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Lee, Kai Mon

SOLUTION STRUCTURES OF YEAST RIBOSOMAL 5S AND 5.8S RIBONUCLEIC ACIDS VIA 500 MHZ PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The Ohio State University

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SOLUTION STRUCTURES OF YEAST RIBOSOMAL 5S AND 5.8S RIBONUCLEIC ACIDS VIA 500 MHz PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By
Kai Mon Lee, B.Tech (Hons.), Grad RIC, M.Sc.

* * * * *

The Ohio State University
1986

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Department of Chemistry
To My Dad and Mom
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER I: INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>A. Objectives</td>
<td>1</td>
</tr>
<tr>
<td>B. Ribosomal RNA Molecules</td>
<td>6</td>
</tr>
<tr>
<td>C. 5S rRNA - Implicated in Protein Biosynthesis</td>
<td>7</td>
</tr>
<tr>
<td>1. Mechanism of Biosynthesis of Protein</td>
<td>7</td>
</tr>
<tr>
<td>2. Involvement of 5S in Protein Biosynthesis</td>
<td>16</td>
</tr>
<tr>
<td>D. Proposed Secondary Base-pairing Schemes of Isolated 5S rRNA</td>
<td>26</td>
</tr>
<tr>
<td>E. Review on Structural Information obtained for 5S rRNA</td>
<td>32</td>
</tr>
<tr>
<td>1. Hydrodynamics and X-ray Scattering</td>
<td>34</td>
</tr>
<tr>
<td>2 Optical Studies</td>
<td>36</td>
</tr>
<tr>
<td>3 Raman and Infrared Studies</td>
<td>38</td>
</tr>
<tr>
<td>4. NMR spectroscopy; $^1$H and $^{19}$F</td>
<td>39</td>
</tr>
<tr>
<td>5. Limited Enzymatic Hydrolysis</td>
<td>40</td>
</tr>
<tr>
<td>6. Chemical Modifications</td>
<td>41</td>
</tr>
<tr>
<td>7. Oligonucleotide Binding</td>
<td>42</td>
</tr>
<tr>
<td>F. Overview on 5.8S rRNA</td>
<td>43</td>
</tr>
</tbody>
</table>
### Chapter II: Isolation and Purification of Yeast Ribosomal 5S and 5.8S Ribonucleic Acids

**A. Introduction**

**B. Experimental Procedures**

1. **Isolation of yeast 5S rRNAs**
2. **Isolation of yeast 5.8S rRNA**
3. **DEAE 32 Ion-Exchange Chromatography**
4. **ZetaPrep DEAE Ion Exchange Chromatography - An alternative to Traditional DEAE-Cellulose Chromatography**
5. **Conventional Soft Gel Sephadex G-75 Chromatography**
6. **Sample Purity Check by Polyacrylamide Gel Electrophoresis (PAGE)**
7. **Mini Gel Electrophoresis - a method for rapid screening of rRNAs**

### Chapter III: High-Speed Preparative-Scale Separation and Purification of Ribosomal 5S and 5.8S RNA's via Sephacryl S-300 Gel Filtration Chromatography

**A. Introduction**
B. Materials and Methods ......................................................... 94
  1. Isolation of Yeast 5.8S rRNA .............................................. 94
  2. Fractionation of RNA's via Sephacryl S-300
     Chromatography .............................................................. 94
  3. Fractionation of RNA's via Sephadex G-75
     Chromatography .............................................................. 99
  4. RNA Sample Preparation ................................................. 99
  5. Calibration Proteins Preparation ..................................... 100
C. Results and Discussions ................................................... 100
  1. Demonstration of Asymmetricity of 5S and
     5.8S rRNA Molecules ...................................................... 100
  2. Comparisons between Sephacryl S-300 and
     Sephadex G-75 Chromatography ........................................ 105
E. Conclusions ........................................................................ 110

CHAPTER IV: WATER SUPPRESSION IN FOURIER TRANSFORM NUCLEAR
MAGNETIC RESONANCE AND NUCLEAR OVERHAUSER
ENHANCEMENTS ........................................................................ 112
A. Introduction ......................................................................... 112
B. Redfield 2-1-4 Pulse Sequence ......................................... 119
C. Binomial 1-3-3-1 Hard Pulse Sequence ................................ 123
D. Base Pairs Assignments from Double-Resonance:
   Homonulear Overhauser Enhancements (NOE) ......................... 124
   1. Theory of NOE ................................................................. 126
   2. Base Assignments via NOE ............................................... 132
E. Experimental NMR Procedures ............................................ 135
   1. Redfield 2-1-4 Method ....................................................... 135
   2. Hard 1-3-3-1 Method ....................................................... 136
   3. Homonuclear Overhauser Enhancement ............................ 136
4. NMR Samples without Mg^{2+} ................................................. 138

CHAPTER V: DEMONSTRATION OF THE GC-RICH COMMON ARM IN
YEAST RIBOSOMAL 5.8S RNA VIA 500 MHz PROTON
NUCLEAR MAGNETIC RESONANCE AND OVERHAUSER
ENHANCEMENTS ................................................................. 140

A. Introduction ................................................................. 140

B. Materials and Methods ..................................................... 143
   1. Isolation and Purification of Yeast 5.8S rRNA ................. 143
   2. Preparation NMR Samples ............................................. 143
   3. NMR Spectroscopy ..................................................... 144

C. Results and Discussions .................................................. 144
   1. Absence of 5.8S rRNA Multimers in NMR samples .......... 144
   2. Multi-stage Unfolding of 5.8S RNA .............................. 149
   3. Calculated Thermal Stabilities of 5.8S rRNA
      Base-paired Helical segments ...................................... 151
   4. Identification of GC-rich arm Base Pairs from
      High-temperature Proton NMR .................................... 153
   5. Assignment of G-C-rich arm base pairs from
      temperature-dependence proton NMR spectra ................... 156
   6. Base-pair Sequencing of the GC-rich Arm via
      Proton NOE Connectivity .......................................... 159

CHAPTER VI: A NOVEL METHOD FOR BASE-PAIR ASSIGNMENTS VIA
SPECIFIC PROTON NMR LINE BROADENING IN
MORPHOLINO-SPINLABELLED RIBOSOMAL 5S RNA AND 500
MHz PROTON HOMONUCLEAR OVERHAUSER ENHANCEMENTS ...... 169

A. Introduction ................................................................. 169

B. Materials & Methods ..................................................... 172
   1. Isolation and purification of yeast 5.8S rRNA .............. 172
   2. Spin-labelling of yeast 5S rRNA with TEMPO-NH2
      (Morpholino spin label) ............................................ 172
3. NMR Samples .................................................. 174
4. EPR Spectroscopy ........................................... 175
5. NMR Spectroscopy ........................................... 175

C. Results and Discussions ................................. 175

1. Identification of the Terminal Base-Pairs from Specific Proton Resonance Broadening on spin-labelled yeast 5S rRNA ................................. 178
2. Confirmation of the assigned terminal base pairs G₁•C₁₂₀ and G₂•U₁₁⁹ by ¹H NOE ................................. 181
3. Base-Pair Sequencing of the Terminal Helix I via Proton NOE Connectivity ................................. 185
4. Identification of G•U Base Pairs .......................... 189
5. Heat-induced melting of yeast 5S ribosomal RNA ...... 192

D. Conclusions .................................................. 194

LIST OF REFERENCES ............................................. 196

x
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Relative sizes of various ribosomal RNAs</td>
<td>4</td>
</tr>
<tr>
<td>Table 2</td>
<td>Reconstitution of 50S ribosomal subunits with different 5S RNA (From Erdmann, 1976)</td>
<td>20</td>
</tr>
<tr>
<td>Table 3</td>
<td>PAGE Stock Solutions</td>
<td>80</td>
</tr>
<tr>
<td>Table 4</td>
<td>Reagents for casting of PAGE slab gels</td>
<td>82</td>
</tr>
<tr>
<td>Table 5</td>
<td>Maximum nuclear Overhauser effects for several pairs of nucleic (Martin et al., 1980)</td>
<td>130</td>
</tr>
<tr>
<td>Table 6</td>
<td>Free energies of stability for individual helices computed from Tinoco rules, Gralla &amp; Crothers, 1973 for three proposed secondary structures of yeast 5.8S ribosomal RNA</td>
<td>154</td>
</tr>
<tr>
<td>Table 7</td>
<td>Assignments for imino proton resonances from the GC-rich arm of yeast 5.8S rRNA, and approximate (+5°C) temperature for onset of melting for that base pair</td>
<td>165</td>
</tr>
<tr>
<td>Table 8</td>
<td>Free energies of stability for individual helices computed from Tinoco rules, Gralla &amp; Crothers, 1973 for three proposed secondary structures of yeast 5S ribosomal RNA</td>
<td>179</td>
</tr>
<tr>
<td>Table 9</td>
<td>Assignments for imino proton resonances from the terminal helix of yeast 5S rRNA, and approximate (+5°C) temperature for onset of melting for that base pair</td>
<td>193</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1: A diagrammatic representation of a prokaryotic ribosome showing all of its major components. [Eukaryotic components are shown in brackets]. From Erdmann et al (1980) ........................................... 2

Figure 2: Nucleotide base pairs commonly occur in RNA, showing the conventional Watson-Crick pairs: (A) The A-U pair; (B) The G-C pair; (C) The non-conventional "wobble" pairing: The G-U pair ......................................................... 8

Figure 3: Schematic diagram of the three-dimensional structure of yeast tRNA^Phe showing the major base pairs, helical stacked, unstacked regions and hairpin loops. From Stryer (1981) ................................................. 9

Figure 4: A schematic view of the initiation process. The formation of the initiation complex and the fully functional 70S ribosome of E. coli. IF-1, IF-2, and IF-3 are the initiation factor proteins. From Lehninger (1975) .................. 12

Figure 5: A schematic view of the elongation process. Protein elongation is shown in the left. EF-T and EF-G are the elongation factors. The right shows the details of the peptidyl transferase reaction, which occurs during elongation. From Lehninger (1975) .................... 14

Figure 6: The location of both 5S and 23S rRNA in ribosome. Top: Two-dimensional view of the ribosome. Bottom: Three-representation of the ribosome and the location of 5S and 23S rRNA. From Tischendorf et al., 1974 ............... 17
Figure 7: The secondary structures for prokaryotic (E. coli) 5S rRNA (left), generalised tRNA (middle), and eukaryotic (yeast) 5S rRNA (right) showing G-A-A-C sequence in prokaryotic 5S rRNA and complementary G-T-U-C sequence in loop IV of most tRNA. Eukaryotic 5S rRNAs contain C-Y-G-A-U sequence .................................................. 21

Figure 8: Proposed secondary structural models for prokaryotic 5S rRNAs. Escherichia coli 5S rRNA models: a, b, c at room temperature, d above 50°C, e, f, g, h, i, k and m. Pseudomonas fluorescens 5S rRNA models: j and l. Filled circles represent the conserved bases in prokaryotic 5S rRNA. From Erdmann (1976) ........................................... 27

Figure 9: Proposed secondary structural models for eukaryotic 5S rRNAs and yeast 5.8S rRNA. KB cell 5S rRNA models: a, b and c. Xenopus laevis 5S rRNA model d, yeast 5S rRNA model e and Chlorella cytoplasmic 5S rRNA model f. A 5.8S rRNA model is shown in g. Filled circles represent the conserved bases in eukaryotic 5S rRNA (a-f). From Erdmann (1976) ........................................... 28

Figure 10: The most popular proposed secondary structural models for 5S rRNA showing similarities and differences ........................................... 30

Figure 11: This model is redrawn from the Fox & Woese model for the secondary structure of 5S rRNA (S. carlsbergensis b). From Luehrsen & Fox (1981) ........................................... 33

Figure 12: The various experimental techniques to probe the secondary structure of 5S and 5.8S rRNAs ........................................... 35

Figure 13: A schematic representation of the location of base-pairing interactions between the 3'-end and 5'-end of mouse 5.8S rRNA with 28S rRNA. From Walker & Pace, 1983 ................. 51

Figure 14: Various proposals for the 5.8S-28S rRNA interactions. From Walker et al., (1983) ................. 53
Figure 15: The three most common proposals for the secondary structure of yeast 5.8S rRNA .............. 55

Figure 16: A flow-diagram of the isolation of procedure for extraction and purification of 5S and 5.8S rRNAs from whole yeast cells ......................................................... 64

Figure 17: A typical DEAE anion-exchange elution profile for the phenol extract of whole yeast cells. The early peak contains proteins, SDS, polysaccharides and nucleotides. The later peak contains a mixture of tRNAs, 5S rRNA and 5.8S rRNA .......... 70

Figure 18: A: A schematic view of the ZetaPrep cartridge construction (From Hou & Mandaro, 1986). B and C: Cartridge flow patterns. Buffers flow through the cartridge by entering the outside cylindrical surface area, penetrating through the spirally wrapped layers to the center core, and then flow out the top side of the cartridge and plastic housing .................................................. 72

Figure 19: A typical elution profile obtained from ZetaPrep DEAE anion-exchange chromatography .................................................... 74

Figure 20: A complete elution profile of yeast rRNA extracted at room temperature from Sephadex G-75 column (150 cm x 5 cm). The rRNA sample was eluted with 10 mM Tris-HCl buffer, pH 7.5 containing 1.0 M NaCl ..................... 78

Figure 21: A complete elution profile of yeast rRNA extracted at 40 to 45°C from Sephadex G-75 column (150 cm x 5 cm). The rRNA sample was eluted with 10 mM Tris-HCl buffer, pH 7.5 containing 1.0 M NaCl ..................... 79

Figure 22: A typical gel scan profile of the polyacrylamide slab gel electrophoresis (14 cm x 16 cm) of 5.8S rRNA purified from Sephadex G-75 chromatography. Purity > 98% ........... 85
Figure 23: Top: A typical gel scan profile of the mini PAGE format (7 cm x 8 cm) electrophoresis of tRNA, 5S rRNA and 5.8S rRNA showing that the three rRNAs were well-resolved from each other. Bottom: Yeast 5S rRNA purified from Sephadex G-75 chromatography. Purity > 98% .......................... 88

Figure 24: A schematic diagram of the LC set-up for Sephacryl S-300 gel filtration chromatography .............................................. 96

Figure 25: A column set-up for packing of 80 cm x 3.2 cm Sephacryl S-300 column .............................. 98

Figure 26: Elution profiles for calibration proteins on 80 cm x 3.2 cm Sephacryl S-300 column under different sample loads and flow rates. Sample loads: 85 mg (Top); 133 mg (Middle) 140 mg (Bottom) ............................ 101

Figure 27: Calibration Curve using the calibration proteins on Sephacryl S-300 ......................... 103

Figure 28: Elution profiles of S. carlsbergensis RNA extracted from whole cells at 40-45°C. Top: Column: 78 cm x 3.2 cm Sephacryl S-300; sample load: 247 mg in 13ml eluent buffer; mobile phase: 10 mM Tris-HCl buffer (pH 7.5, 1.0 M NaCl); Flow-rate: 15 ml/hr; Detection: UV 254 nm; temperature: ambient. Bottom: Column: 144 cm x 5 cm Sephadex G-75; sample: 259 mg in 40 ml eluent buffer; mobile phase: same as above; flow-rate: 30 ml/hr; detection: UV 254 nm; temperature: ambient .................... 107

Figure 29: Gel scans of PAGE on 5.8S rRNA purified from 144 cm x 5 cm Sephadex column (Top), and 80 cm x 3.2 cm Sephacryl S-300 column (Bottom). Purity of 5.8S rRNA > 98% for both columns .................................................. 109

Figure 30: Ring current effects. Top: Induced field ring-current shift effects in stacked nucleotides and their distance. Bottom: The differential upfield shifts on A-U and G-C base pairs in different stacking environments. From Reid et al., (1979) ............. 114
Figure 31: A typical low-field 5S rRNA NMR spectrum in water showing the resonance positions of A·U, G·C and G·U pairs, the ring carbon amino protons (NH2) and the ribose protons ............................................. 116

Figure 32: The A·U, G·C and G·U base pair geometry and the protons which are closest to the imino protons. From Early et al., (1981) ......................... 117

Figure 33: Top: The relation of duration (time) of pulse to width of excitation (frequency). Bottom: The single soft long pulse method for suppression of the intense water signal .................................................. 120

Figure 34: Redfield 2-1-4 pulse sequence. A: A single soft long pulse with a total pulse length of 10t in the time domain and its FT into frequency domain. B: Two 180°C phase-shifted pulses and its FT. C: The resulting 2-1-4 pulse from the addition of pulses in A and in B and its FT. D: Comparison between a single soft long pulse and the Redfield 2-1-4 pulse ......................... 122

Figure 35: Excitation spectrum of the binomial hard 1-3-3-1 pulse sequence. From Hore (1983) ................. 125

Figure 36: An energy level diagram of a two spin system consisting of two nuclear spins, I and S. The subscripts, α and β denote the alignment of the spins against and in the direction of the applied magnetic field respectively. The W terms refer to the transition probabilities between specified energy levels. From Smith (1980) ......................... 127

Figure 37: Plot of the homonuclear NOE in a two-spin system, fI(S) versus log(ωτC). ω denotes NMR larmor frequency, and τC is the molecular correlation time. From Rothner-By (1979) ................................. 131
Figure 38: Characteristic NOE patterns for different base pairs, A-U, G-C, and G-U pairs. Top: NOE difference spectrum of a G.C base pair with broad resonances from the aromatic amino protons in the -6 to -9 ppm region. Second to the top: NOE difference spectrum of an A-U pair which exhibits a narrow resonance from C2-H proton of adenine at about -7 to -8 ppm region. Bottom two: NOE difference spectra of a G-U pair which exhibit two large mutual NOEs with the enhancement produced by irradiation of G imino proton larger that that by irradiation of the U imino proton

Figure 39: Rubin proposed the first secondary structural model for an eukaryotic 5.8S rRNA (S. cerevisae)

Figure 40: The secondary structure of yeast 5.8S rRNA adapted from the model proposed by Nazar, Sitz, and Bush (1975) for 5.8S rRNA based on limited ribonuclease digestion on Novikoff hepatoma 5.8S rRNA

Figure 41: The cloverleaf model derived from laser Raman studies on yeast 5.8S rRNA, and was then adapted to mammalian sequence (Luoma & Marshall, 1978)

Figure 42: 500 MHz proton NMR spectra of yeast 5.8S rRNA at two RNA concentrations. Top: 56 mg/ml. Bottom: 31 mg/ml. All NMR samples contained 95%/5% H2O/D2O, 100 mM NaCl, 1 mM sodium EDTA, 10 mM sodium cacodylate, pH 7.0

Figure 43: 500 MHz proton NMR spectra of yeast 5.8S rRNA at several temperatures

Figure 44: Reversibility of yeast 5.8S rRNA heat-induced unfolding. Top: Initial spectrum at 30°C. Middle: Spectrum for sample which had been heated to 91°C for ca. 30 min and then allowed to cool to 31°C. Bottom: Spectrum for sample which had been maintained at 61°C for 48 hr and then allowed to cool to 30°C
Figure 45. 500 MHz proton NMR difference spectra between yeast 5.8S rRNA spectra acquired at different temperatures. Top: room-temperature spectrum. Middle: Difference spectrum between 61°C and 71°C. Bottom: Difference spectrum between 71°C and 81°C. A negative peak in the difference spectrum corresponds to a resonance which melts away in proceeding from the lower to the higher temperature .......... 158

Figure 46. Proton 500 MHz FT/NMR spectrum (a) and proton homonuclear Overhauser difference spectra (b-f) resulting from irradiation of peaks J, L, B, H, and G at 22°C. In each case, the irradiated peak is denoted by a star (*), and decoupler power is shown at the right ................................................. 160

Figure 47: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, produced as in Figure 46, for peaks E1 and F .......... 162

Figure 48. 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, produced as in Figure 46, for peaks D and C ....... 163

Figure 49: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, for peaks E, F, and C, produced as in Figure 46 but at 55°C ................................................. 166

Figure 50: Reaction scheme for spin-labelling of yeast 5S rRNA with paramagnetic morpholino spin-label (TERMPO-NH2) ................................. 173

Figure 51: EPR spectra of free morpholino spin-label (Top) and the spin-labelled yeast 5S rRNA (bottom) .......................................................... 176

Figure 52: Three proposed secondary base-pairing schemes for 5S rRNA, each adapted to the primary sequence for Saccharomyces carlbergensis ............................................. 177

Figure 53: 500 MHz proton NMR spectra of yeast 5S rRNA at several temperatures .......................... 180
Figure 54: Proton 500 MHz FT/NMR spectra of yeast 5S rRNA in 10 mM Tris-HCl, pH 7.5 buffer containing 100 mM NaCl and 1 mM EDTA at room temperature. Top: Morpholino spin-labelled yeast 5S rRNA. Bottom: Unlabelled yeast 5S rRNA. The arrow indicates resonances where paramagnetic broadening occurred ........................................ 182

Figure 55: Proton 500 MHz FT/NMR spectrum (a) and proton homonuclear Overhauser difference spectra (b-f) resulting from irradiation of peaks L, K, F, A, and C at 22°C. In each case, the irradiated peak is denoted by a star and decoupler power is shown at the right ................................................................. 184

Figure 56: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5S rRNA, produced as in Figure 55, for peaks H, B, P, K, and M ......................................................... 187

Figure 57: The effect of resolution enhancement by negative line-broadening (denoted by LB) on the proton 500 MHz NMR spectrum of yeast 5S rRNA. The same data set was used but enhanced by different values of LB. Top: Resonances B and K resolved into two components ......................................................... 188

Figure 58: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5S rRNA, produced as in Figure 55, for peaks M, N and B1 ................................................................. 190
CHAPTER I
INTRODUCTION

A. Objectives

The ribosome is an extremely vital organelle of all living cells. It functions as a factory for the synthesis of proteins which is a very complex process. Therefore an understanding of the structure and functions of the ribosome is very important to the understanding of the mechanism of biosynthesis of proteins at the molecular and cellular level. However, a ribosome is a highly specialized and complex structure to study by virtually any spectroscopic techniques. For example, the Escherichia coli (E. coli) (prokaryote) ribosomes consist of the two subunits, 50S and 30S (Tissieres & Watson, 1958). And the 50S subunits are found to consist 23S and 5S ribosomal ribonucleic acids (rRNA) and about 34 species of ribosomal proteins, whereas the 30S subunits consist of a 16S rRNA and about 21 species of proteins as shown in Figure 1 (Brimacombe et al., 1976; Erdmann et al, 1980). The ribosomes in the cytoplasm of eukaryotic cells (for example, yeast) are somewhat larger than those of bacteria, and contain at least 70 proteins and 8 rRNA's (Beilka, 1982).

The process of protein synthesis as occurs in the ribosomes is quite well understood through mapping techniques like immune electron
Figure 1: A diagrammatic representation of a prokaryotic ribosome showing all of its major components. [Eukaryotic components are shown in brackets]. From Erdmann et al (1980).
microscopy, neutron diffraction, chemical cross-linking (Lake, 1985) which allows one to map specific ribosomal protein and RNA components and to determine their locations in three dimension. However, the detailed structure and functions of the individual ribosomal components have yet to be established with the exception of tRNA's. The object of the present research investigation is to isolate these individual ribosomal RNA's (rRNA) and then elucidate their secondary structures via high resolution Fourier transform 500 MHz proton nuclear magnetic resonance (FT $^1$H NMR) so as to provide a better understanding of the precise role of the individual ribosomal RNA components in protein biosynthesis at the molecular level and as well at the cellular level.

