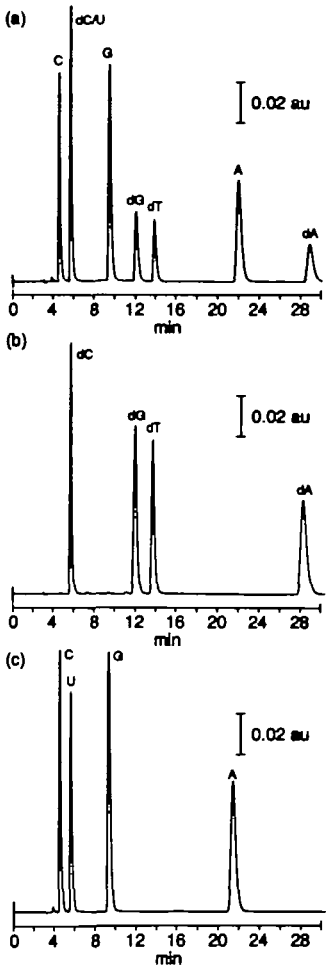

Figure 2. Reverse phase HPLC chromatograms of nucleoside mixtures. A total of 50 μg of nucleoside monophosphates were dephosphorylated with calf intestinal alkaline phosphatase prior to injection onto an Econosphere C8 reverse phase column. Elution was isocratic, with methanol-83.3 mM triethylammonium phosphate, pH 6.0 (4:96 v/v), a flow rate of 1.0 ml/min, and detection at 268 nm at 0.2 absorbance units full scale (aufs). (a) Total nucleotide pool from *M.methylotrophus* cellular lysate, prior to boronate affinity chromatography. (b) Deoxyribonucleotides, which do not bind to the boronate column. (c) Bound ribonucleotides, which are eluted from the boronate column.

was equilibrated with 1 M TEABC and the nucleotides, dissolved in 10 ml of 1 M TEABC, were applied to the column (15). After loading the sample, the column was washed with 1 M TEABC, while collecting 5 ml fractions, until the A_{260} of the eluant dropped below 0.1. To elute bound material, the column was washed with H_2O that had been acidified to pH 4−5 with CO_2, while continuing to collect 5 ml fractions. Elution was continued until the A_{260} of the eluant dropped below 0.1, which usually occurs 100 ml after the start of elution.

Separation of dNMPs from NMPs. Tle nucleotide solution (≤ 2500 A_{260} units) from the P1 digestion was lyophilized and resuspended in 10 ml of 1 M triethylammonium bicarbonate (TEABC) and applied to a boronate affinity column. The deoxyribonucleotides, salts, and other impurities were separated from the ribonucleotides using the boronate affinity chromatography procedure. Fractions of low pH eluant >0.1 A_{260} were pooled, lyophilized, and resuspended in 5 ml of H_2O.

Complete separation of dNMPs from NMPs was verified by reverse-phase HPLC, as shown in Figure 2b,c. Ribonucleotides comprised ~70% of the total nucleotide pool in nucleic acids derived from *M.methylotrophus*. The composition of the ribonucleotide mixture was estimated from the peak areas in Figure 2c, correcting for absorbance differences at 268 and 260 nm, as 22% AMP, 24% CMP, 32% GMP, and 22% UMP. The expected composition of *E.coli* RNA is 26% AMP, 20% CMP, 32% GMP, and 22% UMP (16). This nucleotide composition corresponds to an average extinction coefficient of 11 $cm^2/\mu mol$, and an average molecular weight of 363 g/mol for the monosodium salt of the mononucleotides. Lysis of 4 g wet packed *M.methylotrophus* cells yielded ~33 mg of ribonucleoside monophosphates.

Enzymatic phosphorylation

Preparation of sodium 3-phosphoglycerate. The Na^+ form of AG 50W-X8 was prepared by treating 25 g of resin with three 50 ml exchanges of 1 M NaCl for 15 minutes for each exchange, and three times with H_2O. The Na^+ form of 3-phosphoglycerate was prepared by vigorously stirring 2 g of Ba^{++} 3-phosphoglycerate with 15 ml of a 50% slurry of Na^+-AG 50W-X8 resin for 30 minutes. The resin was removed by filtration and washed three times with 5 ml H_2O. The pH of the combined filtrates was adjusted to 7.5 using 1 M NaOH.