Cells contain three kinds of RNA (Table 1). Messenger RNA (mRNA) is the template for protein synthesis. There is an mRNA molecule corresponding to each gene or group of genes that is being expressed. Consequently, mRNA is a very heterogenous class of molecules. Transfer RNA (tRNA) carries amino acids in an activated form to the ribosome for peptide-bond formation, in a sequence determined by the mRNA template. There is at least one kind of tRNA for each of the twenty amino acids. Table 1 shows the relative sizes of ribosomal RNA molecules. Transfer RNA molecules are approximately 75-80 nucleotides long while 23S rRNA, 16S rRNA, 5S rRNA, and 5.8S rRNA are about 3700, 1700, 120, and 160 nucleotides long respectively. Hence tRNA is the smallest of all the RNA's in the ribosome. Although ribosomal RNA (rRNA) is the major component of ribosomes, its precise role in
### Table 1. Relative sizes of various ribosomal RNAs

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protein synthesis is not yet known. Ribosomal RNA is the most abundant of the three types of RNA. Transfer RNA comes next, followed by messenger RNA, which comprises only 5% of the total RNA. In *E. coli* (prokaryote) ribosome, there are three kinds of rRNA, called 23S, 16S, and 5S rRNA because of their sedimentation behavior. 5.8S rRNA is known to exist only in eukaryotic ribosomes.

Yeast 5S and 5.8S ribosomal ribonucleic acids (rRNA's) are chosen for the present investigation for several reasons: (a) ribosomes typically contain 50 to 60 percent RNA as an integral part of their structures. It is an intriguing problem to understand why this is so, and its elucidation is hence important to the understanding of the structure, function, assembly, and evolution of these ribosomal particles; (b) the three-dimensional structure of the smallest RNA, tRNA has been solved by X-ray crystallography (Rich, 1977; Kim, 1976), and its function is quite well characterized; (c) the next smallest rRNAs are 5S (molecular weight ca. 36,000 daltons) and 5.8S (55,000 daltons; Table 1) whose structures and functions remain unknown, but numerous studies have implicated them to involve in protein synthesis (Erdman, 1976). The relative small size of 5S and 5.8S rRNA molecules have rendered them a good candidate for studies on both ribosomal RNA secondary structure and the chemistry and specificity of protein-RNA interaction; (d) 16S and 23S are too large to be studied by virtually any spectroscopic techniques; (e) preliminary single crystals of 5S rRNA have been reported (Abdel-Meguid, 1983; Morikawa, 1982) but the crystals were of too poor quality to yield useful X-ray structural
information. There is yet report on successful crystallization of 5.8S rRNA. Development of proper conditions for growth of single crystals of 5S and 5.8S rRNAs is another goal in this study; (f) the three-dimensional picture provided from X-ray studies is a static one. For example a single crystal of tRNA^Phe molecules used to obtain electron density maps for structural analysis is about 75% water. Therefore there is a continuing need to carry out studies in a more native aqueous environment in order to substantiate similarities between the crystal structures (not known at the present time for 5S and 5.8S rRNA) and solution structure; (g) since 5.8S rRNA is only associated with eukaryotic ribosomes, yeast being an eukaryote is chosen for the present study. This work represents the first NMR study on any 5.8S rRNA since there is no report of any NMR investigation on 5.8S rRNA, (h) it is generally believed that a universal secondary structure exists for 5S rRNA (Brimacombe & Stiege, 1985; Noller, 1984; Woese et al., 1983; Erdman, 1976). It is interesting to determine whether a universal model exists for 5.8S rRNA as well (Nazar, 1982; Mackay et al., 1982; Erdman, 1976).

B. Ribosomal RNA Molecules

RNA molecules are ribonucleotide polymers constituted of heterocyclic bases adenine (A), guanine (G), uracil (U), cytosine (C), and a small number of modified bases. The primary structure consists of specific sequences of these bases that are interconnected through phosphodiester linkages between 3' and 5' hydroxyls of the ribose moieties. These molecules fold into specific secondary
structures in accordance with the directional hydrogen properties of the individual bases. The most common base pairs in RNA molecules are G•C, A•U, and G•U pairs which are shown in Figure 2. The most stable base pair is the G•C pair which consists of three hydrogen bonds while the A•U and G•C pairs have only two hydrogen bonds.

RNA molecules are single stranded, except in some viruses. Consequently, an RNA molecule needs not have complementary base ratios. In fact RNA sequencing has shown that in most RNA, the proportion of uracil differs from adenine, and the proportion of guanine differs from cytosine. Detailed X-ray analysis of tRNA^Phe indeed has revealed double-helical structures can exist in RNA (Rich, 1977; Kim, 1976) that are produced by the formation of hairpin loops (Figure 3). In these double-helical regions, A forms a complementary pair with U, and G with C. In addition, G can also form a base pair with U, but it is less strong than the G•C pair. Unlike deoxyribonucleic acid (DNA) which is molecule of heredity, the base pairing of RNA hairpin loops is often imperfect. Some of the opposing bases may not be complementary, and one or more bases along a single strand may be looped out to facilitate the pairing of the other. The proportion of helical regions of different kinds of RNA varies over a wide range; a value of 50% is typical.

C. 5S rRNA - Implicated in Protein Biosynthesis

1. Mechanism of Biosynthesis of Protein

The biosynthesis of proteins or translation is the mRNA-directed assembly of amino-acids into polypeptides, that is, proteins.
Figure 2: Nucleotide base pairs commonly occur in RNA, showing the conventional Watson-Crick pairs: (A) The A-U pair; (B) The G-C pair; (C) The non-conventional "wobble" pairing: The G-U pair.
Figure 3: Schematic diagram of the three-dimensional structure of yeast tRNA\(^\text{Phe}\) showing the major base pairs, helical stacked, unstacked regions and hairpin loops. From Stryer (1981).
Messenger RNAs convey the genetic information of DNA, and are the information-carrying link between gene and protein. In addition to mRNA, two other types of RNA namely tRNA and rRNA are involved in the process of protein synthesis.

The overall events of protein synthesis described here is for *E. coli* ribosome (prokaryotic) since they are more intensively studied because of the simplicity of *E. coli*. There is now general consensus concerning the overall structure and morphology of the *E. coli* ribosome (Lake, 1985). In spite of the greater complexity of eukaryotic as compared to prokaryotic ribosomes, many of the general properties and functions are similar in both. The significant differences between prokaryotes and eukaryotes relate to the nature of the mRNAs. In the case of prokaryotes, genes are usually grouped into operons such that transcription (synthesis of RNA from a DNA template) of these operons produces polycistronic mRNAs. Experimental results by Imamoto (1973), and Hamkalo and Miller (1973) showed that the synthesis of the first protein coded for by polycistronic mRNA can be completed before transcription is complete because translation (synthesis of a protein from an RNA template) can commence during their transcription. Eukaryotic chromosomes are bounded by a nuclear membrane which is absent in prokaryotes. Consequently, transcription and translation occur in separate cellular compartments in eukaryotes, namely the nucleus and the cytoplasm, whereas these two processes are closely coupled in prokaryotes.

The *E. coli* (prokaryote) ribosome has a mass of 2,500 kdaltons and has a sedimentation coefficient of 70S. The 70S ribosome
undergoes continual association and dissociation into 50S and 30S subunits during the biosynthesis of proteins. In contrast, an intact eukaryotic ribosome (for example, yeast) sedimenting at 80S. Similar to a prokaryotic ribosome, the 80S ribosome is composed of a large subunit (60S) and a small subunit (40S). The overall machinery of protein biosynthesis involves three basic steps: initiation, elongation, and termination.

(a) Initiation

Initiation process is activated by the dissociation of an inactive 70S ribosome into its two constituent subunits, namely, 50S and 30S. This is followed by the binding of three initiation protein factors called IF-1, IF-2, and IF-3 (Figure 4). Of these three factors, IF-3 is the first to bind to a 30S subunit to form a 30S:IF-3 complex. The formation of this 30S:IF-3 complex appears to prepare the subunit to receive mRNA, which next binds to this complex. To this complex (mRNA:30S:IF-3), is added IF-1, IF-2, guanosine 5'-triphosphate (GTP), and initiator N-formyl-methionyl-tRNA (fMet-tRNA_f in E. coli) to form a 30S initiation complex. IF-3 prevents the 50S and 30S subunits from coming together to form an inactive 70S complex. The function of the other two protein factors is to enhance the binding of fMet-tRNA_f to the mRNA:30S:IF-3 subunit.

The next step is the binding of a 50S ribosomal subunit to the 30S initiation complex to form a 70S initiation complex results in the release of IF-1, IF-2, and IF-3 while the bound GTP is hydrolyzed to guanosine 5'-diphosphate (GDP). The 70S initiation complex is ready
Figure 4: A schematic view of the initiation process. The formation of the initiation complex and the fully functional 70S ribosome of E. coli. IF-1, IF-2, and IF-3 are the initiation factor proteins. From Lehninger (1975).
to commence peptide bond synthesis and polypeptide chain elongation (for reviews, see Lucas-Lenard & Lipman, 1971; Weissbach & Brot, 1974; Leder, 1973; Noller, 1984; Lake, 1985).

(b) Elongation

Following the formation of the 70S initiation complex, fMet-tRNA\(_f\) molecule occupies the P (peptidyl or "donor") site on the 50S subunit and the A (aminoacyl or "acceptor") site is free for occupation by an aminoacyl-tRNA (charged tRNA) (Figure 5). The elongation cycle is comprised of three basic steps: (a) binding of aminoacyl-tRNA (codon) to the A-site of the ribosome recognition, (b) peptide-bond formation between the amino group of the amino acid attached to the incoming aminoacyl-tRNA at the A-site and the carboxyl group of the initiator-tRNA's amino acid (fMet-tRNA\(_f\) in E. coli) at the P-site; (c) translocation of the newly extended peptidyl-tRNA from the A-site to the P-site with the concomitant movement of mRNA so that the next triplet codon is in the proximity to the A-site (figure 5). During this cycle, protein elongation factors, EF-G, EF-Tu, and EF-Ts play an important role. The Tu and Ts abbreviations refer to heat-unstable and heat-stable components of the EF-T complex, respectively.

The first step in the elongation process is the formation of a ternary complex between the incoming aminoacylated tRNA, EF-Tu, and GTP, analogous to the ternary initiation complex between IF-2, GTP, and fMet-tRNA\(_f\) (Figure 5). The complex so produced then binds to the ribosomal A-site with subsequent hydrolysis of GTP resulting in the release of EF-Tu:GDP. The released EF-Tu:GDP is the reconverted to
Elongation

Peptidyl Transferase Reaction

Figure 5: A schematic view of the elongation process. Protein elongation is shown on the left. EF-T and EF-G are the elongation factors. The right shows the details of the peptidyl transferase reaction, which occurs during elongation. From Lehninger (1975).
EF-Tu:GTP by the action of another elongation factor, EF-Ts, which may interact once more with another amino-acylated tRNA.

After the amino-acylated tRNA is bound to the ribosomal A-site, the enzyme called peptidyl transferase catalyzes the peptide bond formation between the free amino group of the incoming amino acid at the A-site and the esterified carboxyl group of peptidyl-tRNA or fMet-tRNA\textsubscript{f} located at the P-site.

The last step in the elongation process is the release of the deamino-acylated tRNA from the A-site and the transfer peptidyl-tRNA (the peptide bound tRNA and mRNA) to the P-site. This shift creates another three base codon to be exposed in the A-site so that the next incoming tRNA can bind to it, thus repeating the whole elongation process. This movement of the ribosome complex along the mRNA is known as translocation. The elongation factor EF-G, which has ribosome-dependent GTPase activity, is necessary for translocation and binds to the 50S ribosomal subunit.

The polypeptide will continue to grow by repetition of the above two steps until the whole protein is completed and the mRNA codon for termination is signaled.

(c) Termination

The elongation cycle is terminated by the appearance of any of three base triplets (termination codon) UAG, UAA, and UGA in the mRNA. Normal cells do not contain tRNAs with anticodons complementary to the termination codons in mRNA to stop these termination signal. These termination codons were first discovered by studies of the genetic
code in bacteria, which indicated that none of them specified an amino acid. Instead these stop signals are recognized by three proteins called release factors, RF 1, RF 2, and RF 3 (Beaudet & Caskey, 1972). RF 1 being more specific for UAA and UAG while RF 2 recognizes UAA and UGA. In the presence of GTP, RF 3 stimulates the binding and release of RF 1 and RF 2.

Termination involves the hydrolysis of the ester bond between the synthesized polypeptide chain and tRNA in the P-site, which is catalyzed by peptidyl transferase whose catalytic specificity is altered by the release factors. The breaking of the ester bond resulting the release of the synthesized protein from the ribosome. The last step is the leaving of tRNA and mRNA from the 70S ribosome which in turn dissociates into its active constituent 30S and 50S subunits as the prelude to the synthesis of another protein molecule. The order in which specific amino-acylated tRNA binds to the A-site in the ribosome determines the amino acid sequence of a protein being synthesized. This is in turn dependent on the sequence of the base triplets of the codon in mRNA. There is at least one kind of tRNA for each of the twenty amino acids. Each tRNA specific for a particular amino acid has an anticodon loop containing three bases which recognizes the complementary triplet codon of mRNA.

2. Involvement of 5S in Protein Biosynthesis

As depicted in Figure 6, a single 5S RNA molecule is located at the center in the groove of the large ribosomal subunit, 50S particle. Erdmann, Fahnestock, Higo, and Nomura (1971) for the first time showed
Figure 6: The location of both 5S and 23S rRNA in ribosome. Top: Two-dimensional view of the ribosome. Bottom: Three-representation of the ribosome and the location of 5S and 23S rRNA. From Tischendorf et al., 1974.
that it is possible to reconstitute functional 50S ribosomal subunits of *Bacillus stearothermophilus* (*B. stearothermophilus*) from their dissociated molecular components. However the reconstituted particle without 5S rRNA displayed greatly reduced activities in (a) polypeptide synthesis directed by mRNA, (b) a poly(U)-dependent synthesis of polyphenylalanine, (c) peptidy transferase activity, (d) the ability to bind the G-factor and GTP, (e) the ability to bind the chain termination codon (triplet), UAA, in the presence of a soluble chain termination factor, RF 1, (d) the ability to participate in poly(U)-dependent nonenzymatic binding of Phe-tRNA. Similar findings from total reconstitution of the 50S subunit from *E. coli* were reported by Dohme and Nierhaus (1976). These reconstitution experiments clearly demonstrate that 5S rRNA is essential for the overall activity of the 50S subunits in protein synthesis, but give little information concerning possible specific functions of 5S rRNA.

Studies have been carried out by total reconstitution of *B. stearothermophilus* (Nomura & Erdman, 1970; Werde & Erdman, 1973) and partial reconstitution of *E. coli* 50S ribosomal subunits with different prokaryotic and eukaryotic 5S rRNAs. Several interesting and important conclusions (Table 2) can be inferred from these experiments: (a) Table 2 show that it is possible to incorporate all prokaryotic 5S rRNAs into prokaryotic 50S ribosomal subunits thus suggesting that the protein sites of those rRNAs are conserved through evolution (Nomura & Erdmann, 1970; Werde & Erdmann, 1973; Hosokawa, 1970; Bellemare et al., 1973), (b) the functional parts of these 5S
rRNAs must also be conserved since reconstitution of 5S rRNAs into B. stearothermophilus yields functional ribosomal particles (Werde & Erdmann, 1973), (c) it is clear from Table 2 that none of the eukaryotic 5S rRNAs can be incorporated into prokaryotic ribosomes (Werde & Erdmann, 1973; Bellemare et al., 1973) indicating differences between prokaryotic and eukaryotic 5S rRNAs are sufficiently significant, for example, to prevent any cross recognition between E. coli 50S subunits and eukaryotic 5S rRNAs (Bellemare et al., 1973)

Early sequence studies of 5S rRNA subsequently lead to the hypothesis that 5S rRNA could play a role in the binding of tRNA to the ribosome by providing a complementary G-A-A-C sequence to the G-T-V-C sequence which is found in loop IV of most tRNA (Figure 7; Forget & Weissman, 1967; Brownlee et al., 1968). In fact, the loop of all prokaryotic tRNAs (Figure 7) participating in protein synthesis contains a G-T-V-C sequence, except eukaryotic initiator and fMet-tRNAf which has a A in place of the 3'-terminal G (Dube et al., 1969). This sequence is matched in one region of the prokaryotic 5S rRNA (for example, E. coli, B. stearothermophilus, Ps. fluorescens) where one of the G-A-A-C sequences has an adjacent C residues at the 5'-end. No such situation exists however in the eukaryotic 5S rRNA. All eukaryotic 5S rRNAs sequenced so far do not possess the sequence G-A-A-C at a specific site but instead the conserved sequence C-Y-G-A-U which is complementary to the G-C-U-A sequence of loop IV found only in eukaryotic initiator tRNAs (Barell & Clark, 1974; Erdmann, 1976). Therefore, it has been concluded that in eukaryotes
Table 2. Reconstitution of 50S ribosomal subunits with different 5S RNA (From Erdmann, 1976)

<table>
<thead>
<tr>
<th>Source of 5S RNA</th>
<th>B. stearothermophilus</th>
<th>E. coli</th>
</tr>
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<tbody>
<tr>
<td>Escherichia coli</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. coli denatured B form</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Bacillus stearothermophilus</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Micrococcus lysodeikticus</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Azobacter vinelandii</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Halobacteria cutirubrum</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>Chorella chloroplast</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Chorella cytoplasmic</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Yeast</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bean</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Wheat germ</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Artemia salina</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Xenopus laevis oocyte</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>HeLa cell</td>
<td>NT</td>
<td>-</td>
</tr>
<tr>
<td>Rat liver</td>
<td>-</td>
<td>NT</td>
</tr>
<tr>
<td>Horse liver</td>
<td>-</td>
<td>NT</td>
</tr>
</tbody>
</table>

A plus sign (+) indicates that 5S RNA is incorporated and a minus sign (-) that is not; NT indicates that this 5S RNA was not tested.
Figure 7: The secondary structures for prokaryotic (E. coli) 5S rRNA (left), generalised tRNA (middle), and eukaryotic (yeast) 5S rRNA (right) showing G-A-A-C sequence in prokaryotic 5S rRNA and complementary G-T-U-C sequence in loop IV of most tRNA. Eukaryotic 5S rRNAs contain C-Y-G-A-U sequence.
the 5S rRNA is involved in initiation of protein synthesis by interacting with fMet-tRNAf and thus should be located at the P-site of the large ribosomal subunit. The proposed role of 5S rRNA in eukaryotes is supported by the fact is the transcript of a special gene of eukaryotic cells, not present in prokaryotes. For prokaryote 5S rRNA, the proposed interaction is specific for tRNA to the A-site (Dohme & Nierhaus, 1976).

Several experimental evidences in favor of the hypothesis have been reported. It has been shown that the binding of the tetranucleotide T-Ψ-C-G to prokaryotic ribosomes inhibits the binding of aminoacyl-tRNA to ribosomes (Ofengand & Henes, 1969; Shimuzu et al., 1970). Chemical modification (cyanoethylation) of its U residue (Ofegand & Henes, 1969) reduced the effectiveness of T-Ψ-C-G as an inhibitor. Second, Erdmann and coworkers (1973) showed that this oligonucleotide sequence forms a complex with 5S rRNA in vitro, which is stabilized by the addition of two ribosomal proteins that by themselves form a functional complex with 5S rRNA. In summary, available experimental data suggest that prokaryotic 5S rRNA binds to elongator-tRNAs at the A-site, whereas eukaryotic 5S rRNA binds the initiator-tRNA at the P-site and eukaryote 5.8S rRNA binds the elongator-tRNAs (Richter et al., 1973; Erdmann et al., 1974; Grummt, 1974; Wool, 1980).

Partial enzymatic hydrolysis with ribonucleases are used to locate unpaired regions of RNA that are at "exposed" positions of the molecule. Feunteun and Monier (1971) demonstrated the E. coli 5S rRNA
inside an intact 50S subunit was protected from hydrolysis by ribonuclease T1 (specific for G-residues) and pancreatic ribonuclease (specific for C-or U-residues). In native *E. coli* ribosomal subunits, 5S rRNA is even protected after extensive trypsin digestion of the ribosome (Feunteun & Monier, 1971), indicating that long stretches of the 5S rRNA are not exposed on the ribosomal surface. These results suggest that 5S rRNA are not exposed on the ribosomal surface and that translation enzymes do not interact directly with 5S rRNA. On the other hand, chemical modification using kethoxal and the carbodimide reagent (1-cyclohexyl-3-(2-morpholinyl)-4-ethylcarbodimide metho-p-toluene) reacts readily with G₄₁, U₄₀, and U₈₇ indicates that G₄₄ of the central C-G-A-A segment is not accessible in both ribosome bound 5S rRNA and isolated 5S rRNA. An interesting question arises since the hypothesis postulates that 5S rRNA binds tRNA through interaction between the C-G-A-A segment in 5S rRNA with the G-T-U-C loop of tRNA, but both partial ribonuclease and chemical modification experiments showed that C-G-A-A region was inaccessible. This seems to suggest that conformational changes in the ribosomal proteins and/or 5S rRNA must take place in order for the postulated interaction to occur.

Among the 35 proteins of the *E. coli* 50S subunit, two proteins L18 and L25 can individually form stable complexes with 5S rRNA. Besides these, protein L5 bind weakly to 5S rRNA (Horne & Erdmann, 1972; Yu & Wittmann, 1973; Garrett, 1974). However, eukaryotic 5S rRNA:protein complexes are not as well characterized as those from the prokaryotes. In contrast to prokaryotic complexes, eukaryotic (yeast)
complex contains only two components, a 5S rRNA molecule and a single larger protein component, YL3 (Yaguchi et al., 1984). Here, the underlying question is whether the proteins merely stabilize the 5S rRNA:complexes or do the proteins significantly alter 5S rRNA conformation either locally or globally?

The binding of ribosomal proteins to 5S rRNA requires close physical contact among the interacting components. Consequently, it is possible that such associations bring about conformational changes in either ribosomal protein or 5S rRNA. The binding of L5, L18, and L25 to 5S rRNA was studied in some detail. Spectroscopic studies with L18 and L25 (Bear et al., 1977; Spierer et al., 1978) and NMR studies with L25 (Kime & Moore, 1983c) indicate that the RNA helices are not disrupted when the proteins bind to 5S rRNA. For both L18 and L25, there is a good evidence that the proteins bind to double-helical structures. In both cases, helices are protected from hydrolysis by cobra venom RNase by the bound proteins, L18 and L25 (Douthwaite & Garrett, 1982), while L18 protects several base-paired guanines from reaction with dimethyl sulfate (Peattie et al., 1981). Although the 5S rRNA does not undergo extensive structural changes when complexed with proteins, L18 does appear to induce a significant alteration in the secondary, and perhaps tertiary structure of 5S rRNA as evident from an increase of 15-25% in amplitude of the 268 nm circular dichroism (CD) band of the RNA. This change is interpreted either as an increase in base-pairing or base-stacking (Bear et al., 1977) or an alteration in the tilting of bases (Spierer et al., 1978) in 5S rRNA.
The CD measurements suggest that the binding of L5 and L25 does not lead to any major changes in the secondary structure of 5S rRNA but L25 has been found to enhance the 5S rRNA thermal stability (Bear et al., 1977; Spiere et al., 1978). Similar findings were also reported by Fox & Wong (1978) that binding of proteins L25, L18, and L5 to 5S rRNA results in an increase in base stacking with an accompanying increase in asymmetry of the bases and a decrease in the conformational stability of the 5S rRNA based on changes in the near ultra violet CD spectra and in the melting profiles. Fox & Wong (1978) postulated that the binding of these proteins to 5S rRNA was necessary to align a part or parts of the 5S rRNA molecule in order to facilitate base-pairing with nucleotide segments(s) of the ribosomal 23S rRNA or the transfer RNA (or both). This is consistent with the finding that 5S rRNA requires the presence of its three binding proteins, L5, L18, and L25, for its attachment to core particles of E. coli ribosome (Yu & Wittmann, 1973).

High resolution NMR studies on 5S rRNA, its L25 binding fragment (residues 1-11, 71-120), and their respective interactions with L25 suggest that all the structural perturbations occur in the 79-85/91-97 helix (Kime & Moore, 1983c). The disappearance of the NOE linking base pairs Ug2•Ag4 with Gg3•Cg3 further supports the above interpretation. In addition several new unidentified low field resonances appear in the 5S rRNA:L25 complex which apparently suggest that hydrogen bonds are involved in the RNA-protein contacts.
In conclusion, 5S RNA undergoes a conformational change as result of the protein binding but the exact nature of the perturbation is poorly understood. It seems reasonable to conclude that much of the architecture of the ribosomal RNAs has evolved to accommodate interactions with ribosomal proteins.

D. Proposed Secondary Base-pairing Schemes of Isolated 5S rRNA

About 350 complete base sequences of 5S rRNA from many organisms have been determined to date (see Erdmann & Wolters, 1986, for recent compilation), of which 216 are derived from eukaryotes and 134 from prokaryotes (Erdmann & Wolters, 1986). The first prokaryotic 5S rRNA to be sequenced was that of E. coli (Brownlee et al., 1967). However, the determination of its secondary structure even it is a relatively small rRNA, has proved to be surprisingly difficult. Although E. coli has only 120 nucleotides (about 40 nucleotides more than a typical tRNA molecule), eight years has elapsed between elucidation of its primary structure (Brownlee et al., 1967) and secondary structure (Fox & Woese, 1975; Nishikawa & Takemura, 1974a,b). Unlike tRNA whose cloverleaf secondary secondary is obvious since its first primary sequence was determined, systematic explorations of all possible secondary structures of 5S rRNA with the help of computer programs indicate no unique base-pairing scheme can be proposed on the basis of the primary structure alone (Richards, 1969; Jordan, 1971b). This is illustrated by the large number of spurious secondary structures (Figures 8, 9) for this molecule as reviewed by Erdmann (1976). The difficulty in deriving a consensus structural model for 5S rRNA is
Figure 8: Proposed secondary structural models for prokaryotic 5S rRNAs. Escherichia coli 5S rRNA models: a, h, c at room temperature, d above 50°C, e, f, g, h, i, k and m. Pseudomonas fluorescens 5S rRNA models: j and l. Filled circles represent the conserved bases in prokaryotic 5S rRNA. From Erdmann (1976).
Figure 9: Proposed secondary structural models for eukaryotic 5S rRNAs and yeast 5.8S rRNA. KB cell 5S rRNA models: a, b and c. Xenopus laevis 5S rRNA model d, yeast 5S rRNA model e and Chlorella cytoplasmic 5S rRNA model f. A 5.8S rRNA model is shown in g. Filled circles represent the conserved bases in eukaryotic 5S rRNA (a-f). From Erdmann (1976).
because the number of base-pairing possibility increases approximately as the second power of the length of the RNA chain. This difficulty is further compounded by the uncertainty in the function of 5S rRNA, subsequently any proposed secondary structure is debatable on grounds of its functional uncertainty in the ribosome.

It is generally believed that a universal secondary structure exists because reconstitution of 5S rRNA from one prokaryote with the remaining constituent ribosomal components from a different prokaryote gives functional ribosome. Consequently, a large number of universal secondary structures have been proposed (Figure 10). None of them are entirely consistent with all the available physical, chemical, and biochemical data. Most of the proposed models for the secondary structure of 5S rRNA (Figure 10) envisage 5S rRNA as a ring-like structure containing four short helical segments, other base-pairing schemes are possible such as the cloverleaf model proposed by Luoma & Marshall (1978a,b). There is one aspect common to all the proposed 5S rRNA models: the 5'-and 3'-ends are base-paired with each other. These models have evolved based on the consideration of the comparative sequence analysis and experimental data as discuss below:

(a) The advent of rapid sequencing techniques has created a large body of information on the primary sequence of 5S rRNA from about 350 different organisms (Erdmann & Wolters, 1986) that enables the construction of secondary structure models for 5S rRNA (Figure 10) on the basis of comparative sequence analysis (Fox & Woese, 1975; Hori & Osawa, 1979; Luehrsen & Fox, 1981; Studnicka et al., 1981; De Wachter
Figure 10: The most popular proposed secondary structural models for 5S rRNA showing similarities and differences.
et al., 1982; Delihas & Andersen, 1982);

(b) information from infrared (IR) and Fourier transform infrared (FT-IR) spectroscopy (Burkey et al., 1983; Chang et al., 1984; Li et al., 1984; Stulz et al., 1981; Appel et al., 1979), ultra-violet (UV) and circular dichroism (CD) (Luoma et al., 1980; Fox & Wong, 1979, Fox & Wong, 1978; Bear et al., 1977; Boedtker & Kelling, 1967; Cantor, 1968; Richards et al., 1972) showing that 5S rRNA contains 25 to 40 base pairs, about two-thirds of which are G·C;

(c) limited enzymatic digestion giving information on the single-stranded and base-paired regions of the molecules (Vigne et al., 1973; Zimmermann & Erdmann, 1978; Vigne & Jordan, 1977); Douthwaite et al., 1979; Pieler et al., 1980; Garrett & Olsen, 1982; Nishikawa & Takemura, 1974; Nichols & Welder, 1979; Ross & Brimacombe, 1979; Vassilko et al., 1981; Douthwaite & Garrett, 1981);

(d) the accessibility of certain bases to specific chemical probes such as kethoxal (G-specific) (Noller, 1974; Litt, 1969); diethyl pyrocarbonate (A-and G-specific) (Peattie & Gilbert, 1980), bisulfite (C-and U-specific) (Woese et al., 1980), dimethylsulfate (C-specific) (Peattie & Gilbert, 1980; Mankin et al., 1981) provide information about single-stranded stacked or unstacked regions;

(e) high-resolution proton nuclear magnetic resonance (NMR) spectroscopy of 5S rRNA providing direct identification of base-pairs especially the use of the powerful NMR technique i.e. homonuclear Overhauser enhancements (NOE) (Shimmel & Redfield, 1980; Reid, 1981, Kime & Moore, 1983a,b; Kime, 1984a,b; Kime et al., 1984; Burns et al., 1980; Kearns & Wong, 1974; Wong et al., 1972);
(f) oligonucleotide probes, which presumably bind to free, unpaired regions of rRNA (Williamson & Brownlee, 1969; Erdmann et al., 1976; Lewis & Doty, 1977; Werde et al., 1978; Backendorf et al., 1981; 
(g) Raman spectroscopy giving information on base pairing in rRNA (Chen et al., 1975; Luoma & Marshall, 1978a,b); 
(h) information on the size and shape of free rRNA in solution is accessible from small angle X-ray diffraction (Osterberg et al., 1976), hydrodynamics studies (Fox & Wong, 1979) and electron microscopy (Tesche et al., 1980).

It should be noted that such important factors as function, protein recognition and interaction are generally not taken into consideration when constructing these models. The majority of the models modifies that of Fox & Woese (1975). Its essential feature is the occurrence of four helices: the "molecular stalk" (positions 1-10 and 110-120), the "weak tuned helix" (18-23 and 60-65), the "common arm base" (31-34 and 48-51) and the "prokaryotic loop" (82-86 and 90-94) as shown in Figures 11. The various secondary base-pairing schemes for 5S rRNA are depicted in Figure 10.

E. Review on Structural Information obtained for 5S rRNA

All the proposed models differ in both the total number of base pairs and the relative proportions of the three main base pair types, G·U, A·U, and G·U (Figure 10). Discriminating between the existing proposed models or new model therefore requires techniques that can provide information on the total base pairing and the nature of the base pairs. Spectroscopic techniques like UV, CD, IR, Raman, NMR all
Figure 11: This model is redrawn from the Fox & Woese model for the secondary structure of 5S rRNA (*S. carlsbergensis* b).
count and identify paired or stacked bases. Almost all the spectroscopic data available are for prokaryotic 5S rRNA (principally, *E. coli*), and only relatively a few performed on eukaryotic 5S rRNA. Using Raman spectroscopy, Luoma & Marshall (1978a,b) provided some earliest evidence for the existence of highly base-stacked helical regions, and base-paired secondary structure for an eukaryotic *Saccharomyces cerevisiae* (bakers' yeast) 5S rRNA. These findings for eukaryotic 5S rRNA are subsequently substantiate by UV, CD and $^1$H FT NMR (Luoma et al., 1980), UV differential thermal melting (Ohta et al., 1983), Fourier transform infrared (FT-IR) (Burkey et al., 1983), infrared (Stulz et al., 1981).

The other techniques such as limited ribonuclease cleavage, chemical modification, oligonucleotide binding have been used to identify single-stranded bases or segments. In addition, gross shape of the rRNA molecule can be obtained from light, low-angle X-ray scattering, hydrodynamics (sedimentation coefficients measurements), and CD experiments.

It is appropriate at this stage to review the structural information obtained so far for isolated 5S rRNA from various techniques. Figure 12 depicts the various physical, chemical, and biochemical techniques used in the study of rRNA.

1. Hydrodynamics and X-ray Scattering

*E. coli* 5S rRNA molecule has a sedimentation coefficient ($S_{20,\text{w}}$) of approximately 4.5S-4.8S which is fairly insensitive to variation of salt (Comb & Zehavi-Willner, 1967; Boedtker & Kelling, 1967). This is
Figure 12: The various experimental techniques to probe the secondary structure of 5S and 5.8S rRNAs.
interpreted as indicative for rigid structure of 5S rRNA molecule. However, the $S$ value of 5S rRNA is strongly concentration-dependent suggesting high asymmetry for this molecule (Visentin et al., 1972). The hydrodynamic conclusions are supported by small-angle scattering data obtained from yeast and *E. coli* 5S rRNA that showed this molecule assumes the shape of a prolate with an axial ratio of 5:1. The high degree of asymmetry of prokaryotic and eukaryotic 5S rRNA is also supported by gel-filtration experiments in this work (see Chapter III).

### 2. Optical Studies

Ultra-violet (UV) absorption, circular dichroism (CD) are sensitive to base stacking and the helical content (i.e. the amount of A•U and G•C pairs). Both UV and CD measurements suggest a high degree of base stacking and pairing. Cantor (1968, 1967) estimated that 40-49 bases in *E. coli* 5S rRNA were in the double stranded regions which means that the secondary structure consists of short helical segments similar to tRNA. Of the 40-49 base-pairs, 28 are G•C base-pairs and 12 are A•U base-pairs. Richards and coworkers (1972) obtained comparable total number of base pairs, 41 and further estimated from UV, CD and infrared absorption studies that the secondary structure of *E. coli* 5S rRNA includes 28 G•C and 13 A•U base-pairs.

In *E. coli*, the UV thermal melting profile exhibits a biphasic hypochromicity (Reynier et al., 1967; Cramer & Erdmann, 1968; Scott et al., 1968). Sea urchin (*S. purpuratus*) (Bellemare et al., 1972) and *B. subtilis* 5S rRNA (Gray & Saunders, 1973) also display biphasic melting behavior, with the exception of *B. stearothermophilus*. From
the total hypochromicity, Gray and Saunders (1973) estimated that the total numbers of base-pairs were 34 for *B. subtilis* and 34 for *B. stearothermophilus*.

For eukaryotic 5S rRNA, a few optical studies have been attempted. From the heat-induced melting of *Saccharomyces cerevisiae* (yeast) monitored by UV and CD, Luoma and coworkers (1980) concluded 35-40 base pairs in native yeast 5S rRNA solution structure in the presence of Mg$^{2+}$, and a 60:40 ratio of G·C to A·U base pairs with a substantial single-stranded contribution to the overall helical content and base stacking. The native structure of yeast 5S rRNA is quite insensitive to Mg$^{2+}$ concentration as evident by small increase in base pairs by 3-4 upon addition of Mg$^{2+}$ to rRNA depleted of Mg$^{2+}$.

The secondary base-pairing scheme proposed by Vigne and Jordan (1977) contains too few secondary base pairs to account for the experimental value of 35-40 base pairs. These optical conclusions are also consistent with the models of Nishikawa & Takemura (1978), Fox and Woese (1975) and Luoma & Marshall (1978a,b).

However, based on optical measurements Maruyama et al (1979) suggested about 30 base pairs in native yeast 5S rRNA conducted under low ionic strength conditions. The low salt may account for the observed differences in total number of base pairs in yeast obtained by Luoma et al 1980 which probably attributed to some melted regions.

It is important to note there are significant variations among the optical results reported because these measurements are based on comparison with model compounds such as synthetic polynucleotides.
(Fresco et al., 1963; Boedtker & Kelling, 1967), and tRNA (Romer et al., 1970; Coutts, 1971; Morikawa et al., 1969; Dourlent et al., 1971). There is subsequently a certain flexibility in interpretation of the results obtained for rRNAs due to the high degree of overlap between spectra of bases or base pairs. Nevertheless, optical methods can provide useful information on the upper limit for the total number of base pairs, and the relative G·C to A·U ratio, but not of sufficient accuracy to distinguish among the various proposed models.

3. Raman and Infrared Studies

Raman spectroscopy is a useful tool for evaluating RNA secondary structure (Luoma & Marshall, 1978a,b), by indicating the degree of "order" in the RNA backbone, the degree of base-stacking, and the amount of base-paired U residues (Thomas, 1975; Peticolas, 1972; Thomas & Hartman, 1973; Chen & Thomas, 1974, Chen et al., 1975). The Raman data for yeast (S. cerevisiae) obtained by Luoma & Marshall (1978a,b) suggest that its phosphodiester backbone was highly ordered, and about 65% of the uracils were base paired. They estimated about 35 base pairs in the native yeast 5S rRNA solution structure which is consistent with value, 35-40 base pairs obtained by optical methods (Luoma et al., 1980).

The infrared temperature spectra between the native and denatured 5S rRNA can be used to determine G·C and A·U base pairs. Burkey and coworkers (1983) estimated 31 base pairs based on FT-IR data which is consistent with optical and Raman findings. For yeast (Saccharomyces carlsbergensis) 5S rRNA, dispersive infrared results were interpreted
to give a total of 46 base pairs (Stulz et al., 1981), greatly exceeding the $^1$H FT-NMR value of 35 for 5S rRNA ($S$. carlsbergensis) in this work and value of 32 for another species of yeast ($S$. cerevisiae) of identical primary nucleotide sequence (Luoma et al., 1980).

4. NMR spectroscopy; $^1$H and $^{19}$F

A large number of NMR studies has been used successfully to determine the total number, types and location of base pairs in tRNA. However, only very little progress has been made to date on 5S rRNA because: (a) there are about 1300 protons in 5S rRNA against about 810 protons in a typical tRNA resulting in greater overlapping of assignable base pair hydrogen bond imino proton resonances in the -9 ppm to -15 ppm region; (b) the higher molecular weight of 5S rRNA leads to broader $^1$H nmr resonances and longer longitudinal magnetic relaxation, $T_1$, (c) the lack of high field NMR spectrometers until the availability of superconducting magnets (300 MHz to 500 MHz) in the past few years, (d) the appearance in the literature of more effective nmr radio frequency (rf) pulse sequences for water suppression.

The first $^1$H NMR investigation on yeast 5S rRNA was reported by Wong and coworkers (1972). Their NMR data gave a total of about 28 base pairs in the presence of Mg$^{2+}$, and this number was reduced to about 21 when Mg$^{2+}$ was removed from the 5S rRNA molecule (Wong et al., 1972). These NMR numbers are too low compared to those obtained by UV, CD, Raman, and FT-IR. This is not surprising since their NMR spectra were obtained at the relatively low applied magnetic field (200 MHz), and the poor resolution precludes any confident on their
findings (Wong et al., 1972). Eight years have elapsed before another NMR work on yeast (S. cerevisiae) at higher magnetic field (360 MHz) (Luoma et al., 1980) appeared in the literature. In the absence of Mg$^{2+}$, simulation of the 5S rRNA spectrum identifies a minimum of about 32 base pairs increasing to a minimum of about 35 base pairs in the presence of Mg$^{2+}$ (Luoma et al., 1980). Similar total number of base-pairing (about 36) on Bacillus licheniformis was reported by Salemink and coworkers (1980). Smith and Marshall (1980) incorporated 5-fluorouracil ($^{19}$FU) place of normal uracil in E. coli 5S rRNA and then obtained the 254-MHz $^{19}$F NMR spectrum of the $^{19}$FU-5S rRNA which indicates a highly rigid molecular framework for the entire molecule in solution. The various NMR techniques used in the study of rRNA are treated in details in Chapter IV.

5. Limited Enzymatic Hydrolysis

A number of ribonucleases have been used as structural probes, including T1-ribonuclease (RNase-T1) specific for G residues, T2-ribonuclease (RNase-T2) for G or A residues, pancreatic ribonuclease (pancreatic RNase) for C or U residues, S1-nuclease for any single stranded region, and cobra venom RNase for double strand. A potential problem in the use of nucleases, particularly single strand-specific ones, is that cleavage of the sugar-phosphate backbone can lead to other rearrangement of the native RNA structure or unfolding of the helices.

For free yeast 5S rRNA the most readily cleaved positions using T1-ribonuclease (T1-RNase) and T2-ribonuclease (T2-RNase) were
positions at \( G_{37}, G_{41}, \) and \( G_{91} \) (Vigne et al., 1973). Secondary sites are at positions 11, 54, 73 and 107 (Nishikawa & Tekemura, 1974a,b,c).

A number of T1-RNase partial digestion studies on isolated E. coli 5S rRNA have suggested that \( G_{41} \) is the first cleavage point, and the next cleavage points occur at positions \( G_{13}, G_{56}, \) and \( G_{86} \) (Jordan, 1971; Vigne & Jordan, 1971; Vigne et al., 1973; Vigne & Jordan, 1977). Positions \( G_{23} \) and \( G_{41} \) were cleaved when T2-RNase (for G or A) was used (Vigne & Jordan, 1977).

From the limited ribonuclease digestion of 5S rRNA isolated from both prokaryotes (Pseudomonas fluorescens and E. coli), and from eukaryotes (yeast and HeLa cells), it appears that region around position 40 is single-stranded in all RNAs tested whether from prokaryotes or eukaryotes (Vigne et al., 1973). However, for eukaryotic 5S rRNAs, a second equally unpaired and accessible region is around position \( G_{89} \).

6. Chemical Modifications

Similar to partial ribonuclease digestion, base-specific chemical probes can be used to obtain information on single-stranded or unstacked regions. The most useful chemical reagents are glyoxal and kethoxal which react specifically with guanine (Staehelin, 1959), methoxyamine with cytosine (Kochetkov et al., 1963), carbodiimides with uridines (Gilham, 1962), monoperphthalic with adenine (Cramer & Seidel 1964). If the chemical modifications are carried in mild conditions, only the most exposed bases will be modified.
Again only relatively few chemical modification experiments on
eukaryotic 5S rRNA have been performed. Kethoxal has been shown to
react preferentially with G₃₀, G₄₁, G₄₉ and G₉₁ corroborating with
findings obtained via partial RNase hydrolysis. Residues G₃₇, G₅₇,
G₈₀, G₈₂ and G₈₅ are also easily accessible.

The most exposed region in *E. coli* is consistently around
residues 40 as evident by the accessibility of C₃₅-C₃₈, C₄₂-C₄₃ and
C₄₇-C₄₉ to methoxyamine (Bellemare et al., 1972) and U₄₀ to
carbodiimide (Lee & Ingram, 1969). It may conclude that C₃₅-C₅₀ is
mostly single-stranded for eukaryotic 5S rRNA.

7. Oligonucleotide Binding

The method of oligonucleotide binding involves the formation of
base-pairs between the single-stranded and exposed segment of 5S rRNA
with complementary sequence of an oligomer. This idea was first
applied to characterize the anticodon loop tRNA^{fMet} (Ulenbeck et al.,
1970) and the findings were only partially in agreement with the known
tRNA structure. Nevertheless, this tool was subsequently used for the
identification of unpaired regions in *E. coli* 5S rRNA (Lewis & Doty,
1970). It is important to note that oligonucleotide binding requires
both single-strandedness as well as good base-stacking. The danger of
the possibility of oligonucleotide forming tertiary base-pairing can be
ignored.

The single-stranded regions identified in oligonucleotide binding
studies for yeast (*S. cerevisiae*) are at positions 15-20, 28-35,
44-50, 65-70, 98-100 and 105-107 which are not in full agreement with
the RNase hydrolysis, and chemical modification findings (Erdmann, et al., 1979). This is not surprising since chemical modification and RNase digestion detect only single-stranded regions but not stacked as opposed to oligomer which identifies helical single-stranded regions. A more open structural model is suggested from the above study as opposed to a compact one indicated by chemical modifications (Werde et al., 1978). The results from oligonucleotide binding on 5S rRNA isolated from rat liver and KB cells imply similar overall structures in pro- and eukaryotic 5S rRNAs (Williamson & Brownlee, 1969).

F. Overview on 5.8S rRNA

The 5.8S rRNA was identified as an integral component of eukaryotic ribosomes in 1968 (Pene et al., 1968; Weinberg & Penman, 1968). This rRNA species was previously designated as 7S, 5.5S, 28S-associated or satellite RNA, but is now generally known as 5.8S rRNA. It is a general consensus that 5.8S rRNA is a universal characteristic of the cytoplasmic ribosomes of all plants and animals (eukaryotes) investigated. It has no counterpart among prokaryotes since 5.8S rRNA is absent from ribosomes of bacteria and blue-green algae (Payne & Dyer, 1972). However, Vossbrink and Woese (1985) reported an exception to this rule in that microsporidian, Vairimorpha necatrix a parasitic eukaryote that contains no 5.8S rRNA.

When isolated under non-denaturating conditions, the 5.8S rRNA is associated with the high molecular weight ribosomal component, 26/28S rRNA (Forget & Weissmann, 1967; Pene et al., 1968; Weinberg & Penman, 1968), the 28S rRNA molecule in mammals, and the 26S rRNA in yeast and
fungi (Lo & Nazar, 1981; Wildeman & Nazar, 1981). The noncovalent association is maintained by hydrogen bonding and base stacking, and the complex is readily isolated. 5.8S rRNA molecule also appears to interact with ribosomal proteins (Metspalu et al., 1978; Nazar, 1978; Ulbrich et al., 1979) but the exact nature of this 5.8S rRNA-protein complex has yet to be established. Unlike 5S rRNA, for isolation of 5.8S rRNA from the whole ribosomes or ribosomal subunits, it is required to disrupt the hydrogen bonds by the gentle treatment with heat or denaturants (urea, formamide). The release of 5.8S rRNA was not accompanied by a loss of proteins from the ribosomes (Nazar, 1978; Giorgini & DeLucca, 1976). These observation implied that the interaction between 5.8S rRNA and 28S rRNA is not significantly influenced and stabilized by ribosomal proteins. The relative ease of reconstituting 5.8S rRNA into 60S subunit probably reflects that the 5.8S-26S rRNA complex (yeast) is stabilized by ribosomal proteins and this interaction probably occurs at the surface of the ribosome (Nazar, 1978). Based on the available information, the region of interaction between 5.8S rRNA and 28S rRNA in mouse ribosome appears to be between the 3'-terminal 20 nucleotides of the 5.8S rRNA molecule and the immediate 5'-end of the 28S rRNA molecule and between the 5'-terminal 20-25 nucleotides of 5.8S rRNA and a region in the 28S rRNA 300-400 nucleotides from the 3'-terminus (Walker & Pace, 1983; Walker et al., 1983; Peters et al., 1982; Michot et al., 1982; Nazar & Sitz, 1980).
The primary structure of 5.8S rRNA has been elucidated in about 38 species (for recent compilation of rRNA sequences, see Erdmann & Wolters, 1986). The first determination of the nucleotide sequence of a 5.8S rRNA was from yeast (S. cerevisiae) by Rubin (1973), later from Naovikoff hepatoma cells (Nazar and coworkers, 1975); both molecules contain 158 nucleotides. Unlike 5S rRNA, modified bases are common in 5.8S rRNA, although 5.8S rRNA of Dictostelium is unmodified. For example, yeast 5.8S rRNA has one pseudouridine (Rubin, 1973), Xenopus borealis (Ford & Mathieson, 1978) and Xenopus laevis (Khan & Maden, 1977) have two pseudouridines and one 2'-O-methylated-guanine and one 2'-methylated uridine, and turtle has one pseudouridine and one 2'-O-methylated guanine. The pseudouridine content in 5.8S rRNA of different mammalian tissues remains relatively constant (Nazar et al., 1975). The role of these modified bases in 5.8S rRNA is unclear.