Enzymatic phosphorylation of NMPs. This procedure was derived from a procedure developed by Whitesides (13). Enzymatic phosphorylation of approximately 83 mg of nucleoside monophosphates to nucleoside triphosphates was performed in a 50 ml three-necked round bottom flask flushed with argon at ambient temperature. The NMPs were added to give a final concentration of 10 mM in a solution containing 15 mM KCl, 75 mM $MgCl_2$, 15 mM dithioerythritol, and 10 mM Na^+ 3-phosphoglycerate (17). The pH of the solution was adjusted to 7.5 with 1 M HCl, and was maintained during the course of the reaction with a pH controller delivering 0.1 M HCl via a peristaltic pump. Synthesis of ATP was initiated by adding 1 μM ATP, 10 units phosphoglycerate mutase, 200 units myokinase, 100 units enolase, and 200 units pyruvate kinase. After 3 hours, when ATP represents >90% of the adenosine nucleotide pool, 0.5 units of guanylate kinase, and 1.0 unit nucleoside monophosphate kinase were added, and the concentration of 3-phosphoglycerate was increased to 20 mM. During the synthesis of CTP, GTP, and UTP, the concentration of phosphoglycerate was increased by 5 mM every 3–4 hours. Complete phosphorylation of all nucleotides occurs within 12–14 hours, and the reaction mixture was stored at −20°C.

Nucleotide analysis. Conversion of NMPs to NTPs was monitored by HPLC, and several chromatograms during the course of a typical phosphorylation are shown in Figure 3. Populations of NMPs, NDPs, and NTPs were analyzed using a 25×4.6 mm Vydac Nucleotide Analysis column. Chromatography was carried out using 0.045 M NH_4COOH, pH 4.6 with H_3PO_4 as solvent A and 0.5 M NaH_2PO_4, pH 2.7 with HCOOH as solvent B, with a linear gradient from 0 to 100% solvent B in 10 minutes and a flow rate of 2.0 ml/min. Sample volumes of 20 μl were injected, and nucleotides were detected at 268 nm with a UV detector.

Nucleotide purification

The phosphorylation reaction mixture was lyophilized, resuspended in 10 ml ice-cold 1 M TEABC, pH 9.5, and purified using the boronate affinity column procedure. This desalts the nucleotides, which is critical for their function in transcription reactions *in vitro*. The lyophilized NTPs were resuspended in 2 ml H_2O and any remaining high molecular weight contaminants were removed by passing the nucleotide solution through a Centricon 10,000 molecular weight cutoff microconcentrator. The concentration of the NTP solution was estimated by UV absorbance at 260 nm.

In vitro transcription

Transcription of TAR RNA. RNA was synthesized by transcription with T7 RNA polymerase (18) from an oligonucleotide template with a single-stranded template region and a double-stranded

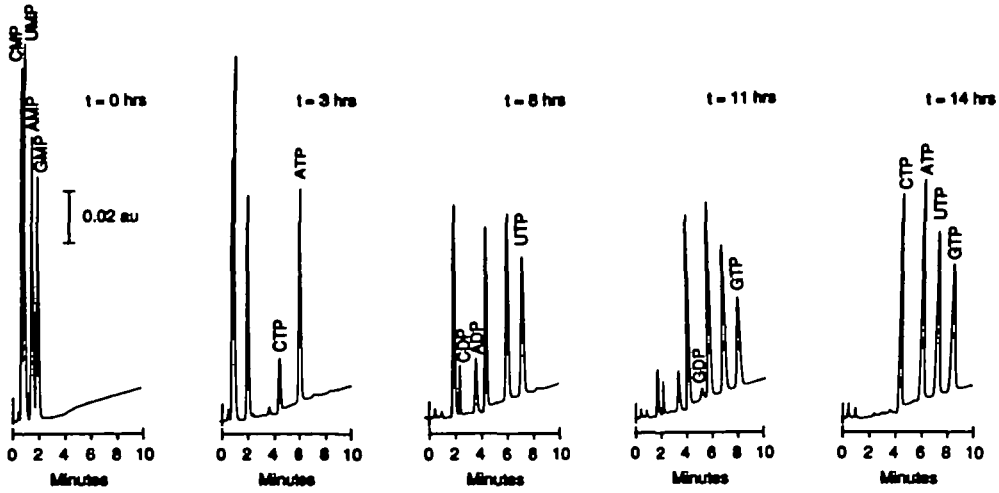

Figure 3. HPLC chromatogams of the reaction timecourse of an enzymatic nucleotide phosphorylation reaction. At the zero timepoint, a catalytic quantity of ATP was added to the reaction, along with myokinase, to initiate the reaction. After three hours, when the vast majority of AMP had converted to ATP, guanylate kinase and nucleoside monophosphate kinase were added to initiate the phosphorylation of the other nucleoside monophosphates. The chromatograms are of 15 nmol of nucleotides injected onto a Vydac nucleotide analysis column using the elution scheme described in the text at a 2.0 ml/min flow rate and detecting at 268 nm at 0.2 aufs.

promoter region. Oligonucleotides were synthesized by standard phosphoramidite chemistry using an automatic DNA synthesizer. A 44 ml transcription reaction was incubated for 4 hours at 37°C in 40 mM Tris·HCl, pH 8.1, 1 mM spermidine, 5 mM DTT, 9.6 mM $MgCl_2$, 0.01% Triton X-100, 80 mg/ml polyethylene glycol (8000 molecular weight), 6.0 mM NTPs (~1.5 mM each), 200 nM each DNA strand, and T7 RNA polymerase (19). Purification and preparation of the RNA for NMR was performed as described elsewhere (20). The optimal added $MgCl_2$ concentration for transcription of the TAR template differed significantly for commercial NTPs (36 mM) and isotopically labeled cellular NTPs (9.6 mM $MgCl_2$). A comparison of reaction products from small scale transcription reactions for commercial and labeled NTPs is shown in Figure 4.

Recycling of nucleotides

A DEAE column with a 17 cm bed height was poured in a 20×2.5 cm glass column, washed with 100 ml of 0.6 M NaCl in TE buffer, and equilibrated with 100 ml of 25 mM NaCl in TE buffer. The buffer from the bottom reservoir of the gel electrophoresis apparatus used to purify the RNA product was saved and loaded onto the DEAE column by gravity. The column was washed with 100 ml of 25 mM NaCl in TE and the bound nucleotide triphosphates and small abortive transcription products were eluted with 0.6 M NaCl in TE buffer while collecting 20 ml fractions. Fractions that contained greater than 0.1 A_{260} units were pooled, digested with nuclease P1, and desalted using boronate chromatography. This resulted in the recovery of 45–50% of the nucleotides used in the transcription reaction as NMPs.

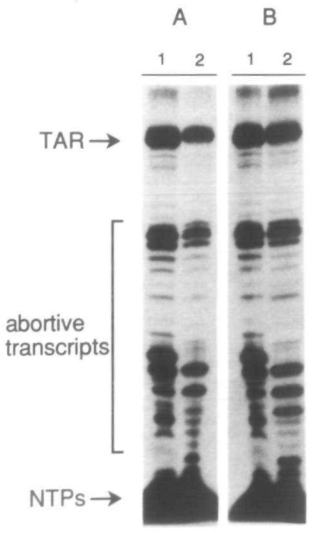

Figure 4. A comparison of *in vitro* transcription reaction of TAR RNA performed with commercial nucleoside triphosphates or labeled NTPs. Small scale transcription reactions were performed as described in the Materials and Methods except that α-^{32}P-labeled CTP was included in the reaction. A) transcription of TAR template in standard transcription buffer using Sigma NTPs (A1) and cellularly derived NTPs (A2). B) Transcription of TAR template using Sigma NTPs in standard transcription buffer (B1) and cellularly derived NTPs in an optimized transcription buffer (B2). Lanes A1 and B1 differ slightly in the pattern of abortive transcripts compared to A2 and B2 due to the presence of GMP in the unlabeled reactions for priming transcription.

Summary. Preparation of sufficient labeled NTPs for synthesis of an NMR sample requires 8 ml of ^{13}C-methanol, which produces ~16 g of wet packed cells. Four lysis procedures are required to process this quantity of cells, resulting in ~165 mg of nucleic acids. Two boronate chromatography steps are required to separate the NMPs from the dNMPs. All of the NMPs can be phosphorylated simultaneously in one reaction (twofold scaleup), provided that the volume is doubled. Two boronate column procedures are required to desalt the resulting NTPs. If the nucleotides and abortive transcripts are recovered and recycled, up to 50% of the input nucleotides can be recovered.