The size of 5.8S rRNA molecules is fairly constant (156-162 nucleotides), except in Drosophila melanogaster and Sciara coprophila. In the latter two cases, the 5.8S rRNA is constituted of two shorter RNAs. One is 30 nucleotides long and is named 2S rRNA (Jordan et al., 1974; Jordan et al., 1976; Pavlakis et al., 1979). The other is 123 nucleotides long and has the same electrophoretic mobility as 5S rRNA in polyacrylamide gels. This particular molecule is often referred to as mature 5.8S rRNA (m5.8S rRNA). In general, 5.8S rRNA molecule displays a limited heterogeneity at the 5'-end. Heterogeneity at the 3'-termini has not been found so far in 5.8S rRNA molecules.
Studies on the nucleotide sequence of 5.8S rRNAs from diverse species indicate that this molecule is unusually more conserved in the primary structure than 5S rRNA (Erdmann & Wolters, 1986). For example, calculated homologies reveal that 5.8S rRNA of *Chlamydomonas reinhardtii* (green algae) shows 78.5% and 63.5% homology with the 5.8S rRNA of yeast and chicken. 5.8S rRNA molecules of mammalian and yeast ribosomes show 75% homologies (Nazar et al., 1975). Turtle differs from that of mammals in one position close to the 5'-end of the molecule only (Nazar & Roy, 1976). The nucleotide sequences of 5.8S rRNA from different plants (broad bean, dwarf bean, tomato, sunflower and rye) are very similar (Woledge et al., 1974). An exception is *Dictyostelium discoideum* (*D. discoideum*) (cellular slime mold) 5.8S rRNA whose nucleotide sequence contains no modified bases and exhibits little homology with other 5.8S rRNAs. These homology data probably reflect that fact that *D. discoideum* diverged from the mainstream of eukaryotic descent at the earliest branch within the 5.8S rRNA phylogeny (Olsen & Sogin, 1982). Altogether, from the determined sequences, it is clear that the structure of 5.8S rRNA is largely conserved during evolution.

**G. Function of 5.8S rRNA**

Although the 5.8S rRNA was identified as an integral part of eukaryotic ribosome about 18 years ago (Pene et al., 1968; Weinberg & Penman, 1968), the function of 5.8S rRNA in the ribosome is still unknown to date. At least two functions for the 5.8S rRNA have been advocated, a role in tRNA binding or subunit interaction (Toots et
It is proposed that eubacterial 5S rRNA and eukaryotic 5.8S rRNA are of the same evolutionary origin and that their functions in ribosomes are similar. All 5.8S rRNA molecules examined so far contain nucleotide sequence, G-A-A-C that is complementary to the T-U-C-G loop of tRNA. It is hence proposed that 5.8S rRNA may involve in the binding of tRNA-binding to the 80S ribosome (Werde & Erdmann, 1977; Ulbrich et al., 1979; Nishikawa & Takemura, 1974c) Furthermore, the binding of E. coli binding proteins L18 and L-25 to yeast 5.8S rRNA but not to yeast 5S rRNA, suggests structural and functional similarities between eukaryotic 5.8S rRNA and prokaryotic 5S rRNA. It is surprising to find E. coli ribosomal proteins do not bind rat liver 5.8S rRNA. This discrepancy may just probably a reflection of the different methods used in the binding studies; with rat liver 5.8S rRNA by affinity chromatography; with yeast 5.8S rRNA by isolation on sucrose gradients of the complex formed from the mixtures of proteins and rRNAs. In affinity experiment, the rRNA is first oxidized with periodate and then coupled by its 3'-terminus to Sepharose through an adipic acid dihydrazide spacer (Burrell & Horowtiz, 1975; Burrell & Horowtiz, 1977). The next step is the passage of ribosomal proteins through the affinity column. Finally, bound-proteins are eluted off from the column and characterized by gel electrophoresis. The 3'-end of rRNA may have been altered by periodate oxidation or coupling to the adipic acid dihydrazide or both
resulting proteins that are associated physiologically with rRNA might not bind to affinity column. Also some proteins might bind factitiously because binding sites have been created by alterations of rRNA structure. It is also possible that initial binding of proteins may alter the conformation of rRNA and creates binding sites for other subsequent proteins. The association of proteins with rRNAs is ionic strength-dependent. For example, real binding can be inhibited by relatively high ionic strength. Conversely, relatively low ionic strength can result in non-specific protein and rRNA interaction. The ionic strength of the binding buffer used in affinity experiments is typically around 0.38. Since at present there are no alternative and comparable experiments to verify results obtained by affinity chromatography, great cautions must be exercised in interpreting or comparing rRNA-protein binding studies. For example, Metspalu et al., (1978) found L18 to bind rat liver 5.8S rRNA, while L19 was reported by Ulbrich and Wool (1979). This discrepancy arises because these two proteins cannot be differentiated by two-dimensional gel electrophoresis alone. L18 and L19 however are easily separated via a combination of one-and two-dimensional gel electrophoresis (Ulbrich & Wool, 1979). In general this limitation reflects the difficulty in characterization of ribosomal proteins precisely.

It is proposed that the common nucleotide sequence, G-A-A-C of eukaryote 5.8S rRNA interacts with a complementary sequence, T-Ψ-C-G at the loop of tRNA during binding of aminoacyl-tRNA to the ribosomal A binding-site. It is observed that the tetranucentide T-Ψ-C-G
inhibits factor-dependent binding of aminoacyl-tRNA to eukaryotic ribosome (Grummt et al., 1974); while the P binding-site of eukaryotic initiator-tRNA is not affected. This observation leads to the hypothesis that eukaryote 5.8S rRNA forms part of the ribosomal A site and participes in the binding of elongator-tRNA. The 5S rRNA forms part of the P site and take part in the binding of initiator-tRNA. The evidence here is very circumstantial. Besides, sequence comparison shows there is no apparent homology between 5S rRNA and 5.8S rRNA molecules (Cedergren & Sankoff, 1975), hence the hypothesis of 5S and 5.8S rRNA having similar functions still awaits to be verified experimentally.

The observation of an alternative form of 5.8S rRNA lead to a suggestion that this molecule may actively participate in the translation mechanism by switching between two conformation states (Lo et al., 1984). Similar function for 5S rRNA has also been speculated. According to this speculation, movement of the ribosome relative to mRNA may have been caused by conformational switches between different secondary conformation of the 5S rRNA molecules, possibly triggered by the binding of ribosomal proteins (Kao et al., 1983; Weidner et al., 1977; Jagadeeswaran & Cherayil, 1980). At present, the role and function of both 5.8S rRNA and 5S rRNA molecules in the ribosome remain an open question.

H. Proposed Models for 5.8S-25S rRNA Complex

Since the discovery that 5.8S rRNA:L-rRNA (large rRNA) complex can be isolated intact (Pene et al., 1968; Weinberg & Penman 1968;
tremendous research activities are directed toward determining the contact sites between 5.8S rRNA and L-rRNA molecules. Pace and coworkers (1977) demonstrated that 1:1 complex with native property could be formed from two isolated 5.8S rRNA and 28S rRNA from mouse by proper annealing. Much of the evidence that 3'-end of 5.8S rRNA interact with L-rRNA come from studies with mouse cells (Pace et al., 1977) and Drosophila (Pavlakis et al., 1979). Later, it was shown that the 5'-end of 5.8S rRNA is also involved in the interaction with L-rRNA (Pavlakis et al., 1979; Nazar & Sitz, 1980). Only recently, conformational-specific nucleases and chemical modification assay reveal that 5'-terminal 20-25 nucleotides and 3'-terminal 40 nucleotides of 5.8S interact with 28S rRNA in mouse cells (Walker et al., 1982; Walker & Pace, 1983). Further confirmation derived from the capability of 5'-terminal 73 nucleotides and 3'-terminal 83 nucleotides of 5.8S rRNA to associate independently with 28S rRNA (Peters et al., 1982). These "half molecules" were derived from 5.8S rRNA molecule from limited enzymatic digestion. There is now a general consensus that the both termini of 5.8S rRNA associate with L-rRNA (Peters et al., 1982; Walker & Pace, 1983; Walker et al., 1982; Olsen & Sogin, 1982; Nazar & Sitz, 1980; Sitz et al., 1981). Figure 13 shows the mouse 5.8S:28S rRNA complex.

However, the location of contact sites in L-rRNA is less defined compared to in 5.8S rRNA. It was noticed that the 5'-end of E. coli 23S rRNA is homologous to eukaryotic 5.8S rRNA (Nazar, 1980; Jacq, 1981). The secondary structure models for E. coli 23S rRNA (Noller et
Figure 13: A schematic representation of the location of base-pairing interactions between the 3'-end and 5'-end of mouse 5.8S rRNA with 28S rRNA. From Walker & Pace, 1983.
al., 1981) and *S. cerevisiae* 26S rRNA (Veldman et al., 1981a,b) which propose base-paired interactions between the 3'-end of 5.8S rRNA and the immediate 5'-end of 28S rRNA and between the 5'-end of 5.8S rRNA and a region in the 28S rRNA about 300-400 nucleotides from the 5'-terminus. Consequently, based on sequence homology comparison between 5.8S rRNA and 28S rRNA (Olsen & Sogin, 1982) and prokaryotic 23S rRNA (Nazar, 1980; Jacq, 1981; Noller et al., 1981; Glotz et al., 1981), several models have been proposed to suggest the nature and locale of contact sites in 28S/25S/23S rRNA (Figure 14). These models for 5.8S:L-rRNA complex (Pavlakis et al., 1979; Kelly & Cox, 1981; Veldman et al., 1981a,b; Michot et al., 1982; Uris et al., 1982, Walker et al., 1982) share two common basic features: (a) both 5'-end and 3'-end of 5.8S rRNA are involved, thus leaving most of the central part of the secondary structure of the 5.8S rRNA molecule intact (Nazar et al., 1975; Ford & Mathieson, 1978; Kelly & Maden, 1980; Olsen & Sogin, 1982; Peters et al., 1982; Uris et al., 1982; (b) the contact sites at L-rRNA are located either within its 3'-end segment (Kelly & Cox; 1981) or 5'-end region (Veldman et al., 1981a,b; Michot et al., 1982; Uris et al., 1982; Figures 13 & 14). These models differ in the amount of secondary features are preserved in the central of the 5.8S rRNA molecule. There is some circumstantial experimental evidence to support the model 1 (Figure 14) in that all of the 5.8S rRNA contact sites in the 28S rRNA are located within several hundred nucleotides of the 5'-end of 28S rRNA (Walker et al., 1983).
Figure 14: Various proposals for the 5.8S-28S rRNA interactions. From Walker et al., (1983).
In contrast, Georgiev and coworkers (1981; 1984) proposed an end to end pairing model which involved the interaction of both 5'- and 3'-ends of yeast 26S rRNA. This model stems from a computer analysis of the complete sequence of 26S rRNA from *S. cerevisiae* (Georgiev et al., 1981) and *S. carlsbergensis* (Veldman et al., 1981) and known sequence of *S. cerevesiae* (Rubin, 1973). Blot-hybridization of \(^{32}P\)-labelled 5.8S rRNA with restriction fragments from *S. cerevesiae* 25S gene, showed strong hybridization signals were obtained with fragments from the 5'-end (nucleotides 1 to 494) and the 3'-end (3066 to 3391) of the gene. However, there are no signals from the fragments from the remaining part of the gene.

Unfortunately, there is no concrete experimental evidence to substantiate any of the proposed contact sites. However, it is certain that 3'-end of 25S rRNA is involved as evident from re-association studies with *Neospora crassa* 25S rRNA (Kelly & Cox, 1981). The evidence concerning the interaction regions between the 5'-end of 5.8S rRNA and L-rRNA is more difficult to evaluate.

I. Proposed Models for Free 5.8S rRNA

Several universal models of free 5.8S rRNA in solution (Figure 15) were deduced from maximal base-pairing using Tinico's rules (Gralla & Crothers, 1978) and the results of partial ribonuclease digestion (Rubin 1973; Nazar et al., 1975; Ford & Mathieson, 1978), laser Raman spectroscopy (Luoma & Marshall, 1978), and thermal melting measurements (Van et al., 1977). All these models contain one common feature, a very stable G•C rich hairpin loop (nucleotides 116-138).
Figure 15: The three most common proposals for the secondary structure of yeast 5.8S rRNA.
Rubin proposed the first secondary structural model for an eukaryotic 5.8S rRNA (S. cerevisiae), which was the first 5.8S rRNA sequence to be determined. In contrast to other models, the 5' and 3'-termini are not paired in Rubin model. Nazar et al (1975) based on the studies on Novikoff hepatoma 5.8S rRNA suggested an alternative model for eukaryotic 5.8S rRNA. The cloverleaf model was derived from laser Raman studies on yeast 5.8S rRNA, and was then adapted to mammalian sequence (Luoma & Marshall, 1978). Ford and Mathieson (1980) constructed a base-pairing scheme for X. laevis 5.8S rRNA. The Rubin and cloverleaf models share a common "helix III"; Nazar and the cloverleaf models share most of a common stalk which pairs the 3'-and 5-termini together. Again similar to 5S rRNA, computer analysis of all base pairing possibility of 5.8S rRNA produces no unique secondary structure can be proposed on the basis of the primary structure alone.

J. Structural Studies on Free 5.8S rRNA

There are fewer studies on free 5.8S rRNA compared to 5S rRNA; about 38 complete base nucleotide of 5.8S rRNA from different species have been determined as opposed to about 350 for 5S rRNA (Erdmann & Wolters, 1986). This section is devoted to reviewing the various data on 5.8S rRNA obtained from different techniques: optical measurements chemical modifications and laser Raman spectroscopy.

1. Optical Measurements

From UV denaturation profiles and ethidium bromide probing, it was estimated that 5.8S rRNA contains a high percentage of G-C pairs
(70% for rat and 60% for yeast), and also there were about twice as many A•U pairs in yeast than in the mammalian rRNA (Van et al., 1977). King & Gould (1970) estimated there were 60% G•C pairs and 40% A•U pairs in mammalian 5.8S rRNA which is consistent with findings of Van et al. (1977). These optical results show a high degree of base pairing in 5.8S rRNA than is possible in the Rubin (1975) model.

2. Partial Ribonuclease Digestion

Enzymatic hydrolysis on yeast (S. cerevesiae) 5.8S rRNA by T1-RNase and pancreatic RNase revealed that primary cleavage sites are located at positions C_{11}, C_{32}, U_{64}, C_{91}, G_{103}, U_{125} and G_{140} (Rubin, 1975). The primary cleavage sites for Novikoff hepatoma 5.8S rRNA are A_{17}, U_{18}, G_{36}, A_{37}, G_{39}, G_{55}, G_{62}, A_{63}, G_{80}, A_{99}, C_{103}, G_{104}, U_{138}, G_{141}, G_{145}, and U_{146} (Nazar et al., 1975). These findings show that regions around positions at 103 and 140 are single-stranded. The nucleotides (1-17) are resistant to nuclease digestion probably suggesting an interaction between 1-16 at the 5'-end and the residues 144-159 at the 3'-end which is consistent with Luoma & Marshall cloverleaf model and Nazar Model. However, these findings do not rule out Rubin model since it was observed that the termini were susceptible to nuclease digestion only when the salt concentration was low (Wildeman & Nazar, 1981). Using nuclease S1 from Aspergillus oryzae which hydrolyzes single-stranded regions, Khan and Maden (1976) conclude that in mammalian HeLa cells the loop (73-81), loop (125-129) of the G•C rich arm and the extreme 3'-end (157-159) are single-stranded. In 5.8S rRNA from S. gairdnerii (trout), T1-RNase cleaves
at G₁₀, G₂₄, G₆₂, G₈₉, G₁₀₄, G₁₀₈, G₁₁₅ and G₁₄₈ (Nazar & Roy, 1978). All the partial ribonuclease digestions are consistent with the proposed cloverleaf and Nazar secondary base pairing models for 5.8S rRNA.

However, plants 5.8S rRNA does not fit either of these models as well as do vertebrates or yeast (MacKay et al., 1980). Attempts to fit Acanthamoeba castellani (amoeboid protist) to Nazar and cloverleaf are equally unsatisfactory (MacKay & Doolittle, 1981). Results obtained on Thermomyces lanuginosus (a thermophilic bacterium), appear to support Nazar model because cleavages are frequently found in regions which are base paired in cloverleaf model, residues 63, 64, 68, 76, 78, 95, 103, 108, and 110 (Wildeman & Nazar, 1981). Also residues 45, 58, 84 and 94 are located in single-stranded region in cloverleaf model but are susceptible to nuclease attack. However, these findings must be viewed with great caution since there is the risk of progressive perturbation of the structure which may render some intrinsically inaccessible residues to be accessible to nuclease action. At present there are no criteria such as biological activity to gauge whether the structure has been perturbed seriously.

3. Chemical Modifications

Sodium bisulphite is used to probe exposed cytidine residues by converting it into uridine via intermediate adduct formation and deamination (Shapiro et al., 1970; Hayatsu et al., 1970; Goddard & Schulman, 1972). Results from bisulphite modification on HeLa 5.8S rRNA revealed that C₁, C₁₉, C₂₁, C₂₃, C₅₀, C₅₂, C₇₈, C₈₃, C₁₀₀, C₁₀₃, C₁₂₇, C₁₂₈, and C₁₅₈ (Kelly et al., 1978). As expected cytidine
residues located at the loop regions are significantly modified. Kelly and Maden (1980) obtained similar findings on HeLa using a combined carbodiimide (reacts prefentially with unpaired uridine residues and slightly with guanine residues) and bisulphite probes. The findings from chemically modified 5.8S rRNA are in agreement with the enzymatic digestion data as discussed earlier.

4. Raman Experiments

Laser Raman investigation of yeast 5.8S rRNA by Luoma & Marshall led to the proposal of cloverleaf model. The Raman data suggest that yeast 5.8S rRNA has a highly ordered backbone and a high degree of base pairing, greater 70% base paired uridine residues involving significant U stacking, an extensive G·C stacking, and only moderate A stacking (Luoma & Marshall, 1978). Rubin model has only 44% of its U residues in the base paired region which is inconsistent with Raman data. However, these findings do not exclude Nazar model.

F. Summary of Present Work

Ever since the elucidation of the primary structure of 5S rRNA and 5.8S rRNA, structure analysis of rRNA and its correlation to molecular function has become a very active field of research. There have been a progression of physical, chemical and biochemical studies aimed at determining the detailed structure of 5S and 5.8S rRNA. Although many structures for eukaryotic and prokaryotic 5S rRNA and 5.8S rRNA have been proposed based on comparative sequence analysis, physical, and accessibility measurements, there is as yet no
general consensus on a universal secondary structure for any of the 5S and 5.8S rRNA.

The goal of this investigation is to decide among the various proposed models of 5S and 5.8S rRNAs via high resolution 500 MHz proton NMR. The present work involved the isolation and purification of 5S and 5.8S rRNAs and determination of their solution structures using $^1$H NMR and synthesis of novel spin-labels. This work represents the first proton NMR studies on any 5.8S rRNA and provided the most direct evidence for the existence of the common G+C-rich arm in 5.8S rRNA through a combination of proton (500 MHz) homonuclear Overhauser Enhancements (NOE) and the temperature-dependence proton 500 MHz NMR experiments (see Chapter V).

A new and novel approach to identifying terminal base pairs is reported here in Chapter VI. The yeast 5S rRNA was labelled with a paramagnetic morpholino nitrooxide spin-label at the 3'-terminus which produced highly selective $^1$H NMR line broadening according to the electron-proton separation. Comparison of the high field $^1$H NMR spectrum (500 MHz) of the nitrooxide-labelled 5S rRNA in H$_2$O with the $^1$H spectrum of the unlabelled 5S rRNA revealed the terminal-stem base pairs, from the specific broadening of nmr resonances resulting from their proximity to the labelled-3'-terminus. The hence identified terminal-stem base pairs provided an excellent starting point for NOE experiments from which I demonstrated the existence of the terminal helix in yeast 5S rRNA. This work constitutes a novel approach to structural elucidation of RNA's based on paramagnetic broadening.
Obtaining RNA samples requires the use of a full range of chromatographic techniques (e.g., gel filtration, ion-exchange, gel electrophoresis). This work developed a high speed, economical, preparative, size exclusion chromatography via Sephacryl S-300 for isolation and purification of 5S and 5.8S rRNAs (see Chapter III). This method substantially reduces purification time compared to the traditional Sephadex chromatography.

This work also developed a chromatographic technique for separation of proteins, nucleotides and polysaccharides from rRNA mixture via the use of pre-packed and ready-to-use DEAE cartridge (AMF ZetaPrep DEAE 250). The simplicity of the cartridge approach eliminates the need for time-consuming preparation of DEAE ion-exchange media and column packing, thus reducing RNA purification time (see Chapter II). Set-up time is minimal since cartridges are merely placed in housings, connected to a pump and equilibrated.

In addition, this work introduces the use of mini 7 cm x 8 cm slab gel for rapid electrophoresis of RNA samples which is useful for routine screening of RNA samples. The mini gel electrophoresis system has several advantages over conventional 14 cm x 18 cm gel format: (a) electrophoresis time is half of that of the large gel system; (b) minimal tank buffer and hence lower cost, since less chemicals are needed; (c) mini gels require only smaller amount of the expensive electrophoretic grade acrylamide resulting in substantial saving in operating cost; (d) glass plates replacements are inexpensive (80¢ per 8 cm x 10 cm glass plate versus $6.50 per 16 cm x 18 cm plate.
A. Introduction

Holley et al., (1961) demonstrated that unbroken, whole yeast cells released their low-molecular RNAs (tRNAs, 5S rRNA and 5.8S rRNA) when permeabilized with phenol. Since then many variations of the basic phenol permeabilization method have been reported. In the isolation of low-molecular weight RNAs, these methods have the advantages over phenol extraction of disrupted cells. First, by changing the conditions of extraction, permeabilization can be selective for low-molecular RNAs. For instance, if permeabilization of yeast (Saccharomyces cerevisiae) is carried out at a temperature of 40-45°C, the molar amount of 5.8S rRNA released is equal to that of the 5S rRNA. Second, since cell disruption is not required in this procedure, hence only intact tRNAs, 5S rRNA, 5.8S and small amount of larger RNAs (for example, 26S rRNA) can leak out of the cells while DNA and most of the rRNAs remain inside the cells due to their large size.

Phenol serves the purpose of increasing the porosity of the cell membrane to extraction of lipid and proteins, and also inhibiting ribonuclease activity. However, it was observed that phenol alone was
not sufficient to inhibit ribonuclease as subsequent purification resulted in degradation of the rRNA. Laskov et al (1959) suggested the use of ethylenediamine tetraacetate (EDTA) to reduce ribonuclease activity.

5S and 5.8S rRNAs were isolated from yeast cells according to the procedure described by Rubin (1975). The general scheme for the isolation and purification of yeast 5S and 5.8S rRNAs is essentially similar, and is outlined in Figure 16. Isolation is carried out via phenol extraction of whole, unbroken yeast cells. This is followed by purification of 5S and 5.8S rRNAs from proteins and tRNAs through DEAE (Diethylaminoethyl) ion-exchange and gel-filtration chromatography.