NMR spectroscopy. NMR experiments were performed on a Varian VXR 500 MHz spectrometer with an indirect detection probe. NMR data were transferred to a Silicon Graphics 4D35TG+ workstation and were processed with FELIX version 1.0 from Hare Research Inc. For the DQF-COSY, 2048 and 256 complex points were accumulated in t_2 and t_1, respectively. Data were zero-filled to 4096 points in both dimensions after apodization. For the 3D-HCCH-COSY, 512 complex points were accumulated in t_3, 32 complex points in t_2, and 48 complex points in t_1. The sweep widths were 3000 Hz in ω_3, 3000 Hz in ω_2, and 3000 Hz in ω_3. Linear prediction of 12 additional points was performed in t_2 prior to apodization, and data were zero filled to 128 points in t_1 and t_2.

RESULTS

In order to generate large quantities of labeled nucleotide monomers, we took advantage of the abundance of RNA present in the bacterial cell. It is estimated that 20% of the dry weight of rapidly dividing *E.coli* is RNA (16), mainly in the form of ribosomal RNAs, and that 3% of the dry weight is chromosomal DNA. Labeled monomers can be prepared by isolation of total nucleic acids from bacterial cells grown on isotopic precursors, followed by conversion of the cellular RNA into a useable form. Here we discuss the practical aspects of each step of our protocol.

Cell growth

The growth of *E.coli* on glucose-minimal salts medium is straightforward (21, 22). The medium can be substituted with ^{15}N-ammonium salts and/or ^{13}C-glucose to produce uniformly labeled bacterial mass with high efficiency. For incorporation of ^{15}N only, *E.coli* is definitely the organism of choice. Optimization of a glucose-minimal salts medium for conversion of ^{15}N into biomass reduces the cost of the isotope to a small fraction of the total cost of preparing the nucleotides (data not shown). However, for ^{13}C incorporation, the cost of the labeled glucose is quite high. On a per mole of carbon basis, ^{13}C-methanol is the most inexpensive isotopic source for carbon labeling, and we explored the possibility of exploiting methylotrophic bacteria for the preparation of labeled RNAs.

There are two classes of methylotrophic bacteria that are distinguished by the pathway used to incorporate methanol carbons into cell material. The choice of methylotrophic strain for isotopic labeling requires consideration of the consequences of these two pathways. In the serine pathway (23), the net reaction for assimilation of methanol is conversion of two moles of formaldehyde and one mole of CO_2 into one mole of 3-phosphoglycerate (23). The net reaction in the hexulose monophosphate (HMP) pathway (23) is conversion of three moles

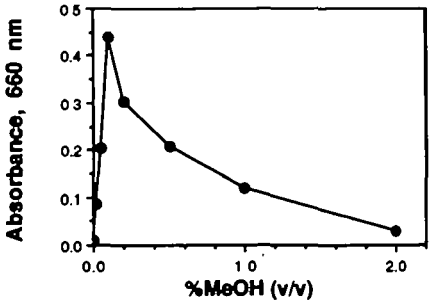

Figure 5. Yields of *M.methylotrophus* in batch culture as a function of methanol concentration. Cultures were grown to saturation (48 h) and the absorbance read at 660 nm.

of methanol into dihydroxyacetone phosphate, and, in contrast, no carbon is incorporated into metabolic pathways from CO_2. There is a significant danger of dilution of the input isotope by incorporation of atmospheric CO_2 in serine pathway organisms such as *Pseudomonas AM1* or *Pseudomonas extorquens*, but not in HMP pathway organisms. Thus, our choice of methylotrophic strain was *Methylophilus methylotrophus* (ATCC 53528), which is an obligate methylotroph using the HMP pathway.

M.methylotrophus grows relatively rapidly on a methanol minimal salts medium, with a doubling time of ~2 hours. Most of the literature concerning growth of this organism relates to continuous flow culture in bioreactors or fermenters, but fermenter growth was not tested, since the high flow aeration usually employed might volatilize the methanol. For optimal isotopic labeling, complete conversion of the isotope source into biomass was insured by growing cells to stationary phase in shake flasks. The growth of *M.methylotrophus* was optimized for the highest production of biomass per input gram of methanol by growing one liter cultures containing increasing amounts of methanol, as shown in Figure 5. Above 0.1% methanol, cell yields dropped dramatically, and therefore 0.1% was chosen as the optimal methanol concentration for high yield growth. Under these conditions, ~2 g of wet packed cells are obtained per liter of culture.

The effects of other components of the medium was also examined. These organisms are very sensitive to phosphate concentration, and it was found that low phosphate media gave better growth rates (23). Addition of a trace metal supplement containing EDTA as a chelating agent was found to inhibit growth. Neither an increase in the amount of trace metal supplement nor addition of vitamins (biotin, B12, thiamine, pantothenate, riboflavin, folic acid, nicotinamide) to the medium had any effect on yields. A variety of growth media and conditions are reported in the literature (11) and we have observed a considerable range of growth rates under different conditions. The media reported here consistently gave the best results for the present application.

Cultures of *M.methylotrophus* just entering stationary phase sometimes cannot be harvested by centrifugation, as they do not form packed cell pellets. In this case, the cells remain suspended in solution as a translucent flocculent mass. Lysis of this flocculent mass produces a great deal of non-nucleic acid material during isopropanol precipitations, and results in very low yields of cellular nucleic acids. This problem appears to be related to the

Table I.

Organism	Limiting Nutrient	g cells / g isotope	mg NMPs / g cells	mg NMPs / g isotope
E.coli	^{13}C-glucose + ^{15}N-ammonium sulfate	2.9	20	58
E.coli	^{15}N-ammonium sulfate	6.4	7	45
M.methylotrophus	^{13}C-methanol	2.5	10	25

maintenance of *M.methylotrophus* on agar plates. Cultures derived from glycerol stocks give rise to less flocculent material than cultures derived from single colonies on agar plates. When flocculence is observed, aging of the stationary phase cultures for 24 h at room temperature without agitation resulted in cultures that are readily harvested by centrifugation. Closely related methylotrophs are known to secrete heteropolysaccharides during logarithmic growth (24). The room temperature aging of saturated cultures may permit the organisms to consume the polysaccharides after the methanol is exhausted from the medium. ^{13}C NMR analysis of the medium after cell harvest revealed no detectable ^{13}C-labeled compounds.

The yield of biomass per input g of carbon is similar for *E.coli* and *M.methylotrophus* (Table I), and the major difference in yield is in the amount of nucleic acids obtained per g of cells. This may in part be due to the lower nucleic acid content (16%) in *M.methylotrophus* (25). Although the yield of NMPs from *M.methylotrophus* are 42% of that for *E.coli*, there is approximately a 600% price difference for the carbon source. The yield of biomass from *E.coli* grown on ^{13}C-glucose is, in our experience, 50–70% of yields from identical runs with unlabeled glucose. This is certainly not due to an isotope effect, but rather probably reflects the chemical purity of the glucose. No difference in yields is observed for *M.methylotrophus* grown on labeled or unlabeled methanol. For economic production of ^{15}N-labeled nucleotides, *E.coli* is the recommended organism, while for production of ^{13}C or ^{13}C/^{15}N-labeled nucleotides, *M.methylotrophus* is more economical.

Cell lysis

The highest yields of cellular nucleic acids were conveniently obtained by direct lysis of bacterial cells in hot phenol–chloroform solutions; elevated temperatures gave higher yields than room temperature lysis. Good yields of nucleic acids are correlated to minimizing the volume of the white proteinaceous interface layer obtained in the phenol extraction. Increasing the amount of cell mass per unit volume in the extraction reduces yields. Protease treatment greatly reduces the interfacial layer, and increases the yields of nucleic acids slightly, but we do not routinely use protease.

Use of a Waring blender during the first phenol extraction reduced yields by shearing the nucleic acids. However, the blender treatment during the second and third phenol extractions increases the yield of these two extractions because it breaks up the inclusion layer efficiently. Isopropanol precipitation gives comparable yields to ethanol precipitation, but offers the advantage that only an equal volume of isopropanol is required for precipitation. Use of sodium acetate for the precipitation greatly increases the yield compared to sodium chloride.