In the present work, a new and high-speed DEAE ion-exchange chromatography was developed for rRNA purification via ZetaPrep DEAE. There is no report so far on the use of this innovative ZetaPrep DEAE method for purifying rRNA. This method offers shorter purification time and low cost for large-scale purification of rRNA. Also a new method for rapid screening of 5S rRNA and 5.8S rRNA samples via mini gel format electrophoresis was developed.

B. Experimental Procedures

I. Isolation of yeast 5S rRNAs

Brewer's yeast (Saccharomyces carlsbergensis) cells were generously provided by the Annheuser-Busch Brewery, Inc., Schrock Road, Columbus, Ohio. About 510 grams (gms) of yeast cells were suspended in 1600 ml of buffer containing 20 mM EDTA, 0.1 M sodium acetate, and 1% sodium dodecyl sulfate (SDS) (pH 5.0) in a 4000 ml beaker.
Figure 16: A flow-diagram of the isolation procedure for extraction and purification of 5S and 5.8S rRNAs from whole yeast cells.
One-half volume of 90% phenol was added, and the mixture was stirred vigorously for 2 hours at room temperature. A 3.5 inches long magnetic stirring bar was used. The aqueous/phenol phases were separated by centrifugation at 9800 × g for 15 minutes at 5°C on Beckman Model J2-21 centrifuge. The top aqueous phase was removed via siphoning using a 50 ml plastic disposable syringe with a short Tygon tubing attached to it. The cell pellet was saved and washed repeatedly with deionized water to remove residual proteins. The washed cell pellet was kept in the freezer for the subsequent isolation of 5.8S rRNA. The aqueous phase was reextracted with equal volume of phenol. RNA was allowed to precipitate at 5°C after addition of 2.5 volume of cold 95% ethanol. It took 4-5 days for the crude RNA precipitate to settle as brownish, sticky RNA mixture onto the bottom of the beaker. The precipitated RNA consists of tRNAs, 5S rRNA, 5.8S rRNA and high molecular weight rRNA's.

2. Isolation yeast 5.8S rRNA

The cell pellet obtained from extraction of 5S rRNA was used in the isolation of 5.8S rRNA. The whitish, gluey cell pellet was suspended in 1000 ml of buffer containing 20 mM EDTA, 0.1 M sodium acetate, and 1% SDS (pH 5.0) in a 4000 ml beaker. One-half volume of 90% phenol was added, and the mixture was stirred vigorously for 2 hours at temperature of 40-45°C. The resulting whitish suspension was too viscous to be stirred effectively via magnetic stirrer even when a 3.5 inches long magnetic stirring bar was employed. A variable-speed and high torque mechanical stirrer (Talboy Model 102) fitted
with a stainless steel zig-zag paddle was found capable of stirring
the viscous suspension effectively. The temperature was maintained
via an electric hot plate (Thermolyne, Nuova II). The aqueous/phenol
phases were separated by centrifugation at 9800 x g for 15 minutes at
5°C using Beckman Model J2-21 centrifuge. The top aqueous layer was
removed via siphoning with a 50 ml syringe fitted with a short Tygon
tubing. The aqueous phase was reextracted with equal volume of
phenol. RNA was allowed to precipitate at -5°C after addition of 2.5
volume of cold 95% ethanol.

3. DEAE-32 Ion-Exchange Chromatography

The proteins and polysaccharides were removed from crude RNA
mixture by using DEAE-32 cellulose ion-exchange (Whatman, England)
chromatography before further fractionation via gel filtration
chromatography (Sephadex 75 or Sephacryl S-300). Specifically, the
cationic DEAE-cellulose initially binds solubilized polyanionic RNAs
(negatively charged polyphosphate backbone), allowing elution of
dissolved non-ionic polysaccharides, and small molecules through the
DEAE-cellulose column. RNAs and other anions bind to DEAE-cellulose
as a function of their size. The bound anions such as nucleotides,
SDS and proteins because their weaker electrostatic interaction with
the exchanger, are first released by washing the column with 0.3 M
sodium chloride (NaCl). The stronger binding of tRNAs, 5S rRNA, and
5.8S rRNA requires their elution with higher salt concentration,
1.0 M sodium chloride.
Step I. DEAE-32 cellulose ion-exchanger preparation

About 100 grams of DEAE-32 cellulose was suspended in 1500 ml of 0.5 N hydrochloric acid solution (HCl) and left standing for 30 minutes. The HCl was decanted off and the ion-exchanger was washed repeatedly in a filter funnel with deionized water till the pH of the filtrate registered 4.0. The pH of the filtrate was monitored via pH paper (EM Science, ColorpHast™). The ion-exchanger was resuspended in 1500 ml of 0.5 N sodium hydroxide (NaOH) solution and left standing for 30 minutes. The NaOH solution was decanted off and ion-exchanger was then washed in a filter funnel with 1000 to 1500 ml of deionized water. The washing was stopped when the pH of the filtrate was about 8.0. The resin was next suspended in 800 ml of 10 mM Tris-HCl (Tris(hydroxymethyl) aminomethane-hydrochloride) buffer containing 1.0 M NaCl and 10 mM magnesium chloride (Mg²⁺), pH 7.5. The pH of the suspension was then adjusted to pH 7.5 with sodium hydroxide solution, and vacuum filtered. The ion-exchanger cake was resuspended in 1000 ml of Tris-HCl, 1.0 M NaCl, 10 mM Mg²⁺, pH 7.5 buffer and allowed to settle for 45 minutes. The purpose of the settling step was to remove very fine divided cellulose which might clog the column when it was packed and resulted slow column flow rates.

Step II. Packing of DEAE-32 cellulose column

The DEAE-cellulose was ready for packing. A thick slurry of DEAE-cellulose was packed into a 5 cm wide x 11 cm long glass column (BioRad). About 30 gm to 50 gm of DEAE-32 cellulose was used in each purification. The column was washed with 10 mM Tris-HCl, pH 7.5
buffer containing 1.0 M NaCl and 10 mM Mg$^{2+}$ until absorbance at 260 nm ($A_{260}$) of the eluent was less than 0.05. It was then equilibrated with at least 3 volume of the 10 mM Tris-HCl, pH 7.5 buffer containing 0.3 M NaCl and 10 mM Mg$^{2+}$. This column was ready for use. The column was then connected to a Liquid Chromatography (LC) system consisting of a UV detector fitted with preparative flow cell (2-mm light path and 260 nm filter (Gilson Model 111), a fraction collector (Gilson FC-220), and a chart recorder (Zipps & Zonen BD 4). Any unused DEAE-cellulose was stored in Tris-HCl, pH 7.5, 1.0 M NaCl, 10 mM Mg$^{2+}$ buffer at 5°C. A trace of chloroform was added to inhibit microbial growth.

Step III. Removal of proteins, polysaccharides, SDS from rRNA via DEAE-cellulose chromatography

This step is applicable to both 5S and 5.8S rRNA. Most of the ethanol-water solution was siphoned off and the remaining RNA precipitate-ethanol-water mixture was poured into 50 ml disposable conical Polypropylene centrifuge tubes (Falcon Model 2098) and centrifuged at 3500 x g for 20 minutes using a table-top centrifuge (IEC Model HN-S II). The resulting RNA pellet was thoroughly drained of ethanol and dried by blowing over the pellet with nitrogen gas filtered through a Millex-FG50 filter unit (Millipore Corporation). The dried rRNA pellet was dissolved in minimal amount of 10 mM Tris-HCl, pH 7.5 buffer containing 0.3 M NaCl, 10 mM Mg$^{2+}$ to give a yellowish rRNA solution.
The rRNA solution was applied to the DEAE-cellulose column equilibrated with 0.3 M NaCl, 10 mM Mg²⁺, Tris-HCl, pH 7.5 buffer. The column was washed with the same buffer until the A₂₆₀ was reduced to about 0.1. The flow rate was unimportant since this was a stepwise chromatography. A high flow rate was chosen to lessen elution time. Finally, the tRNAs, 5S rRNA and 5.8S rRNA were released from the column by washing the column with 1.0 M NaCl, 10 mM Mg²⁺, 10 mM TRIS-HCl, pH 7.5 buffer. A typical elution profile from DEAE chromatography is shown in Figure 17. The first peak corresponds to the elution of proteins, SDS, nucleotides and polysaccharides follows by a second peak containing a mixture of rRNA consisting mainly of tRNAs, 5S rRNA and 5.8S rRNA. Fractions from peak 2 were pooled together and its rRNA concentration was determined from its A₂₆₀. The rRNA was then precipitated by addition of 2.5 volume of 95% ethanol pre-cooled to -10°C. The rRNA was allowed to flocculate and settle at 5°C. The rRNA is stable in ethanol for a few months.

4. ZetaPrep DEAE Ion Exchange Chromatography - An alternative to Traditional DEAE-Cellulose Chromatography

The preparation of DEAE cellulose media in Step I (Section 3) together with the packing and equilibration of DEAE-cellulose column in Step II (Section 3) are time consuming processes. The innovative ZetaPrep DEAE ion-exchange chromatography was first introduced by AMF Incorporation to the market in 1985. Since the pre-packed, self-contained and ready-to-use ZetaPrep DEAE devices come ready to equilibrate, thus eliminating the time spent preparing DEAE media and
Figure 17: A typical DEAE anion-exchange elution profile for the phenol extract of whole yeast cells. The early peak contains proteins, SDS, polysaccharides and nucleotides. The later peak contains a mixture of tRNAs, 5S rRNA and 5.8S rRNA.
pouring the column. ZetaPrep DEAE resin has high surface area and large pore size thus allowing the penetration of large biomolecules (larger than 300 Angstrom) into the resin pore, hence both adsorption sites located on the exterior and interior can be utilized. Because of high porosity ZetaPrep DEAE has high protein binding capacity, for example the protein binding capacity for ZetaPrep-250 cartridge is 10-15 grams of bovine serum albumin (BSA). The high flow rate for ZetaPrep-250 (125 ml per minute) means shorter processing time. There is no report so far on the use of ZetaPrep DEAE chromatography for rRNA purification. Because of the elimination of prior DEAE media and column preparation, high protein capacity, fast flow rate due to the semi-rigid structure, and competitively low cost, I decided to evaluate the use of ZetaPrep DEAE for rRNA purification in place of traditional DEAE-cellulose ion-exchange chromatography.

ZetaPrep DEAE media is a solid phase matrix composed of three materials: a cellulose support for mechanical strength, vinyl polymers as functional group carriers and a cross-linker for controlled porosity and dimensional stability. Its fast flow rate derives from its unique cartridge flow pattern and short "bed depth". Figure 18 shows the vertical and horizontal cross-sectional view of a ZetaPrep cartridge and housing. Buffers and sample flow through the cartridge by entering the outside cylindrical surface area, penetrating through the spirally-wrapped layers to the center core, and then flow out the top side of the cartridge and housing. This unique design provides a shorter elution path than conventionally packed column.
Figure 18: A: A schematic view of the ZetaPrep cartridge construction (From Hou & Mandaro, 1986). B and C: Cartridge flow patterns. Buffers flow through the cartridge by entering the outside cylindrical surface area, penetrating through the spirally wrapped layers to the center core, and then flow out the top side of the cartridge and plastic housing.
The ZetaPrep-250 DEAE device was set up such that the inlet Tygon tubing was attached to the bottom housing connector and the outer Tygon tubing to the top side housing connector. The inlet tubing was connected to a peristaltic pump (Rainin Rabbit pump) and the outlet tubing to a UV photometer (Gilson Model 111) fitted with a 260 nm filter and a preparative flow cell (2-mm light path). Complete swelling and equilibration of the DEAE cartridge was achieved by passing 10 mM Tris-HCl, pH 7.5 buffer containing 0.3 M NaCl and 10 mM Mg\(^{2+}\) until the effluent pH was the same as the buffer. A flow rate of 150 ml/hr was used which was limited by the capability of the pump. The rRNA solution (see Step III) was flowed into the cartridge. The cartridge was washed with 0.3 M NaCl buffer until its A\(_{260}\) was reduced to 0.2. Finally, the cartridge was washed with 1.0 M NaCl buffer to elute off tRNA, 5S rRNA, and 5.8S rRNA. The elution profile (Figure 19) obtained from ZetaPrep-250 DEAE device was the same as that from classical DEAE-cellulose (Figure 17). The rRNA was then precipitated by addition of 2.5 volume of 95% ethanol pre-cooled to -10°C. The rRNA was allowed to flocculate and settle at 5°C. This work shows that ZetaPrep DEAE ion-exchange is suitable for removal of proteins, SDS, polysaccharides and nucleotides from RNA mixture.

The ZetaPrep cartridge can be reused following regeneration and re-equilibration. The cartridge was regenerated by the washing with 1 M acetic acid (pH 2.7) followed by 0.05 M Na\(_2\)HPO\(_4\) with 1.0 M NaCl (pH 8.2). The above cycle of washes was repeated 1-2 times (3-4 cartridge volumes each) until the effluent O.D\(_{280}\) stabilized at the baseline reading. The cartridge was then re-equilibrated with the
Figure 19: A typical elution profile obtained from ZetaPrep DEAE anion-exchange chromatography.
starting elution 0.3 M NaCl, 10 mM Mg$^{2+}$, 10mM Tris-HCl buffer, pH 7.5 until the effluent was at pH 7.5. The regenerated cartridge was stored at 5°C for future use.

In summary, the ZetaPrep DEAE chromatography has proved to be a very attractive alternative method for rRNA purification compared to traditional DEAE method because the former offers shorter rRNA purification time due to its faster flow rate, no prior DEAE media preparation and column packing. Set-up time is minimal since ZetaPrep cartridges are merely placed in housings and equilibrated.

5. Conventional Soft Gel Sephadex G-75 chromatography

The final purification of 5S rRNA and 5.8S rRNA was accomplished via gel filtration chromatography on Sephadex G-75 column. The RNA mixture isolated from DEAE ion-exchange chromatography contains essentially three main components: a large amount of tRNAs (about 26,000 MW), a small amount of 5S rRNA (about 35,000), and a small amount of 5.8S rRNA (about 52,000). In gel filtration or size-exclusion chromatography, molecules are separated according to their size in a column packed with a porous gel medium. Molecules larger than the largest pores in the swollen gel beads cannot penetrate the gel and are eluted first. Smaller molecules can enter the gel to varying extent depending on their size and shape, hence accordingly retarded on their passage through the column. Molecules are hence eluted in the order of decreasing molecular size.

Sephadex is a bead-formed, cross-linked dextran gel which swells in water and aqueous salt solutions. By varying the degree of cross-
linking, gels of different porosities, and fractionation range can be achieved. Sephadex G-75 was chosen for purification of 5S rRNA and 5.8S rRNA because it has the right fractionation range of 3,000 to 80,000. Because of low resolving power of Sephadex, very long columns are required to fractionate rRNAs. Consequently the total separation time via Sephadex chromatography is typically lasting 3 to 5 days.

The Sephadex G-75 gel medium (Pharmacia) was prepared according to the manufacturer's instructions. The gel was swelled in 10 mM Tris-HCl buffer, pH 7.5 containing 1.0 M NaCl. The 5 cm x 150 cm glass column was purchased from Spectrum Medical Industries, Inc., Los Angeles, and thoroughly cleaned before packing. The column was packed with Sephadex G-75 in accordance to the instructions supplied by Pharmacia. The column was completely equilibrated by passage of at least 6000 ml of degassed buffer (1.0 M NaCl, 10 mM Tris-HCl, pH 7.5). The column was used in conjunction with a LC system comprised of a Beckman double-beam UV photometer (Model 153) with a fixed (254 nm) spectral filter and equipped with a preparative flow cell of 0.5-mm path length with an internal volume in measuring cell of 2µl (Beckman Instruments, Inc, Fullerton, CA, USA), a Fractomette™ Alpha 200 fraction collector (Buchler Instruments, Inc, Fort Lee, NJ, USA), and a Linear chart recorder. The buffer was degassed under vacuum.

Most of the ethanol-water solution was siphoned off and the remaining RNA precipitate-ethanol-water mixture was poured into 50 ml disposable conical Polypropylene centrifuge tubes (Falcon Model 2098) and centrifuged at 3500 x g for 20 minutes using a table-top IEC Model HN-S II centrifuge. The resulting RNA pellet was thoroughly drained
of ethanol and dried by blowing over the pellet with nitrogen gas filtered through a Millex-FG 50 filter unit. The dried rRNA pellet was dissolved in 30-60 ml (1-2% bed volume) of the eluting buffer and filtered through a Millex-GV 0.22 micron filter unit (Millipore, Bedford, MA, USA) before applying to the column. Flow rates along with other chromatographic conditions are given in the Figure legends. Figure 20 shows the separation profile of the RNA extracted at room temperature. The RNA species were well separated from each other. The early eluting fraction (peak 1) is large rRNA and some 5.8S rRNA, while peak 2 corresponds to purified 5S rRNA and the last large peak 3 corresponds to tRNAs. Fractions from peak 2 (5S rRNA) were pooled together and precipitated with 2.5 volumes of pre-cooled 95% ethanol. Chromatography of RNA sample extracted at 40-45 °C from yeast (Figure 21) showed essentially the same elution profile as that obtained at the room temperature (Figures 20). However the peak 2 corresponding to 5.8S rRNA (Figure 21) has increased substantially in the sample extracted at 40-45 °C. Under this temperature conditions the extraction was still specific for 5S rRNA, 5.8S rRNA and tRNA but high amount of cells' high molecular rRNAs was released. Chapter III will discuss the development of a high-speed, preparative gel filtration method for purification of 5S rRNA and 5.8S rRNA via Sephacryl S-300.

6. Sample Purity Check by Polyacrylamide Gel Electrophoresis (PAGE).

Polyacrylamide gels are formed by polymerization of acrylamide and N,N'-methylene-bisacrylamide (BIS). By themselves the acrylamide monomer and cross-linker are stable, but polymerize in the presence of
Figure 20: A complete elution profile of yeast rRNA extracted at room temperature from Sephadex G-75 column (150 cm x 5 cm). The rRNA sample was eluted with 10 mM Tris-HCl buffer, pH 7.5 containing 1.0 M NaCl.
Figure 21: A complete elution profile of yeast rRNA extracted at 40 to 45°C from Sephadex G-75 column (150 cm x 5 cm). The rRNA sample was eluted with 10 mM Tris-HCl buffer, pH 7.5 containing 1.0 M NaCl.
an added free radical system. One method is the addition of a free radical initiator, ammonium persulfate along with a catalyst N,N,N',N'-tetramethylethylenediamine (TEMED).

For the present application, a discontinuous PAGE system was used because the resolution obtainable in a discontinuous system is much higher than that of a continuous one. In the discontinuous system a non-restrictive large pore gel, called stacking is layered on top of a separating gel. Each gel is made with a different buffer, and the tank buffers are different from the gel buffer. In contrast, continuous system has only a single, continuous separating gel and uses the same buffer in the tank and the gel, thus a little easier to set up.

(a) Preparation of PAGE stock solutions

The stock solutions for PAGE were prepared according to Table 3.

Table 3. PAGE Stock Solutions.

<table>
<thead>
<tr>
<th>Stock Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Monomer Solution (30%T, 2.7% Bis)</td>
<td>Acrylamide 58.4 g, Bis 1.6 g, Water to 200 ml</td>
</tr>
<tr>
<td>2. Running gel buffer (1.5 M Tris-Cl pH 8.8)</td>
<td>Tris 36.3 g, Adjust to pH 8.8 with HCl, Water to 200 ml</td>
</tr>
<tr>
<td>3. Stacking gel buffer (0.5 M Tris-Cl pH 6.8)</td>
<td>Tris 3.0 g, Adjust to pH 6.8 with HCl, Water to 50 ml</td>
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4. 8 M Urea and 12 mM EDTA solution.

<table>
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<th>Quantity</th>
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<td>Urea</td>
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<tr>
<td>EDTA</td>
<td>2.2 g</td>
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<td>Water</td>
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</table>

5. Initiator (10% ammonium persulfate)

<table>
<thead>
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<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulfate</td>
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</tr>
<tr>
<td>Water</td>
<td>to 5.0 ml</td>
</tr>
</tbody>
</table>

6. Tank buffer (20 mM Tris-acetate pH 8.0 and 4 M Urea and 1 mM EDTA)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>2.4 g</td>
</tr>
<tr>
<td>Urea</td>
<td>240.2 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.4 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 2000 ml</td>
</tr>
</tbody>
</table>

7. Stain stock (1% methylene Blue)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>to 200 ml (stir and filter)</td>
</tr>
</tbody>
</table>

8. Stain solution (0.2% methylene Blue, 0.4 M sodium acetate and 0.4 M acetic acid)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene Blue</td>
<td>100 ml</td>
</tr>
<tr>
<td>0.4 M sodium acetate</td>
<td>16.4 g</td>
</tr>
<tr>
<td>0.4 M acetic</td>
<td>16.6 ml glacial acetic acid</td>
</tr>
<tr>
<td>Water</td>
<td>to 500 ml</td>
</tr>
</tbody>
</table>

(b) Gel apparatus

A vertical slab gel fabricated unit (Hoefer Model SE 600) was purchased from Hoefer Scientific Instruments, San Francisco. The
SE 600 gels are 14 cm wide x 16 cm long. Each gel was wide enough to run twenty samples at a time. Spacers and combs was 0.75 mm thick. Power supply (Hoeffer PS 1200) for electrophoresis was also purchased from Hoefer. The power supply offers constant voltage, constant current and constant power capability.

(c) Preparation of running and stacking gels

Table 4. Reagents for casting of PAGE slab gels.

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Separating gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer solution</td>
<td>10.0 ml</td>
<td>1.67 ml</td>
</tr>
<tr>
<td>Running gel buffer</td>
<td>7.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>-</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>8 M Urea, 12 mM EDTA</td>
<td>12.5 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
<td>0.005 ml</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>0.15 ml</td>
<td>0.0 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>30.0 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Final gel concentration</td>
<td>10%T 0.9Bis</td>
<td>5%T 0.4Bis</td>
</tr>
</tbody>
</table>

(d) Preparation of Separating Gel

The SE 600 Vertical Slab Gel Unit was assembled in the casting mode. In a 50 ml vacuum flask, 30 ml of separating gel solution was mixed according to Table 4. The ammonium persulfate and the TEMED was left out and a small magnetic stir bar was added. Dissolved gases
were removed by evacuating the stoppered flask for 10 minutes with a water aspirator while stirring with a magnetic stir bar. The TEMED and ammonium persulfate were added at this stage and the solution was mixed well by gently swirling. The solution was pipetted into the sandwich to a level about 4 cm from the top. Water was layered over the gel solution to ensure flat interface and to prevent oxygen in the air from interfering with the polymerization reaction. The gels were allowed to stand for several hours. A total volume of 30 ml gel solution was sufficient to cast two 14 cm x 16 cm x 0.7 mm slab gels.

(e) Preparation of Stacking Gel

In a 50 ml vacuum flask, 10 ml of stacking gel solution was combined according to Table 4. Dissolved gases were removed under vacuum as described above. The top of the solidified gels was thoroughly rinsed with deionized water. The TEMED and ammonium persulfate were then added to the degassed stacking solution. The remainder of the sandwich was filled to the top with stacking gel solution. A comb was inserted into each sandwich with great care to ensure that no bubbles were trapped below the teeth of the comb. Oxygen will inhibit polymerization and will cause a local distortion in the gel surface at the bottom of the well. The stacking gel typically took about one hour to polymerize completely.

(f) Sample Preparation

The RNA precipitate was collected by centrifugation in 1.5 ml conical microcentrifuge tubes on a Fisher Model 59A Microcentrifuge. The residual ethanol was removed from the pellet by draining and
drying under filtered nitrogen gas. The RNA was then dissolved in sample buffer [10% (w/v) sucrose, 5 M urea, 0.5% SDS, and 0.05% phenol blue] and heated for 1 to 2 minutes at 65°C in a constant temperature water bath (Neslab Model Endocal RTE-9B). The RNA sample was cooled to room temperature and stored at 5°C for future use.