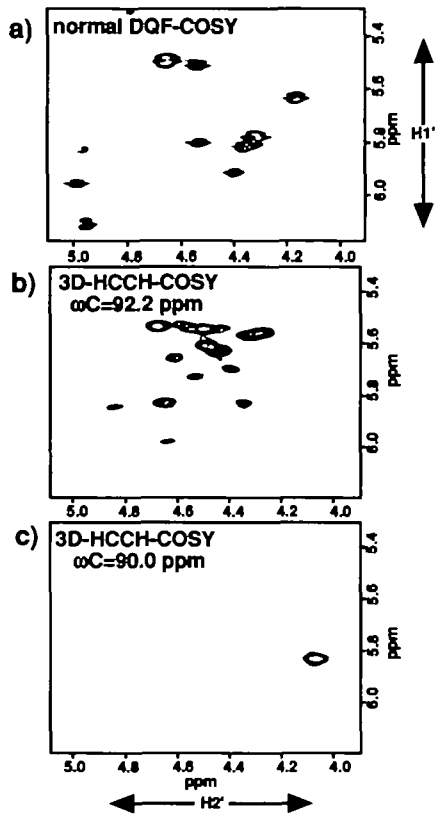

Figure 6. NMR spectra of TAR RNA. a) The region of a DQF-COSY spectrum of unlabeled TAR containing cross peaks between H1′ and H2′ protons. The magnitude spectrum was taken to permit direct comparison to the 3D spectra. b) The same regions as in (A) of a slice from a 3D-HCCH-COSY spectrum of u-^{13}C-TAR through ω_C = 92.2 ppm. c) Slice of the same region through ω_C = 90.0 ppm.

Nucleic acid hydrolysis

P1 nuclease rapidly degrades both DNA and RNA into 5′ nucleoside monophosphates. DNA is digested much more slowly than RNA, and the hydrolysis of DNA is greatly enhanced by a denaturation step. We favor using large amounts of nuclease and a short digestion period since dephosphorylation of the nucleotides has been observed during prolonged P1 nuclease digestions. The digestion is readily monitored by reverse phase HPLC analysis of aliquots that are dephosphorylated prior to injection.

Nucleotide separation

We have avoided the use of DNase in the lysis procedure to permit isolation of the deoxyribonucleotides as a byproduct, and to avoid the expense of using large quantities of RNase free DNase. Boronate affinity chromatography rapidly and efficiently separated deoxyribonucleotides (dNMPs) from ribonucleotides (NMPs), and resulted in desalting and purification of the NMPs. Boronates form covalent complexes with 1,2 cis-diols at high pH that are readily hydrolyzed at low pH. There are several commercially available boronate affinity media based on *m*-aminophenylboronic acid attached to different polymeric resins. Of these, we have found the only satisfactory resin for this purpose to be Affi-gel 601, a polyacrylamide based resin. Other resins were unacceptable because of low nucleotide capacity, or failure to bind NMPs. Affi-gel 601 does suffer from slow deterioration due to hydrolysis of the support, releasing *m*-aminophenylboronic acid. This compound gave a slight yellow color to the nucleotide preparations, but has no adverse effects on subsequent RNA synthesis.

Binding of NMPs requires high ionic strength, high pH (>9.0), and low temperatures, and dNMPs are not retained under these conditions. The dNMPs are collected in the flow-through as a byproduct, and the NMPs are eluted from the column using water that has been acidified with CO_2. Although the volume for elution is higher than if a buffer were used, a subsequent desalting step is avoided after concentration of the eluted NMPs. Affi-gel 601 undergoes large (~100%) volume changes upon change of pH and ionic strength, and use of a short, wide column is recommended to avoid reduction of flow rate.

Separation of dNMPs from NMPs is readily monitored by reverse-phase HPLC of dephosphorylated aliquots of the column fractions. NMPs from this procedure are essentially free of dNMPs. The dNMP fraction contains a small (1–2%) amount of NMPs that leak through during the boronate chromatography. Typical HPLC traces of the total nucleotide pool from P1 nuclease digestion input into the boronate chromatography, and the resulting dNMP and NMP pools, are shown in Figure 2. There is no evidence for detectable quantities of modified nucleotides that might be expected from tRNAs.

The chemical and isotopic purity of the ribonucleotides were verified by ^{1}H NMR spectroscopy. Several milligrams of ^{13}C-labeled CMP was purified by HPLC and the ^{1}H NMR spectrum recorded. The H6 proton is split into a doublet by the large (~200 Hz) coupling to ^{13}C6. The isotopic purity of the nucleotides was estimated as 98% by integration of the residual singlet from H6 coupled to a ^{12}C6 (data not shown).

Enzymatic phosphorylation

A general method for preparation of NTPs from cellular RNA has been reported by Whitesides (13). We have employed this method in conversion of isotopically labeled NMPs to NTPs. A series of nucleotide kinases is used to complete the phosphorylation of NMPs to NDPs, and the final phosphorylation to NTP can be effected with either pyruvate kinase or acetate kinase. High concentrations of phosphoenol pyruvate (PEP) or acetyl phosphate (AcP) are used to drive the equilibrium toward NTPs.

Of the several possibilities for the phosphorylation of NDPs, we have settled on pyruvate kinase, where PEP is generated *in situ* from 3-phosphoglycerate using phosphoglycerate mutase and enolase. Both acetyl phosphate and PEP as ultimate phosphate donors give comparable results. We favor use of PEP primarily because a large quantity of precipitate formed during phosphorylations performed with acetate kinase, although phosphorylation still went virtually to completion. Since PEP is expensive compared to the enzymes and substrates required for *in situ* PEP generation, and both methods give comparable results, we favor the *in situ* method.

The enzymatic phosphorylation proceeds in two steps. First, ATP is generated from AMP using adenylate kinase and pyruvate kinase. The reaction is initiated by a catalytic amount (<0.1%) of unlabeled ATP. After several hours, ATP synthesis is complete, and the remaining nucleotide monophosphate kinases are added. The rate of phosphorylation of the remaining

nucleotides is much more rapid when the total nucleotide concentration is kept at or below 10 mM. Some inhibition of the kinases may occur at high nucleotide concentrations. For the conditions we use, complete phosphorylation of all four NMPs to NTPs occurs in 14 hrs. The enzymatic phosphorylation is monitored by ion exchange HPLC on a Vydac nucleotide analysis column. Five timepoints during a typical phosphorylation reaction are shown in Figure 3.

Nucleotide purification

The NTPs from the enzymatic phosphorylation reaction are conveniently desalted and purified using boronate affinity chromatography. The binding of NTPs to the column is weaker than for NMPs, and consequently the elution volume for NTPs is usually smaller. NTPs do hydrolyze at pH 9.5 at an appreciable rate, and it is critical to chill high pH nucleotide solutions on ice at all times. If the total time at high pH is kept to a minimum ($\sim$2 hrs), hydrolysis is $<5\%$. NTPs taken directly from the phosphorylation reaction give no activity in transcription reactions. Desalted nucleotides exhibit increased transcription efficiency, but are still somewhat less efficient than commercial NTPs (*vide infra*). Finally, to ensure that the nucleotides are free from proteins, in particular RNases, the NTPs are filtered through a 10,000 molecular weight cutoff filter.

In vitro transcription

Isotopically labeled NTPs prepared as described were used in large scale *in vitro* transcription reactions using T7 RNA polymerase to prepare milligram quantities of RNAs for NMR studies. A small scale transcription of TAR RNA is shown in Figure 4A using commercial NTPs (lane 1) and ^{13}C-labeled NTPs (lane 2), and the yield of full length transcript is 2–3 fold lower. Reoptimization of the Mg^{++} concentration in the transcription reaction increased the efficiency of the labeled transcription reactions. Some Mg^{++} may be complexed by the NTPs during the boronate affinity chromatography, and therefore, less Mg^{++} is required in the transcription reactions than is typical. Comparison of the transcription efficiency of commercial and isotopic nucleotides under optimized conditions is shown in Figure 4B, and the yields are very similar. Also, consideration must be given to the yield of transcript per mole of input NTP. For the TAR template, the amount of transcript increases only slightly as the labeled NTP concentrations are increased from 1 mM to 4 mM. Thus the optimal production of transcript per mole of NTP is obtained from lower NTP concentrations than are often used in unlabeled transcriptions.

Recycling of nucleotides

For large scale preparations of RNAs for NMR, only 5–10% of the input nucleotides are incorporated into full length transcripts. RNAs from large scale transcriptions are typically purified by preparative electrophoresis. Electrophoresis should be continued until the product RNA band is close to the bottom of the gel, ensuring that most of the abortive transcripts elute from the gel into the bottom buffer tank. The abortive transcripts and unincorporated nucleotides can then be recovered from the large volume of electrophoresis buffer by adsorption to a DEAE chromatography column. Elution of the nucleotides and oligonucleotides from the column is followed by digestion with P1 nuclease. This results in a mixture of NMPs and NTPs that can be completely phosphorylated using the enzymatic procedure.

DISCUSSION

We have developed a method for the preparation of isotopically labeled ribonucleotides, and using these reagents, we have synthesized u-^{13}C-TAR. We have previously investigated the structure of unlabeled TAR using 2D-^{1}H-NMR methods (9). To demonstrate the utility of uniform isotopic labeling of RNA, we have performed a 3D-heteronuclear NMR experiment on u-^{13}C-labeled-TAR.