(e) Running the Gel

The combs were carefully removed from the gels. Each well was rinsed with distilled water and then filled with tank buffer. The sample was layered onto well with the aid of a Hamilton Syringe. The gel was run at a constant power of 10 Watts per gel slab. The gels were maintained at constant temperature by connecting the heat-exchanger to a water tap. Typically the electrophoresis took 5-6 hours to complete under these conditions.

(g) Staining and Destaining Gels

The sandwiches were disassembled and the gels were fixed in 1.0 M acetic acid for 1 hour. For visualization of RNAs, the gels were stained in methylene blue stain solution in a tray for 1 hour. Destaining was accomplished by repeated washing the stained gels with water until RNA bands became visible. Prolonged destaining in water could result in disappearance of stained RNA bands which could be revisualized by restaining. For rapid destaining, a Hoefer Slab Gel Diffusion Destainer (Model SE 530) was used.

The gels were scanned at 550 nm via a gel scanning device attached to Beckman DU8 spectrophotometer fitted a Gel Scan COMPUSET™
Figure 22: A typical gel scan profile of the polyacrylamide slab gel electrophoresis (14 cm x 16 cm) of 5.8S rRNA purified from Sephadex G-75 chromatography. Purity > 98%.
program. A typical gel scan along with the scanning parameters were shown in Figure 22. This particular scan showed that 5.8S rRNA was at least 99% pure. For storage, slab gels after electrophoresis were preserved using Hoefer Slab Gel Dryer (Model SE 540). The Dryer works on the principle of dry-heat vacuum. The gels were dehydrated and bonded to filter paper or porous cellophane membrane in about 1 hour.

(7) Mini Gel Electrophoresis - a method for rapid screening of rRNAs

A method for rapid electrophoresis of RNA samples would be useful for routine sample screening since two conventional 14 cm x 16 cm gels typically takes about 5-6 hours to run (see section 6). This section is devoted to evaluation of the use of minature slab gel 7 cm x 8 cm for rapid electrophoresis of RNA.

The SE 250, the "Mighty Small II" (Hoeffer), is a mini vertical slab gel unit intended for rapid electrophoresis of protein or nucleic acid samples of small volumes. This unit allows running of two gels simultaneously. Compare to the SE 600 Vertical unit, the buffer requirements for SE 250 are minimal - 75 ml for each upper chamber (versus 400 ml-700 ml in SE 600 unit) and 150 ml (versus 4200 ml in SE 600 unit) for the common lower chamber. Assembly of the SE 250 unit is easy. To date there is no report on the use of mini 7 cm x 8 cm slab gels for rapid and routine screening of 5S rRNA and 5.8S rRNA.

The following protocol for running mini slab gels is similar to the procedures described for 14 cm x 16 cm slab gel in section 6. The 250 unit was assembled in accordance to manufacturer's instructions. A Multiple Gel Caster (Hoeffer Model SE 215) was used for casting of
several gels at one time thus reducing time for gel polymerization. Up to ten 7 cm x 0.75 mm thick gels could be cast simultaneously using the Multiple Caster. The preparation of denaturating gels was as described in section 6. The separating gel was about 5 cm high, the stacking gel, 2.5 cm high. Procedures for sample treatment and application can be found in section 6. Unused gels were wrapped with Saran plastic food wrap and stored at 5°C for up to 2 weeks without loss of resolution for future use. The electrophoresis was carried out at 40 mA Constant Current (20 mA/gel). The electrophoresis typically took 2 to 3 hours to complete. The gel was fixed with 1.0 M acetic acid and stained with methylene blue. It was destained with water. Figure 23 (Top) clearly shows that the three RNA species namely tRNA, 5S rRNA and 5.8S rRNA were well-resolved from each other and the separating power of the mini gel system was comparable to that of the larger gel system. The bottom gel scan in Figure 23 showed that yeast 5S rRNA purified from Sephadex G-75 column was greater than 98%. This work demonstrates that resolving power of mini slab gel is adequate for routine and rapid analysis of rRNAs.

In summary, the main advantages of 7 cm x 8 cm mini gel format over the larger 14 cm x 16 cm gel format for routine rapid separation and visualization of 5S rRNA and 5.8S rRNA are: (a) shorter electrophoresis time (2 to 3 hours versus 5 to 6 hours); (b) minimal tank buffer (75 ml for each upper chamber versus 400 ml-700 ml in SE 600 unit, and 150 ml versus 4200 ml in SE 600 unit for the common lower chamber) and hence lower cost, since less chemicals are needed;
Figure 23: Top: A typical gel scan profile of the mini PAGE format (7 cm x 8 cm) electrophoresis of tRNA, 5S rRNA and 5.8S rRNA showing that the three rRNAs were well-resolved from each other. Bottom: Yeast 5S rRNA purified from Sephadex G-75 chromatography. Purity > 98%.
(c) small gels require only half the amount of the expensive electrophoretic grade acrylamide resulting in substantial saving in operating cost; (d) glass plates replacements are inexpensive (80¢ per 8 cm x 10 cm plate versus $6.50 per 16 cm x 18 cm plate); (d) the use of Multiple caster ensures a ready supply of identical gels; (e) SE 250 vertical mini slab gel unit is very easy to set up.
CHAPTER III

HIGH-SPEED PREPARATIVE-SCALE SEPARATION AND PURIFICATION
OF RIBOSOMAL 5S AND 5.8S RNA'S VIA SEPHACRYL S-300
GEL FILTRATION CHROMATOGRAPHY

A. Introduction

The secondary base-paired structure of 5S and 5.8S RNA has been extensively investigated using techniques such as limited nuclease digestion, oligonucleotide binding, binding to ribosomal proteins, chemical modifications, and optical spectroscopy. All of these techniques require only microgram quantities of RNA. More recent structural studies of these RNAs have been based on physical techniques which require much larger amounts of purified RNA: 10 mg (or 15 mg) per sample of 5S rRNA (or 5.8S rRNA) for proton Fourier transform nuclear magnetic resonance (FT/NMR) spectrometry (Burns et al., 1980; Luoma et al., 1980; Salemink et al., 1981; Kime & Moore, 1983a,b,c; Chang & Marshall, 1986a; Chen & Marshall, 1986; Li & Marshall, 1986), 20 mg/sample for \(^{19}\)F FT/NMR (Marshall & Smith, 1980), 50 mg/sample for \(^{31}\)P FT/NMR (Burkey & Marshall, unpublished results), and 20 mg/sample for differential scanning calorimetry (Li & Marshall, 1985; Chang & Marshall, 1986b). Development of proper conditions for
growth of RNA single crystals for X-ray diffraction analysis may require very large amounts of pure RNA (>100 mg). Although the three-dimensional structure of tRNA has been determined crystallographically (Kim, 1976; Rich, 1977), and confirmed in solution via proton NMR (Reid, 1981) there is as yet no report of successful crystallization of 5.8S rRNA. Preliminary single crystals of 5S rRNA have been reported (Abdel-Meguid et al., 1983; Morikawa et al., 1982) but the crystals were too poor in quality to yield useful X-ray structural information. Thus, there is a continuing need for a rapid, economical method for isolation and purification of multi-milligram quantities of ribosomal RNA's, preferably from initial sample loads of several hundred milligrams of mixed RNA's.

As discussed in Chapter II, "ISOLATION AND PURIFICATION OF YEAST RIBOSOMAL 5S AND 5.8S RIBONUCLEIC ACIDS", ribosomal 5S and 5.8S RNA can be purified via gel filtration column chromatography on Sephadex G-75/G-100 (Li et al., 1984; Monier & Fuenteun, 1971) or by electrophoresis with polyacrylamide gels (Rubin, 1975). However, separations obtained by gel electrophoresis are time-consuming and often result in preparations that are contaminated by polyacrylamide gel material. In addition, electrophoretic separation methods are typically limited to a load capacity of about 50 mg of RNA. Often fractionations obtained by electrophoresis usually result in a substantial loss of materials.

Conventional soft gel materials (such as Sephadex) for aqueous gel filtration have been available for many years for the separation and purification of ribosomal RNA's at either analytical or
preparative scale. However, isolation of milligram quantities of RNA typically requires a very large column, 150 cm long and 2-5 cm diameter (Li et al., 1984; Monier & Feunteun, 1971). Moreover, because soft gels compress readily, they cannot tolerate the high pressure needed for high flow rate. Therefore, RNA separations can take several days of continuous elution.

The feasibility of employing macroporous supports for high performance size exclusion chromatography (HPSEC) of proteins was demonstrated in the mid 1970s using coated or derivatized silicas (Regnier & Noel, 1976; Chang et al., 1976). Recent improvements in the efficiency of aqueous size exclusion supports have lead to HPSEC to offer performance equal to or greater that of soft gels with typically a 100-fold reduction in separation times (Wehr & Abbot, 1979). Low molecular weight RNA samples at microgram (Uchiyama et al., 1981) to 2 milligram (Dornburg et al., 1986) loads recently have been fractionated via high performance size exclusion chromatography (HPSEC) on TSK G 3000/4000 SW columns packed with rigid macroporous silica-based micro-particulates. The silica-based packings have 250 Angstrom and 500 Angstrom pore size, respectively.

HPSEC currently suffers from several major limitations. First, the inherent instability of silica at neutral and alkaline pH limits the column lifetime when physiological buffers are used as the mobile phase. Second, the modest load capacity of analytical columns limits their applicability for large-scale (>5 mg) preparative separations, since the prohibitive cost of preparative HPSEC columns (a Bio-Sil
TSK-250 60 cm x 5.5 cm gel filtration column costs $12,000.00, is available from Bio-Rad Laboratories and associated equipment places them out of reach for most laboratories. Third, separations on HPSEC columns such as TSK-GEL G 3000/4000 SW (Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) are normally conducted under RNA denaturing conditions. The mobile phase contains a detergent such as sodium dodecyl sulfate (SDS) (Uchiyama et al., 1981; Dornburg et al., 1986) whose subsequent removal from the eluted purified RNA fractions requires extensive dialysis. Moreover, the use of denaturing agents may cause undesirable (and possibly irreversible) RNA structural changes.

Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) is a gel material with several features that are potentially attractive for separation and purification of ribosomal 5S and 5.8S RNA's: semi-soft gel with reasonably high mechanical strength, rapid flow rate, and relatively low cost. The stability of the matrix at higher temperatures enables chromatography to be performed in a wide range of temperatures, for instance at 65 °C for the separations of RNA fragments. Sephacryl is prepared by covalently cross-linking allyl dextran with N,N'-methylenediacrylamide to give a rigid gel with a carefully controlled range of porosity. In this work, I investigated the utility of Sephacryl S-300 for preparative (greater than 100 mg) and rapid fractionation of low molecular weight ribosomal RNA's. Sephacryl S-300 was chosen because of its optimal fractionation range of $5 \times 10^3$ to $1 \times 10^6$ for separation of rRNAs. It is important to note that size exclusion columns which give good separation for proteins do not necessarily mean they can resolve RNAs as well.
B. Materials and Methods

1. Isolation of Yeast 5.8S rRNA

Brewer's yeast (Saccharomyces carlsbergensis) cells were generously provided by the Anheuser-Busch Brewery, Inc., Schrock Road, Columbus, Ohio. The isolation protocol was as described in Chapter II. 5.8S rRNA was extracted from yeast cells according to the Rubin procedure (Rubin, 1975). The yeast cells were suspended in a buffer containing containing 20 mM EDTA, 0.1 M sodium acetate, and 1% SDS (pH 5.0). One-half volume of phenol was added, and the mixture was stirred vigorously for 2 hr at 40-45°C. After the phases were induced to separate by centrifugation at 9800 x g, the aqueous phase was removed and reextracted with an equal volume of phenol. RNA was allowed to precipitate at -20°C after addition of 2.5 volume of 95% ethanol. The precipitated RNA consists of 5.8S rRNA, 5S rRNA, tRNA's, and high molecular weight rRNA's. Proteins and other RNA's were first removed from crude RNA mixture by using DEAE-32 ion-exchange (Whatman) chromatography before further fractionation via Sephadex.

2. Fractionation of RNA's via Sephacryl S-300 Chromatography

The liquid chromatography (LC) equipment consisted of an ultraviolet absorbance detector (ISCO Model UA5) equipped with a preparative flow cell of 1 mm light path and 3.7 microliter capacity, a fraction collector (Retriever II™) fitted a diverter valve and a peristaltic pump (WIZ™) to supply solvent at a constant flow rate. All of the LC equipment was purchased from ISCO, Lincoln, Nebraska, USA. Gore-Tex™ macroporous polytetrafluoroethylene (PTFE) 2.0 micron
pore size tubing (Anspec, Ann Arbor, MI) was used to remove any air bubbles from the eluent before entry to the column. The column eluent was monitored at 254 nm. A schematic diagram in Figure 24 depicts the set-up of the LC system.

The chromatographic column (90 x 3.2 cm) fitted with an adjustable adaptor and the packing reservoir were purchased from Amicon, Danvers, MA, USA. The use of adaptor allows simple and reproducible sample application, completely eliminating disturbances of the gel bed and protecting the column from insoluble particles. A water jacket was used to maintain a constant operating temperature. The column of Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden) was packed according to the instructions supplied by the manufacturer. The column and packing reservoir were mounted vertically. About 150 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl was added to the column and any air trapped in the bed support was removed. About 70 ml of eluent (10% of total column volume) was left in the column. The Sephacryl S-300 gel was suspended in eluent buffer (three times the volume of settled gel). The gel was allowed to settle and excess buffer was decanted off. The Sephacryl S-300 was re-suspended in a volume of eluent buffer in a 2 liter Buchner vacuum flask to make a slurry containing about 70% settled gel and 30% excess liquid such that the slurry was not so thick as to retain air bubbles. Dissolved gases were removed by evacuating the stoppered flasks with water aspirator until no evolution of gases were observed. The flask was swirled gently occasionally to facilitate degassing.
Figure 24: A schematic diagram of the LC set-up for Sephacryl S-300 gel filtration chromatography.
The degassed Sephacryl slurry was carefully poured into the column and the gel was allowed to settle for about 10 minutes. The packing reservoir was filled to the top with eluent carefully to avoid disturbing the gel. The packing reservoir was immediately connected to the ISCO WIZ peristaltic pump as shown in Figure 25. The gel was packed using downward flow at a constant flow rate of 145 ml per hour. After about two bed volumes (about 2000 ml of eluent), eluent in the packing reservoir was first siphoned off followed by the removal of the reservoir. The column was filled to the top with eluent before insertion of the flow adaptor so as to avoid air being trapped under the adaptor nylon net. The peristaltic pump was connected to the top of the column for downward chromatography. The column was washed adequately and equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl (approximately 1500 ml) at a constant flow rate of 80 ml per hour. The column was ready for use and connected to the ultraviolet absorbance detector (ISCO Model UA5) equipped with a preparative flow cell of 1 mm light path and 3.7 microliter capacity (Figure 24). Sample was applied via a disposable Luek-lock type syringe connected to a 3-way valve as shown in Figure 24.

The buffer was degassed and filtered through a Model FG-256 HPLC solvent filter-degasser (LAZAR Research Laboratories, Inc., Los Angeles, CA, USA), fitted with a 0.45 micron cellulose nitrate membrane filter (Whatman, Maidstone, England). The buffer was prepared in a 4 liter beaker and filtered and degassed directly to a amber storage bottle. The bottle was immediately sealed with parafilm.
Figure 25: A column set-up for packing of 80 cm x 3.2 cm Sephacryl S-300 column.
3. Fractionation of RNA's via Sephadex G-75 Chromatography

A 5 x 150 cm Sephadex G-75 (Pharmacia) column was used in conjunction with a LC system comprised of a Beckman double-beam UV photometer (Model 153) equipped with a fixed (254 nm) spectral filter and a preparative flow cell of 0.5 mm path length and an internal measured cell volume of 2 microliter (Beckman Instruments, Inc, Fullerton, CA, USA), and a Fractomette™ Alpha 200 fraction collector (Buchler Instruments, Inc, Fort Lee, NJ, USA). The glass column was purchased from Spectrum Medical Industries, Inc, Los Angeles, CA, USA). The Sephadex column was packed under hydrostatic pressure without a pump. The effluent was monitored at 254 nm. Constant flow rate was achieved by adjusting the buffer reservoir height. The Sephadex column was equilibrated by passage of the three bed volumes of the same elution buffer as for Sephacryl S-300. The buffer was degassed and filtered via a LAZAR HPLC solvent filter-degasser. Samples were pipetted onto the column carefully with minimal disturbance to the gel bed.

4. RNA Sample Preparation

An RNA sample (ca 247-259 mg) from which protein had previously been removed via DEAE-32 chromatography was dissolved in 15-30 ml of eluting buffer (10 mM Tris-HCl, 1.0 M NaCl, pH 7.5) and filtered through a Millex-GV 0.22 micron filter unit (Millipore, Bedford, MA, USA) before application to the Sephacryl S-300 column via a syringe connected to a 3-way valve. In contrast, RNA sample (40-50 ml) was pipetted onto the Sephadex G-75 column. Flow rates along with other chromatographic conditions are shown in the Figure legends.
5. Calibration Proteins Preparation

A mixture of calibration proteins consisting of catalase from bovine liver (232,000 MW), albumin from bovine serum (67,000 MW), ovalbumin from chicken egg (43,000 MW), chymotrypsinogen A from bovine pancreas Type II (25,000 MW), and cytochrome C from horse heart (12,384 MW) was prepared by dissolving the proteins in the elution buffer. All proteins were purchased from Sigma Chemical Company, St Louis, MO. The sample was clarified through a Millex-GV 0.22 micron filter unit to remove insoluble proteins. Blue Dextran 2000 (2 x 10^6 MW) (Pharmacia) was used for determining the column void volume. Blue Dextran has limited solubility in the elution buffer.

C. Results and Discussions

1. Demonstration of Asymmetry of 5S and 5.8S rRNA Molecules

The Sephacryl S-300 column was calibrated via running calibration proteins through the column. The column void volume was determined from the elution volume of Blue Dextran 2000 which has a molecular weight of 2,000,000 daltons. Figure 26 shows the elution profiles for calibration proteins on Sephacryl S-300 under varying flow rates and sample loads. The effluent was monitored at 254 nm. Despite that about 100 mg of proteins was loaded, a 0.01 Absorbance setting was used on the UV monitor because a preparative flow cell of 1 mm light path was used. The 80 cm x 3.2 cm Sephacryl S-300 column was capable of resolving a mixture of proteins up to 132 mg at a flow rate of 40 ml/hr as evident from the middle chromatogram in Figure 26. When the
Figure 26: Elution profiles for calibration proteins on 80 cm x 3.2 cm Sephacryl S-300 column under different sample loads and flow rates. Sample loads: 85 mg (Top); 133 mg (Middle); 140 mg (Bottom).
flow rate was increased to 80 ml/hr, resolution decreased for example cytochrome C (peak 6) and chymotrypsinogen A (peak 5) was not as well resolved compared to that obtained at 40 ml/hr. Even at the flow rate of 80 ml/hr, resolution was acceptable for preparative separations.

A calibration curve (Figure 27) was constructed by plotting $K_{av}$ versus log molecular weights. The use of $K_{av}$ has several advantages over other elution parameters such as $V_e$, $V_e/\bar{V}_d$: (a) it is less sensitive to errors which may be introduced as a result of variations in column preparation and column dimensions, (b) it does requires the unreliable determination of the gel internal volume ($\bar{V}_i$) as is required with $K_d$. $K_{av}$ for each protein is given by the equation

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$ (1)

where $V_e$ = elution volume of protein

$V_0$ = column void volume = elution volume for Blue Dextran 2000

$V_t$ = total bed volume

Figure 27 demonstrates that good linearity can be obtained for the relationship between elution volumes and molecular weights of proteins at a flow rate rate of 40 ml/hr on Sephacryl S-300 column. However this linear relationship is not valid for rigid elongated rod-like molecules or non-globular molecules or molecules with extended conformation because of these molecules cannot penetrate the pore of the gel beads. The calibration proteins are all globular.

5S rRNA, 5.8S rRNA and tRNA deviate from the linear calibration curve as shown in Figure 27 with greatest deviation for 5.8S follow by
Figure 27: Calibration Curve using the calibration proteins on Sephacryl S-300.
5S and tRNA. Transfer RNA form L-shaped tertiary structures which account for its non-linear behavior. The extended structure of tRNA molecule is confirmed by X-ray diffraction (Rich, 1977; Kim, 1976). Figure 27 shows that 5S and 5.8S rRNA are even more asymmetric than tRNA. These findings are in agreement with the results from the X-ray scattering at small angles from dilute solutions of yeast and E. coli 5S rRNA which indicate that the 5S rRNA molecule is prolate with an axial ratio of about 5 to 1 (Connors & Beeman, 1972). Boedtker & Kelling (1967) and Comb & Zehavi-Willner (1967) concluded from their sedimentation studies that 5S rRNA is more asymmetric than tRNA which support the results in this work. Also Figure 27 shows that 5.8S rRNA is a more extended molecule than tRNA and 5S rRNA molecules. The conclusions drawn from this gel filtration experiments are that both 5S rRNA and 5.8S rRNA are elongated molecules with 5.8S rRNA having more extended structural features.

In size exclusion chromatography, the resolving power is strongly dependent on the support pore volume ratio (i.e., the ratio of internal pore volume to intersitial volume, $V_i/V_0$). The column with the greater pore volume ratio will provide the best resolution with all else equal (column efficiency, column dimensions). Most macroporous silicas have pore volume ratios from 0.5 to 1.5, somewhat less than that obtainable with traditional cross-linked dextran gel filtration supports like Sephacryl or Sephadex which have the pore volume ratios of up to three (Regnier & Gooding, 1980). From the calibration curve, the apparent molecular weight determined for 5S
rRNA is 104,713 daltons, for 5.8S rRNA is 380,189 daltons, for tRNA is 43,000 daltons. The apparent molecular weights are much larger than their actual respective molecular weights of 35,000, 55,000 and 25,000 daltons. This suggests that support pore volume ratio is a very important factor in selecting column packing materials for RNA fractionation which is in agreement with findings of Regnier & Gooding (1980). Because of the extended structure of RNA, high gel porosity is essential for the penetration of RNA molecules. This explains why not all support materials are suitable for fractionation of rRNAs even though they have high resolving power for proteins.

2. Comparisons between Sephacryl S-300 and Sephadex G 75 Chromatography

All cellular RNAs with the exception of viral RNAs are single-stranded nucleic acids that fold into specific globular conformations depending on their nucleotide sequences in saline solutions. The RNA globular conformation is determined by the pH, ionic strength and temperatures. Intramolecular base pairing and globular conformation are stabilized at neutral pH, low temperature, and in saline solutions of high ionic strength. Although Mg$^{2+}$ which normally stabilizes the globular conformation but it also contributes to peak-broadening. This broadening effect is attributed to interactions among the RNAs, eluent and the column matrix. Additions of denaturants such as urea, SDS or guanidine hydrochloride to the elution buffers can greatly improve resolution but subsequent removal of denaturants from the eluted purified RNA fractions requires extensive dialysis. Moreover, the use of denaturing agents may cause undesirable (and possibly
irreversible) RNA structural changes. The present fractionation via Sepahcryl was performed under non-denaturating conditions. Fractionations are usually more efficient when RNA samples are dissolved in solution of high ionic strength.

Sephacryl S-300 and Sephadex G-75 elution profiles for mixed RNA's extracted from yeast are shown in Figure 28. With Sephacryl S-300 (Figure 28, upper chromatogram), the three smallest RNA species (i.e., 5.8S RNA, 5S RNA and tRNA's) are well resolved. High molecular weight rRNA species eluted at the void volume from the three low molecular weight rRNA species. The Sephadex G-75 chromatogram (Figure 28, bottom chromatogram) shows three well-resolved peaks corresponding to 5.8S RNA, 5S RNA, and tRNA's. The high molecular weight rRNAs are not seen in this particular sample, which was isolated from a different cell lot. From one cell lot to another, major differences in the relative amounts of the various RNA's were observed. The retention volumes for either column were conserved for various RNA sample loads, indicating that RNA adsorption to the column is negligible. Sample recovery was typically greater than 90%, with minor losses arising from the RNA precipitation step.