NMR of u-^{13}C-labeled TAR

Two problems encountered in NMR analysis of RNAs in particular are spectral crowding in the ribose proton region of the spectrum, and unobserved cross-peaks in homonuclear COSY experiments due to small coupling constants. Both of these problems are alleviated by the use of heteronuclear methods. An expansion of the H1′-H2′cross peak region of a DQF-COSY spectrum of TAR is shown in Figure 6a. This region should contain one cross peak from each residue, but only 10 peaks are visible. This results from the small coupling constant between H1′ and H2′ in the C3′-*endo* sugar conformation usually observed in RNA. The only cross peaks usually observed in this region are from sugars in the C2′-*endo* conformation. The same region from two slices of different ^{13}C chemical shifts from a 3D-HCCH-COSY spectrum of u-^{13}C-TAR is shown in Figure 6b,c. In this experiment, the coherence transfer occurs through three large one-bond coupling constants that are independent of the sugar conformation, thus all 31 cross peaks can be observed. In Figure 6b, many cross peaks are visible that are not present in Figure 6a. The dispersion of ^{13}C chemical shifts relieves spectral crowding in proton correlated spectra, as illustrated in Figure 6c, where only one cross peak is observed at that ^{13}C chemical shift.

CONCLUSIONS

We have presented a general and detailed method for the preparation of isotopically labeled ribonucleotides and RNAs. The ability to incorporate isotopic labels into RNA will enable use of powerful heteronuclear 3D- and 4D-NMR methodology for structure determination. These methods have aleady made an important contribution to protein structure, and with the availability of labeled RNAs can now be applied to the rapidly increasing number of interesting RNA structural problems.

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REFERENCES

1. Clore,G.M. and Gronenborn,A.M. (1991) *Prog. NMR Spectrosc.* **23**, 43–92.
2. Ikura,M., Kay,L.E. and Bax,A. (1990) *Biochemistry* **29**, 4659–4667.
3. Clore,G.M., Kay,L.E., Bax,A. and Gronenborn,A.M. (1991) *Biochemistry* **30**, 12–18.

4. Nikonowicz,E.P. and Pardi,A. (1992) *Nature* **355**, 184–186.
5. Nikonowicz,E.P. and Pardi,A. (1992) *J. Am. Chem. Soc.* **114**, 1082–1083.
6. Santoro,J. and King,G.C. (1992) *J. Mag. Reson.* **97**, 202–207.
7. Polson,A.G., Crain,P.F., Pomerantz,S.C., McCloskey,J.A. and Bass,B.L. (1991) *Biochemistry* **30**, 11507–11514.
8. Clore,G.M., Bax,A., Driscoll,P.C., Wingfield,P.T. and Gronenborn,A.M. (1990) *Biochemistry* **29**, 8172–8184.
9. Puglisi,J.D., Tan,R., Calnan,B.J., Frankel,A.D. and Williamson,J.R. (1992) *Science* **257**, 76–80.
10. Beardsmore,A.J , Aperghis,P.N.G. and Quayle,J.R. (1982) *J. Gen. Microb.* **128**, 1423–1439.
11. Long,A.R. and Anthony,C. (1990) *Methods Enzymol.* **188**, 216–222.
12. Hamel,J.F., personal communication.
13. Haynie,S.L. and Whitesides,G.M. (1990) *Appl. Biochem. Biotech.* **23**, 205–220.
14. Lim,C.K and Peters,T.J. (1989) *J. Chromatogr.* **461**, 259–266.
15. Schott,H., Rudloff,E., Schmidt,P., Roychoudhury,R. and Kossel,H. (1973) *Biochemistry* **12**, 932–938.
16. Neidhardt,F.C. (1987) In Neidhardt,F.C. (ed.), *Escherichia coli and Salmonella typhimurium*. American Society for Microbiology, Washington, DC.
17. Simon,E.S., Grabowski,S. and Whitesides,G.M. (1990) *J. Org. Chem.* **55**, 1834–1841.
18. Milligan,J.F., Groebe,D.R., Witherell,G.W. and Uhlenbeck,O.C. (1987) *Nucleic Acids Res.* **15**, 8783–8798.
19. Grodberg,J. and Dunn,J.J. (1988) *J. Bacteriol.* **170**, 1245–1253.
20. Wyatt,J.R , Chastain,M. and Puglisi,J.D. (1991) *Biotechniques* **11**, 764–769.
21. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
22. Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
23. Anthony,C. (1982) *The Biochemistry of Methylotrophs*. Academic Press, London.
24. Stirling,D.I., Eur. Pat. Appl. no. 231585.
25. Byrom,D. and Ousby,J.C. (1975) *Microbial Growth on C1 Compounds*. The Society of Fermentation Technology, Tokyo, Japan.