It is clear from the chromatograms (Figure 28) that phenolic extraction of intact cells leads to preparations in which the high ratio of tRNA to 5S rRNA and 5.8S rRNA makes the purifications of 5S and 5.8S rRNAs very difficult. However the present purification route often yields 5S and 5.8S rRNA preparations with greater than 90% purity without successive chromatographies on Sephacryl S-300 or
Figure 28: Elution profiles of *S. carlsbergensis* RNA extracted from whole cells at 40-45°C. Top: Column: 78 cm x 3.2 cm Sephacryl S-300; sample load: 247 mg in 13 ml eluent buffer; mobile phase: 10 mM Tris-HCl buffer (pH 7.5, 1.0 M NaCl); Flow-rate: 15 ml/hr; Detection: UV 254 nm; temperature: ambient. Bottom: Column: 144 cm x 5 cm Sephadex G-75; sample: 259 mg in 40 ml eluent buffer; mobile phase: same as above; flow-rate: 30 ml/hr; detection: UV 254 nm; temperature: ambient.
Sephadex G 75. The gel scans shown in Figure 29 clearly demonstrate that 5.8S rRNA purified from the smaller Sephacryl S-300 column is
tparable to that obtained from the 4.5 times larger Sephadex G 75
column and also its separation time was 2.5 times shorter.

The present study demonstrates the feasibility of using Sephacryl
S-300 for simple, rapid, economical, high-resolution, high-yield
separation of ribosomal RNA's, based upon sample loads as large as 250
mg or more. Despite of the shorter column employed, the Sephacryl
S-300 fractionation of 5.8S RNA, 5S RNA, and tRNA's compares favorably
to that obtained with a 4.5 times larger Sephadex G-75 column, and was
obtained 2.5 times faster (34 hr vs. 88 hr). 5S rRNA is not quite as
well resolved by Sephacryl S-300 as by Sephadex G-75 in the
chromatograms of Figure 28, but either chromatogram is adequate for
preparative purposes. Use of two Sephacryl S-300 columns in tandem
should in principle extend the RNA sample load to 500 mg.

The main advantages of Sephacryl S-300 over Sephadex for rRNA
fractionation can be summarized as follows: shorter separation times,
higher sample load, and smaller column volume (and thence lower cost,
since less packing material is needed). Compared to preparative gel
electrophoresis, gel filtration chromatography gives poorer separative
resolution of 5S rRNA and 5.8S rRNA, but can handle an order of
magnitude higher sample load without the problems of RNA binding to
the gel, and/or RNA denaturation. Although high performance size
exclusion chromatography (HPSEC) offers higher resolving power than
Sephacryl, preparative-scale HPSEC is much more expensive, both in
Figure 29: Gel scans of PAGE on 5.8S rRNA purified from 144 cm x 5 cm Sephadex column (Top), and 80 cm x 3.2 cm Sephacryl S-300 column (Bottom). Purity of 5.8S rRNA > 98% for both columns.
initial cost and in replacement of contaminated columns. For example a Bio-Sil TSK-250 60 cm x 5.5 cm preparative gel filtration column costs $12,000.00, is available from Bio-Rad Laboratories versus $196.00 for 750 ml of Sephacryl S-300 Superfine gel media which is sufficient to pack a 80cm x 3.2 cm column.

E. Conclusions

This work presents a rapid and economical alternative to Sephadex and high performance size exclusion chromatography (HPSEC) for preparative-scale separation and purification of low molecular weight RNA's: 5.8S RNA, 5S RNA, and tRNA's. These three RNA species can be well resolved from each other and from higher molecular weight RNA species via Sephacryl S-300 gel filtration chromatography under mild eluting conditions: 10 mM Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl. For a sample load of about 250 mg, the resolving power of a Sephacryl S-300 column (78 x 3.2 cm) is comparable to that of a 4.5 times larger Sephadex G-75 column (144 x 5 cm). Moreover, the total separation period is 2.5 times shorter for the Sephacryl method. Up to 500 mg or more of crude ribosomal RNA mixtures could be separated via two Sephacryl S-300 columns operated in tandem.

In addition Sephacryl S-300 gives good linear relationship between molecular weights against elution volumes. The present gel filtration experiments demonstrate the asymmetricity of 5S rRNA, 5.8S rRNA and tRNA molecules. The findings also suggest that 5.8S rRNA molecule has more extensive extended structural features than 5S rRNA.
which in turn more than tRNA. The asymmetricity of 5S rRNA is in agreement with the conclusions from small angles scattering of X-ray on yeast and *E. coli* 5S rRNA (Connors & Beeman, 1972), and from sedimentation studies (Boedtke & Kelling, 1972). Many studies have shown that 5.8S rRNA has different conformation depending of the salt conditions.
A. Introduction

5S and 5.8S rRNA molecules are approximately 120 and 160 nucleotides long and their respective masses are 35,000 and 55,000 daltons. The molecular formula for a typical 5S rRNA (for example E. coli 5S rRNA) is

\[ \text{C}_{1143}\text{H}_{1301}\text{N}_{465}\text{O}_{958}\text{P}_{120} \]

It is clear from the molecular formula that it is difficult to perform \(^1\text{H}\) NMR studies on 5S and 5.8S rRNA molecules because there are about 1300 protons in 5S rRNA molecule and about 1730 protons in 5.8S rRNA molecule. If each of the protons were to give rise to one individual distinct resonance, there would be 1300 and 1730 resonances making NMR assignments virtually impossible. In addition, NMR line-width increases with molecule size which further complicates the matter. The tumbling time of a molecule is directly related to the its molecular weight, hence the linewidths are linear function of molecular size. This linear relationship breaks down for rigid elongated molecules or molecules with rapid local motion independent
of the global tumbling time. In high-molecular weight RNAs, the resonances are too broad to resolve. Therefore, the large number of proton resonances coupled with their broad linewidths led to severe overlapping and unresolved NMR spectra for 5S and 5.8S rRNA molecules.

The breakthrough came when Kearns et al (1971) observed a number of partially resolved proton resonances in the extreme low-field tRNA spectrum in water (i.e., -15 ppm to -9 ppm). These protons resonate at such low-field region originate from base pair hydrogen-bond imino protons. In non-hydrogen bonded systems the imino protons exchange rapidly with water molecules hence do not produce a resonance. However base pair hydrogen-bond imino protons exchange sufficiently slow (greater than 5 msec) that a resonance is detected. The dispersion of chemical shifts between -9 ppm and -15 ppm has been assigned to the upfield sequence-dependent ring current contributions of nearest nieghbor and (to a lesser extent) next-nearest neighbor base pairs. The effect of ring current effects is shown in Figure 30. The free flow of electrons in a purine or pyrimidine placed in a static magnetic field generate a local induced magnetic field that perturbs the resonance position of nearby protons. The magnetic lines of force arising from the induced "ring current" are shown in Figure 30. The methyl group in the lower nucleotide is shifted downfield because the induced magnetic field is in the same direction of the static field (Figure 30). In contrast, the hydrogen-bond proton in the upper base pair is upfield by the induced field of the lower nucleotide which opposes the external static field at this point in
Figure 30: Ring current effects. Top: Induced field ring-current shift effects in stacked nucleotides and their distance. Bottom: The differential upfield shifts on A-U and G-C base pairs in different stacking environments. From Reid et al., (1979).
space. The ring currents of A and G are much more stronger than for pyrimidines i.e C and U (Giessner-Prettre & Pullman, 1970), and hence the spectrum is sequence dependent as shown in Figure 31. The magnitudes of the fields vary as \((1-3 \cos^2 \Theta) \times \frac{1}{R^3}\), where R is the distance away from the center of the base (Bovey, 1969). Hence the effects of the ring current shifts are short range and highly directional. Because of the \(\frac{1}{R^3}\) distance-dependent factor nearest neighbor interactions dominate. Because of the angular dependence, resonances from the protons located directly above the plane of the molecule are shifted upfield, whereas resonances from protons located in the plane of the molecules are shifted downfield. This accounts for the observation that resonances from aromatic protons appear at much lower fields than do resonances from aliphatic protons (Bovey, 1969). In addition, the resonances from the exchangeable ring nitrogen protons start out at lower fields than aromatic protons and move even further downfield, away from the position water resonance. This further downfield shift of resonances is due hydrogen bonding which can be attributed to removal of electron density from the protons involved in the hydrogen bond (Drago et al., 1970; Pimentel & McClellen, 1971; Jaszunski & Sadlej, 1973).

Each G•C and A•U base pair contains one hydrogen-bond imino proton while there are two such protons for a G•U base pair (Figure 32). One would expect to observe one proton resonance from each G•C and A•U base pair and two resonances from a G•U base pair in the -15 ppm to -8 ppm region of the spectrum (Figure 31). An A•U base pair
Figure 31: A typical low-field 5S rRNA NMR spectrum in water showing the resonance positions of A·U, G·C and G·U pairs, the ring carbon amino protons (NH2) and the ribose protons.
Figure 32: The A-U, G+C and G+U base pair geometry and the protons which are closest to the imino protons. From Early et al., (1981).
typically resonates around -15 ppm to -13.5 ppm, a G•C base pair resonates between -13.5 ppm and -12 ppm, and a G•U base pairs resonates at -12 ppm to -11 ppm (Figure 31). In addition to A•U and G•C base pairs resulting from hydrogen bonds with different chemical shifts, nearby stacking interactions are also reflected in the chemical shifts. From the measurement of chemical shifts positions, intensities and linewidths, NMR provides information about the types and number of bases in a particular environment, and the lifetime of a particular base pair. In this manner, studies of rRNA in H₂O can provide a wealth of useful structural information such as all the secondary and tertiary hydrogen bonds. This makes NMR such a powerful and definitive tool in the investigation of rRNA secondary structure.

In order to observe these exchangeable imino protons, ¹H NMR spectra must recorded in H₂O rather than D₂O because such protons undergo rapid deuterium exchange. A typical RNA nmr sample contains about 110 M water protons and the RNA protons are usually less than 1 mM. The detection of ¹H NMR signals from dilute RNA molecules in aqueous solutions presents several major obstacles: (a) the spectrum of interest is obscured by the intense water resonance; (b) severe dynamic range problems since both intense H₂O signal and weak RNA signal are present together in the free induction decay (FID); (c) inadequate ADC (analog-to-digital converter) resolution; (d) rounding errors in discrete FT; (e) problems with phasing and baseline curvature; (f) digitization problems imposed by a limited computer word length. Various experimental techniques of water suppression are
available to circumvent the above difficulties associated with intense 
H$_2$O signal include tailored excitation (Tomlinson & Hill, 1973), 
correlation spectroscopy (Dadok & Sprecher, 1974), rapid scan FT NMR 
Gupta et al., 1974), selective excitation (Redfield, 1978; Morris & 
Freeman, 1978; Wright et al., 1981; SKlenar & Starcuk, 1982; Plateau & 
Gueron, 1982; Hore, 1983a,b). In general only those approaches 
involving selective pulse sequence that excite only the region of 
interests and has zero spectral density at the H$_2$O resonance are 
useful in the study of rapidly exchangeable protons. This is because 
such methods do not involve perturbation of the exchangeable proton 
resonances by processes such as magnetization transfer. Among such 
methods include single long pulse (Alexander, 1961), composite soft 
2-1-4 pulses (Redfield et al., 1975), hard time-shared 1-3-3-1 pulse 
sequence (Hore, 1983a,b; Turner, 1983).

For the present $^1$H NMR studies on 5S and 5.8S rRNA, only Redfield 
2-1-4 composite soft pulse (Redfield et al., 1975) and 1-3-3-1 hard 
pulse (Hore, 1983a,b; Turner, 1983) were employed.

B. Redfield 2-1-4 Pulse Sequence

The duration of the pulse in FT NMR determines the width of 
excitation in frequency units as shown in Figure 33. If the pulse is 
ininitely short, the excitation bandwidth is infinitely wide. A wide 
region is excited when a short pulse (i.e., hard pulse) is used. In 
contrast if the the pulse time is increased (i.e., long pulse), only a 
small area is excited but greater energy concentrated over that region 
because the excitation amplitude (peak to peak power) is concentrated
Figure 33: Top: The relation of duration (time) of pulse to width of excitation (frequency). Bottom: The single soft long pulse method for suppression of the intense water signal.
over a small fraction of the time-domain excitation period. Hence Figure 33 shows that in order to record $^1H$ NMR spectra of RNA in $H_2O$, a long soft pulse is desirable. Ideally if the rf (radio frequency) is centered at 12.2 ppm and a correct pulse duration is chosen (in this case 360 microseconds), the spectral density is exactly zero at the center of the water resonance (Figure 33). This single long pulse although can lead to substantial water suppression as only the "wing" of the water resonance is excited but suffers from two major limitations. First, in the final spectrum the water line is still intense. Second it fails to provide a flat power to excite the area of interests causing nonlinearity of phases and baseline curvature.

Redfield et al., (1975) achieved a better water nulling by dividing a long pulse into ten segments by a ratio of 2-1-4-1-2 and having the third and seventh phase-shifted changed by 180° as shown in Figure 34. This pulse sequence can be viewed as a simple long pulse of amplitude $H_1$, minus two short pulses of amplitude 2$H_1$. Even with this pulse sequence, the water resonance is only suppressed 500-1000 times. In practice, instrumental imperfections such as errors in producing exact 180° phase-shifted pulses, spatial inhomogeneity of the static $B_0$ field and $B_1$ inhomogeneity, thus reduce the effectiveness of water nulling. In addition it requires that the carrier frequency to be at 0.97τ away from the water resonance. Since the spins resonating exactly at the carrier frequency are flipped 90°, and the water spins are not flipped at all, it is obvious that other spins in between will be flipped by various angles. Hence
Figure 34: Redfield 2-1-4 pulse sequence. A: A single soft long pulse with a total pulse length of 10\(t\) in the time domain and its FT into frequency domain. B: Two 180° phase-shifted pulses and its FT. C: The resulting 2-1-4 pulse from the addition of pulses in A and in B and its FT. D: Comparison between a single soft long pulse and the Redfield 2-1-4 pulse.
a linear phase correction restores the proper phase at the center position of each peak correction, but fails to correct for dispersion components far from the centers of peaks, and consequently leads to base-line undulation results from phase shift of the intense residual water peak. In practice, it is necessary to optimize the "4" pulse and the last "2" pulse length, phase shifts, delays and transmitter frequency in order to achieve maximal water suppression.

C. Binomial 1-3-3-1 Hard Pulse Sequence

The maximum suppression of the water peak obtainable via 2-1-4 pulse sequence is a factor of 500-1000 which still leaves a large residual water resonance in the resulting spectrum. Since intense residual water signal leads to a severely distorted base-line, a new pulse technique for water suppression is sought. Recent reports have discussed the use of a new pulse sequence designed to selectively suppress excitation of an intense water signal while allowing simultaneous detection of peaks at either side of the water peak. This sequence is known as 1-3-3-1, uses a series of four pulses of relative lengths 1,3,3,1 with a short interpulse length interval. The second and fourth pulses in the sequence are shifted by 180° relative to the first and the third pulses. The rf carrier frequency is set to fall directly on the water peak. Thus the sequence is

\[ \alpha(x) - \tau - 3\alpha(-x) - \tau - 3\alpha(x) - \tau - \alpha(-x) - \text{ACQ} \]  

The centers of the low field and high field are given \( \frac{1}{2}\tau \), while the effective angle at these points is generated by the sum of the individual pulses, or \( 8\alpha \). The major advantage of the 1-3-3-1 method
is the ease of implementation since the rf carrier is set so as to fall directly on the water peak. Another advantage is that solvent cancellation is not sensitive to the exact intensity of the pulses, for which long-term stability is therefore not necessary. However in the Redfield 2-1-4 method, a variation of the pulse amplitude in the 2-1-4 sequence changes spectral position which corresponds to cancellation. In FT-NMR, strong and short pulses are often more easily obtainable than weak and long ones. Using 1-3-3-1 method, spectral regions on both sides of the solvent are excited similarly and can therefore be observed in the same experiments.

The Fourier transform of the 1-3-3-1 pulse sequence is shown in Figure 35. It is clear from Figure 35 that the 1-3-3-1 method produces sufficiently flat power over the region of interests and broad flat near-zero spectral density at the the water resonance position. This method produces a more superior water suppression up to a factor of 35,000 than the 2-1-4 pulse sequence.

D. Base Pairs Assignments from Double-Resonance: Homonuclear Overhauser Enhancements (NOE)

Nucleic acids which have been investigated by proton homonuclear Overhauser enhancement experiments include tRNA species among which are tRNA^Phe (Johnston & Redfield, 1979) and tRNA^Asp (Roy & Redfield, 1981) from yeast, and tRNA^Val from E. Coli (Hare & Reid, 1982a,b). This method has since been employed for identification of several base paired secondary structural segments of ribosomal 5S RNA's from prokaryotes (Kime & Moore, 1983a,b; Kime et al., 1984) and this work
Figure 35: Excitation spectrum of the binomial hard 1-3-3-1 pulse sequence. From Hore (1983).
reports the first NMR and NOE studies on 5.8S rRNA from yeast.

1. Theory of NOE

The Overhauser effect is the change in integrated intensity of the resonance signal(s) of one set of nuclei as a result of the saturation of another set of nucleic by a strong resonant RF field. It is a dipole-dipole phenomenon in which magnetization is transferred through space from proton to an adjacent proton. It is caused by changes in the populations of nuclear polarization of the monitored nucleic coupled to the saturated nucleic, caused by spin-lattice relaxation ($T_1$) mechanism. The NOE is best understood in terms of spin relaxation of a two spin states (Noggle & Schrimer, 1971).

The energy level diagram for a two spin-system, $I$ and $S$ in which the spins $I = S = 1/2$ is shown in Figure 36. $I$ and $S$ are not J coupled but are in proximity to one another that dipole-dipole coupling between the two will contribute to spin-lattice relaxation. The spins can be aligned against the static magnetic field, indicated by a $\alpha$ subscript, or in the direction of the static field (the $\beta$ subscript), with latter being the energetically more favorable situation. The four possible transition probabilities needed to describe spin relaxation during an NOE experiment are indicated in Figure 36. $W_I$ represents the single quantum transition probability that spin $I$ will go from from $I_\alpha$ to $I_\beta$ (or $I_\beta$ to $I_\alpha$) while the state spin of $S$ remains unchanged. $W_S$ represents the single quantum transition probability for spin $S$ when spin $I$ remains unchanged.
Figure 36: An energy level diagram of a two spin system consisting of two nuclear spins, I and S. The subscripts, α and β denote the alignment of the spins against and in the direction of the applied magnetic field respectively. The W terms refer to the transition probabilities between specified energy levels. From Smith (1980).
superscript indicates the spin undergoing the quantum transition. The zero quantum transition probability, $W_0$, is for both spins $I$ and $S$ undergoing transitions of the type $I_\alpha S_\beta \rightarrow I_\beta S_\alpha$ or $I_\beta S_\alpha \rightarrow I_\alpha S_\beta$. Lastly, $W_2$ is the two quantum transition probability for the two spins aligned in the same direction to relax simultaneously in the same direction. That is, transition of the type $I_\alpha S_\alpha \rightarrow I_\beta S_\beta$ or $I_\beta S_\beta \rightarrow I_\alpha S_\alpha$. The resonance of nucleus $S$ is saturated so the spin population of levels 1 equals 2 and 3 equals 4. This results in perturbation of spin populations in all the energy levels from their equilibrium values prior to saturation. Consequently the system will attempt to return to the equilibrium populations in those levels. $W_1$ causes relaxation between the energy levels $1 \rightarrow 2$ and $3 \rightarrow 4$ while $W_2$ between $1 \rightarrow 3$ and $2 \rightarrow 4$. Neither $W_1$ or $W_2$ transitions are effective in altering populations levels since these levels are at equilibrium. However, $W_2$ attempts to re-establish the equilibrium population by increasing the population of energy level 4 through a corresponding decrease in energy level 1. This transition leads to a higher population in the lower spin state of nucleus $I$ and, consequently, to an net enhancement of the absorption intensity of spin $I$. $W_0$ will redistribute the populations between energy levels 2 and 3 which at equilibrium are equal. The results are a decrease in the population of energy level 3 and an increase in the population of energy energy 2. Its net effect is to decrease the intensity of the nmr resonance of the $I$ spins. Hence $W_0$ and $W_2$ transitions have opposing effects when one of the spins is saturated.
The fractional enhancement of the integrated intensity of spin I when spin S is saturated is given:

\[ f_I(S) = \frac{W_2 - W_0}{2W_1 + W_0 + W_2} \cdot \frac{\gamma_S}{\gamma_I} \]  

where \( \gamma_S \) and \( \gamma_I \) are the respective gyromagnetic ratios of spins S and I. The numerator, \( W_2 - W_0 \), is called the cross-relaxation term. The change in intensity is the result of cross-relaxation between the observed and the irradiated nucleic. The magnitude of cross-saturation of any particular neighbor depends on its proximity to the saturated proton (\( r^{-6} \)) and on the number of proximal protons in the immediate vicinity that are being simultaneously cross-saturated. The marked distance dependence (\( r^{-6} \)) limits the first-order NOE (in the absence of further spin diffusion) to distance in the 1-4 Å range, and it is used mainly to relate pairs of resonances that may be greatly separated in the spectrum but are very close to each other in space.

The fractional enhancement can be expressed in terms of molecular correlation time, \( \tau_C \) (Balaram et al., 1972) is given:

\[ f_I(S) = \frac{5 + \omega^2 \tau_C^2 - 4 \omega^4 \tau_C^4}{10 + 23 \omega^2 \tau_C^2 + 4 \omega^4 \tau_C^4} \]  

For small molecules in which the dipole-dipole relaxation mechanism is the exclusive means of relaxation, the \( f_I(S) \) is given:

\[ f_I(S) = \frac{\gamma_S}{2\gamma_I} \]
For protons the maximum enhancement will be 0.5 or 50%, the maximum intensity observable is $1 + 0.5$ of normal, and the effect is quite small. If $\gamma$'s are very different, as in the case for example that $\text{H}^1$ is irradiated and another nucleus observed, then the enhancement can be substantial as shown in Table 5 for maximum nuclear Overhauser effects for several pairs of nucleic.

Table 5. Maximum nuclear Overhauser effects for several pairs of nucleic (Martin et al., 1980)

<table>
<thead>
<tr>
<th>Irradiate</th>
<th>$1^\text{H}$</th>
<th>$13^\text{C}$</th>
<th>$15^\text{N}$</th>
<th>$19^\text{F}$</th>
<th>$29^\text{Si}$</th>
<th>$31^\text{p}$</th>
<th>$1^\text{H}$</th>
<th>$13^\text{C}$</th>
<th>$19^\text{F}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observe</td>
<td>$1^\text{H}$</td>
<td></td>
<td>$13^\text{C}$</td>
<td>$15^\text{N}$</td>
<td>$19^\text{F}$</td>
<td>$29^\text{Si}$</td>
<td>$31^\text{p}$</td>
<td>$1^\text{H}$</td>
<td>$13^\text{C}$</td>
</tr>
<tr>
<td>$f_1(S)_{\text{max}}$</td>
<td>0.5</td>
<td>1.99</td>
<td>-4.93</td>
<td>0.53</td>
<td>-2.52</td>
<td>1.24</td>
<td>0.47</td>
<td>1.87</td>
<td>0.5</td>
</tr>
<tr>
<td>$1 + f_1(S)_{\text{max}}$</td>
<td>1.5</td>
<td>2.99</td>
<td>-3.93</td>
<td>1.53</td>
<td>-1.52</td>
<td>2.24</td>
<td>1.47</td>
<td>2.87</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Since $\gamma$ can be negative for example $15^\text{N}$ or $29^\text{Si}$, the enhancement can be negative which gives rise to negative signal.

For large molecules such as 5S or 5.8S rRNA molecules, the NOE is negative. In such cases, $\omega^4 \tau_c^4 > 1$ and according to equation 3, the $f_1(S)$ is negative. For homonuclear proton-proton interactions, the limiting negative enhancement is -1, which means the nmr resonance signal is completely disappeared. The negative NOE for 5S and 5.8S rRNA molecules are more understood from the plot of NOE intensity versus $\log \omega \tau_c$ (Figure 37) with $\omega$ is the larmor frequency (ca. $3 \times 10^9$ rad/sec at 500 MHz) and $\tau_c$ of the molecule (ca $1 \times 10^{-8}$ sec for 5S rRNA). For 5S rRNA molecule, $\log \omega \tau_c$ is about 1.5 which shows that
Figure 37: Plot of the homonuclear NOE in a two-spin system, $f_I(S)$ versus $\log \omega \tau_c$. $\omega$ denotes NMR larmor frequency, and $\tau_c$ is the molecular correlation time. From Rothner-By (1979).
the NOE is not only negative but also very small and close to complete nulling of the NOE signal. For slow rotation ($\tau_C > 7.1 \times 10^{-10}$ sec) $f_1(S)$ is negative ($\log \omega \tau_C = 0.32$) since transition from positive $f_1(S)$ to negative $f_1(S)$ occurs at about $\log \omega \tau_C = 0.08$. Fast tumbling ($\tau_C < 7.1 \times 10^{-10}$ sec) where $W_2 > W_0$ will results in positive enhancement that is an increase in the signal due to I spins when the S spins are saturated.

2. Base Assignments via NOE

Redfield and cowokers pioneered the use of NOE for investigation of the secondary and tertiary of tRNA's, who for the first time observed NOE's between the two hydrogen bonded imino-protons in the G•U basepair present in the acceptor stem of yeast tRNA$^{Phe}$ (Johnston & Redfield, 1978). There are several excellent review articles on the application of NOE to RNA (Reid, 1981; Redfield & Kunz, 1979).

What kind of information are obtainable from NOE experiments? First, the primary NOE from an irradiated base pair hydrogen-bond imino proton to other protons in the same base establishes the identity of the base pair type (A•U, G•C, G•U). For example the two hydrogen-bonded ring NH protons (imino protons) in a G•U pair are strongly dipolar coupled due to their close spatial proximity (ca. 2.5 A; bottom figure of Figure 32) and both protons typically exchange slowly ($< 5$ s$^{-1}$) with H$_2$O. Hence, saturation of either the G or U imino proton in a G•U pair produces a large mutual NOEs as shown in the lower two spectra of Figure 38. The enhancement produced by irradiation of the G imino proton is larger than that by irradiation
Figure 38: Characteristic NOE patterns for different base pairs, A•U, G•C, and G•U pairs. Top: NOE difference spectrum of a G•C base pair with broad resonances from the aromatic amino protons in the -6 to -9 ppm region. Second to the top: NOE difference spectrum of an A•U pair which exhibits a narrow resonance from C2-H proton of adenine at about -7 to -8 ppm region. Bottom two: NOE difference spectra of a G•U pair which exhibit two large mutual NOEs with the enhancement produced by irradiation of G imino proton larger that that by irradiation of the U imino proton.
of the U imino proton (Hurd & Reid, 1979; Johnston & Redfield, 1981; Hare & Reid, 1982a). This diagnostic NOE behavior is very useful for identification of resonances belonging to G•U pair in the nmr spectrum. Each A•U pair has a adenine aromatic proton (C2-H proton) near in space to the uridine imino proton (ca. 3 Å; Top, Figure 32) Irradiation of an A•U (second to the top spectrum, Figure 38) imino proton exhibits a narrow and fairly large NOE resonance from the C2-H proton of adenine at about 7 - 8 ppm, as well as other broader resonances from the aromatic amino protons in the 6 - 9 ppm region. In a Watson-Crick type G•C pair only the amino protons (NH2 protons) are spatially close to the guanine imino proton (ca. 2.4 -2.6 Å; middle figure in Figure 38). A G•C is easily distinguishable from A•U and G•U pair since saturation of a G•C imino proton produces NOE to broad amino protons in the -6 to - 9 ppm region as shown in the topmost spectrum of Figure 38. The broad aromatic NH2 proton resonances are due relaxation of 14N. The above discussions show that each base pair type i.e. A•U, G•C and G•U exhibits a unique and distinguishable intra-basepair primary NOE pattern thus enables identification of base type in 5S rRNA and 5.8S rRNA using NMR spectroscopy.

Second the marked distance dependence (r^-6) limits the second-order NOE (in the absence of further spin diffusion) to distance in the 1 -4 Å range, hence can identify the hydrogen-bonded imino protons from adjacent base pairs i.e. base-pairs above and below the irradiated base pair. Hence inter-basepair secondary NOEs can be used
to relate pairs of resonances that may be greatly separated in the spectrum but are very close to each other in space. For example the top two NOE difference spectra in Figure 38, clearly establish a connection between an A-U and a G-C, from the small interpair "secondary" NOE observed for either peak when the other is irradiated. Thus by sequentially irradiating the imino protons connected by NOEs, one obtains a linear "base pair sequence", provided that all of the resonances are well-resolved and single. The NOE-determined base pairs segment provides information on the secondary as well as tertiary structures of 5S and 5.8S rRNAs. Unquestionably NOE is a very powerful and definite tool in the investigation of rRNA since intra-base "primary" and inter-base "secondary" NOEs provide the most direct identification of base-pair type and base-pair sequence respectively.

E. Experimental NMR Procedures

1. Redfield 2-1-4 Method

NMR spectra at 500MHz (11.75 tesla super-conducting magnet) were acquired on a Bruker AM-500 FT-NMR spectrometer with quadrature detection and four-phase cycling, without sample spinning, at room temperature (23°C). Normal (i.e non-NOE) spectra were obtained via a modified Redfield 2-1-4 pulse sequence (Redfield & Kunz, 1979), to flip over the downfield protons without affecting water protons. The carrier frequency was centered at -15.0 ppm (i.e. 5200 Hz from the water resonance frequency at -4.8 ppm). A total pulse length of 196 microsecond was used in conjunction with an acquisition time of 0.1
second. Transients were digitized to 16 bits resolution and accumulated into 4K channels of 24 bit memory. A relaxation delay of 0.5 second between successive acquisitions was used. The duration of the "4" pulse and final "2" pulse were adjusted with the "magic knobs" to give maximal water suppression. Usually, 2000 to 10,000 scans were signal-averaged and all final spectra gave a baseline that required only slight baseline flateining. Before Fourier transformation and phase correction, spectra were resolution enhanced by Lorentzian to Gaussian transformation. Chemical shifts are measured relative to the solvent H\textsubscript{2}O peak and converted to the DSS (3-trimethylsilyl-l-propane sulfonic acid) reference from an independent calibration. Downfield shifts are defined as positive.

2. Hard 1-3-3-1 Method

The non-NOE spectra were also acquired via a 1-3-3-1 pulse sequence (Hore, 1983a,b) with carrier frequency centered at the water resonance with a 50 microsecond delay between pulses, and "1" flip angle equals 11.25° (or 8 microsecond; a 90° pulse equals 64 microsecond). Usually 2000 scans were sufficient to give adequate signal/noise. The final spectra were usually sufficiently flat that require minimal baseline correction.

3. Homonuclear Overhauser Enhancement

Nuclear Overhauser enhancements were carried out using the following pulse sequence:

\[ [(RD - Dec(t_1, \omega_A) - FID(+))_{32} - (RD - Dec(t_1, \omega_{off}) - FID(-))_{32}]_n \]
The first cycle of this sequence started with a selective pre-irradiation pulse of duration $t_1$ (0.5 to 0.6 second) was applied by the $^1$H decoupler at the chosen resonance frequency $\omega_R$. This was followed by a "Redfield 2-1-4 observation pulse" and the resultant FID is recorded in a 4 K channel. A 2 millisecond delay was inserted between switching off the pre-irradiation and the triggering the observe pulse, the Redfield 2-1-4 pulse. The second cycle was exactly equal to the first except that the pre-saturation pulse was applied at an off resonance position $\omega_{off}$. The NOE difference spectrum was obtained by alternate addition and subtraction of time-domain data set with simultaneously alternation between on-resonance and off-resonance irradiation. The NOE data were stored in 4 K data set.

A total of 16,000 time-domain transients (8,000 on-resonance and 8,000 off-resonance) were typically acquired in order to observe distinguishable small "secondary" inter-base NOEs from background noise in the final NOE difference spectrum.

Relatively low decoupler power (0.50-0.05 mW, corresponding to a bandwidth of 21.4-7.6 Hz) was used for selective saturation (pre-irradiation period 0.5 - 0.6 second, to prevent extensive spin diffusion) of the resonance of interest, with off-resonance irradiation at 18 ppm. NOE difference spectra were apodized to give a line-broadening of 10 Hz in order to enhance the signal-to-noise ratio. In some cases NOE difference spectra were subjected to exponentially time weighted resolution enhancement to resolve components of a multi-peak envelope. NOE experiments were carried out
at 22°C unless otherwise stated, in order to prevent too rapid chemical exchange of hydrogen-bonded imino-protons with water protons.

4. NMR Samples without Mg$^{2+}$

Most of the ethanol-water solution was siphoned off and the remaining RNA precipitate-ethanol-water mixture was poured into 50 ml disposable conical Polypropylene centrifuge tubes (Falcon Model 2098) and centrifuged at 3500 x g for 20 minutes using a table-top IEC Model HN-S II centrifuge. The resulting RNA pellet was thoroughly drained of ethanol and dried by blowing over the pellet with nitrogen gas filtered through a Millex-FG50 filter unit (Millipore Corporation). The dried yeast 5S rRNA or 5.8S rRNA pellet was dissolved in about 1 to 2 ml buffer containing 10 mM EDTA, 100 mM NaCl, 10 mM sodium cacodylate, pH 7.0, and heated to 65°C for 10 min to remove Mg$^{2+}$ and other metal ions especially paramagnetic metal ions. Heating was conveniently carried out in a constant temperature water-bath (Neslab Model Endocal RTE-9B). The solution was allowed to cool slowly to room temperature and then dialyzed against 1 mM EDTA, 100 mM NaCl, and 10 mM sodium cacodylate, pH 7.0, at 5°C with one change of buffer. The dialysis tubing was purchased from Spectrum Medical Industries, Inc., Los Angeles. The tubing was boiled in deionized water for several minutes followed by rinsing thoroughly with deionized water to remove residual sulfur and any ribonuclease that might be present. The dialyzed yeast 5S and 5.8S rRNA samples were concentrated via AMICON "Centricon 10" microconcentrator (AMICON Corporation, Danvers, MA) to give a final concentration of 20 mg/ml to 63 mg/ml for 5S rRNA.
and 56 mg/ml for 5.8S rRNA. D$_2$O (Merck) was added to give a final 5% D$_2$O concentration, to provide a 2D field-frequency lock signal. All NMR 5S rRNA and 5.8S rRNA samples were checked for degradation by PAGE electrophoresis. Only samples with greater than 88% purity were used for NMR experiments.

About 0.5 to 0.6 ml of the dialyzed and concentrated RNA samples depleted of Mg$^{2+}$ were pipetted into 5 mm NMR tubes. Vortex plugs were not used. The 5 mm NMR tubes were purchased from Wildmad (Royal Imperial Model 528 PP) and from Norell (Ultra-Precision Model 508 UP). Both NMR tubes gave satisfactory performance for high-resolution 500 MHz $^1$H FT-NMR experiments however with the Norell tubes priced considerably cheaper. All NMR tubes were boiled in deionized water containing "Liquinox" detergent for 1 hour and then rinsed very thoroughly with deionized water. The cleaned tubes were then baked in an electric oven at 110 to 120 °C for several hours. This was to ensure the removal of any ribonuclease that would degrade RNA into fragments. The NMR samples can be stored at 5 °C for several months without any degradation.
A. INTRODUCTION

5.8S rRNA belongs to a class of small RNA molecules (including transfer-RNA (tRNA)) that function in protein synthesis at the ribosome. The structure and function of the smallest RNA, tRNA, is relatively well understood for two reasons. First, the three-dimensional structure of tRNA has been determined crystallographically (Rich 1977; Kim, 1976) and confirmed in solution via proton NMR (e.g., Reid, 1981). Second, tRNA is normally found in the free cytoplasm; thus the tRNA solution structure should give a good starting point for describing tRNA bound to the ribosome during peptide chain elongation. In contrast, 5.8S rRNA is normally bound to the ribosome, and its three-dimensional structure has not yet been attempted crystallographically or via proton NMR.

Eukaryotic 5.8S rRNA is thought to be hydrogen-bonded to higher molecular weight ribosomal RNA's (in yeast, to the 25S rRNA from the large ribosomal subunit (Lo & Nazar, 1981)). 5.8S rRNA can be
released from whole ribosomes or ribosomal subunits by application of
either heat or denaturants (urea, formamide, dimethylsulfoxide)
without simultaneous release of ribosomal proteins (Nazar, 1978;
Giorgini & Delucca, 1976). Nazar (1978) showed that yeast 5.8S rRNA
can be reconstituted efficiently into 60S ribosomal subunits from the
same species. The ease with which 5.8S rRNA can be dissociated and
reincorporated back into ribosomal subunits suggests that 5.8S RNA is
probably located on or near the surface of the ribosome (Lo & Nazar,
1982; Liu et al., 1983). The region of interaction between 5.8S and
28S rRNA in mouse ribosomes appears to be between the 3'-terminus of
the 5.8S rRNA molecule and a Watson-Crick complementary segment of the
28S rRNA molecule, and is probably stabilized by the presence of a
GC-rich arm near the 3'-terminus of 5.8S RNA (Walker & Pace, 1983;
Walker et al., 1982; Peters et al., 1982; Nazar & Sitz, 1980).
Although the role of 5.8S RNA in protein synthesis remains as elusive
as that of other rRNA's, it has been suggested that 5.8S rRNA is
involved in the binding of aminoacyl-tRNA to the ribosome (Ulbrich,
1982; Erdmann, 1976).

The first determination of the primary structure of a 5.8S rRNA
was first reported by Rubin (1973) for yeast. About 38 5.8S rRNA
nucleotide sequences have since been reported (Erdmann & Wolters,
1986). All 5.8S rRNA molecules have 157-160 nucleotides, contain
modified nucleotides, and shown a high degree of sequence homology.

The presence of 5.8S rRNA secondary structural segments can be
inferred from limited nuclease digestion (Fujiwara & Ishikawa., 1982;
Wildeman & Nazar, 1981; Darlix & Rochaix, 1981 Mackay & Doolittle, 1981; Mackay et al., 1980; Olsen & Sogin, 1982; Khan & Maden, 1976; Nazar et al., 1975), Raman spectroscopy (Luoma & Marshall, 1978a,b), chemical modifications (Liu et al., 1983; Lo & Nazar, 1982; Kelly & Maden, 1980; Kelly et al., 1978), and optical and fluorescence experiments (Van et al., 1977). Based upon consideration of maximal base pairing using Tinoco rules (Gralla & Crothers, 1973), several models for the secondary structure of 5.8S rRNA in solution have been proposed (Rubin, 1973; Nazar et al., 1975; Luoma & Marshall, 1978a; Ford & Mathieson, 1978; Uris et al., 1982). All of these models are largely compatible with the above-listed experimental results. Based upon the above experiments, it has not yet been possible to establish a universal secondary structure for ribosomal 5.8S RNA's.

Proton NMR and homonuclear Overhauser enhancement (NOE) experiments constitute the method of choice for identification and assignment of base pairs in RNA's in solution. First demonstrated for tRNA's (Johnston & Redfield, 1981; Heerschap et al., 1982; Heerschap et al., 1983a,b; Hare & Reid, 1982 a,b; Roy & Redfield, 1983), the method has since been employed for identification of several base-paired secondary structural segments of ribosomal 5S RNA's from both prokaryotes (Kime & Moore, 1983a,b; Kime et al., 1984; Chang & Marshall, 1986a) and eukaryotes (Li & Marshall, 1986; Chen & Marshall, 1986). This work reports the first NMR and NOE studies on 5.8S rRNA, including temperature-dependent melting experiments. Evidence for the existence of the common G·C-rich arm is presented here.
B. Materials and Methods

1. Isolation and purification of yeast 5.8S rRNA

Brewer's yeast (Saccharomyces carlsbergensis) cells were generously provided by the Anheuser-Busch Brewery, Inc., 700 Schrock Road, Columbus, OH. 5.8S rRNA was extracted from the cells according to the procedure of Rubin (1975). Specifically, yeast cells were suspended in a buffer containing 20 mM EDTA, 0.1 M sodium acetate, and 1% SDS (pH 5.0). One-half volume of phenol was added, and the mixture stirred vigorously for 2 hr at 40 to 45°C. The phases were separated by centrifugation at 9800 x g, and the aqueous phase removed and reextracted with an equal volume of phenol. RNA was then allowed to precipitate overnight at -20°C after addition of 2.5 volumes of 95% ethanol. The precipitated RNA consisted of tRNA's, 5S rRNA, 5.8S rRNA, and other large rRNA's. Purification via ion-exchange (DEAE-32) and gel filtration (Sephadex G-75) chromatography yielded 5.8S rRNA of purity >92% as determined from 10% acrylamide gel electrophoresis.

2. Preparation NMR Samples

Yeast 5.8S rRNA was dissolved in 10 mM EDTA, 100 mM NaCl, 10 mM sodium cacodylate, pH 7.0, and heated to 65°C for 10 min to remove metal ions. The solution was allowed to cool slowly to room temperature and then dialyzed against 1 mM EDTA, 100 mM NaCl, and 10 mM sodium cacodylate, pH 7.0, at 5°C with one change of buffer. The final RNA concentration was adjusted to 56 mg/ml by concentrating the dialyzed RNA sample via ultrafiltration via an Amicon Centricon. D2O
was added to give a final 5% D2O concentration, to provide a 2D field-frequency lock signal. For details refer to Chapter II.

3. NMR Spectroscopy

For experimental details refer to Chapter IV.

C. Results and Discussions

Figures 39, 40 and 41 shows three proposed secondary base-pairing schemes for 5.8S rRNA, each adapted to the primary nucleotide sequence of *S. cerevisiae*. Laser Raman investigations of 5.8S rRNA led to the proposal of cloverleaf secondary structure for eukaryotic 5.8S rRNA (Luoma & Marshall, 1978). Originally the Nazar model was proposed for Novikoff hepatoma ascites based on maximal base pairing and the results of partial ribonuclease digestion (Nazar et al., 1975). The Rubin and cloverleaf models share a common "helix III"; the Nazar and cloverleaf models share most of a common stalk which pairs the 3'-and 5'-termini together; and all three models share a common G.C-rich arm variously labeled as "IV" or "e". Helix I in Rubin model is not base-paired as opposed to the other models.

1. Absence of 5.8s rRNA multimers in NMR samples

Figure 42 (top) shows the proton NMR spectrum of yeast 5.8S rRNA (ca. 1 mM = 56 mg/ml), in 10 mM cacodylate, 100 mM sodium chloride, 1 mM EDTA, pH 7.0, in the absence of Mg²⁺. The broad "humps" labeled as A (13.5-14.5 ppm) and K (10.8-11.2 ppm) correspond to the respective regions in which AU and GU base-pair hydrogen-bond imino protons are normally found. In order to ascertain whether these broad
Figure 39: Rubin proposed the first secondary structural model for an eukaryotic 5.8S rRNA (S. cerevisiae).

Figure 40: The secondary structure of yeast 5.8S rRNA adapted from the model proposed by Nazar, Sitz, and Bush (1975) for 5.8S rRNA based on limited ribonuclease digestion on Novikoff hepatoma 5.8S rRNA.
Figure 41: The cloverleaf model derived from laser Raman studies on yeast 5.8S rRNA, and was then adapted to mammalian sequence (Luoma & Marshall, 1978).
Envelopes were due to homogeneous broadening arising from long rotational correlation times associated with RNA aggregates resulting from too-high RNA concentration, the RNA sample was diluted to a concentration of 31 mg/ml. The close similarity between the proton NMR spectra of the dilute sample (Figure 42, bottom) and the concentrated sample (Figure 42, top) shows that the broad spectral features at A and K are due either to superposition of several unresolved resonances or rapid exchange of base-pair protons with H₂O rather than to RNA aggregation. Rapid exchange might be expected from rapid opening and closing of the least stable helix (e.g., helix I with 7 A·U and 3 G·U base pairs in the cloverleaf or Nazar models of Figures 39, 40, 41), particularly since Mg²⁺ had been removed from our RNA NMR samples. This is in accord with limited pancreatic and T₁ ribonuclease digestion (Wildeman & Nazar, 1981) that helix I in isolated 5.8S rRNA exists only under high salt conditions in the presence of Mg²⁺. Further evidence come from intermolecular contacts involving the 3'-and 5'-termini of 5.8S RNA in the 5.8S/28S complex from mouse (Walker & Pace, 1983; Pace et al., 1977; Walker et al., 1983; Walker et al., 1982).

The terminal "stem" helix (labeled as "I" or "a" in the models) is predicted to be weakly paired (< 5 kcal/mole stability free energy) from the calculations of the relative thermal stabilities of various base-paired arms of the 5.8S rRNA secondary structure using Tinoco's rules (see section 3 for details). These calculations are in full agreement with the above nmr conclusions for rapid opening and
Figure 42: 500 MHz proton NMR spectra of yeast 5.8S rRNA at two RNA concentrations. Top: 56 mg/ml. Bottom: 31 mg/ml. All NMR samples contained 95%/5% H2O/D2O, 100 mM NaCl, 1 mM sodium EDTA, 10 mM sodium cacodylate, pH 7.0.
closing of the weakly base paired terminal helix under the relatively low salt conditions (100 mM NaCl) used in this NMR experiments.

2. Multi-stage unfolding of 5.8S RNA

Differential scanning calorimetry (DSC) has revealed multi-stage heat-induced unfolding of tRNA's (Privalov & Filimonov, 1978) and 5S RNA's (Li & Marshall, 1985; Chang & Marshall, 1986b). Analysis of the DSC curves into a sum of two-state melting stages yields component enthalpies of melting, some of which can be interpreted in terms of thermodynamic stabilities calculated from Tinoco rules (Gralla & Crothers, 1973) for particular base-paired helices in those molecules. The differential thermal melting curve for yeast 5.8S RNA in the absence of Mg$^{2+}$ has been analyzed into 3-5 transitions (Maruyama et al., 1983); however, the component enthalpies of melting for each stage correlate poorly with those calculated for individual helices of any of the base-pairing schemes shown in Figures 39, 40, 41. In both the DSC and differential thermal melting experiments, one monitors a single parameter (heat capacity or optical absorbance) which is a sum of effects from the whole molecule. From 500 MHz proton NMR at several temperatures, on the other hand, one can monitor RNA unfolding simultaneously at each of the many (ca. 15-20) resolved peak positions in the spectrum.

Figure 43 shows the stepwise melting of yeast 5.8S rRNA, as reflected in the stability of its base-pair hydrogen bonds detected via 500 MHz proton NMR spectrometry. The close similarity between NMR spectra taken before and after exposure of yeast 5.8S rRNA to high
Figure 43. 500 MHz proton NMR spectra of yeast 5.8S rRNA at several temperatures.
temperature (91°C for 30 min or 61°C for 48 hr) is clearly evident in Figure 44. Since the NMR spectrum is highly sensitive to any changes in base-pairing, the results of Figure 44 show that a near-identical base-pair structure is regained after heating and cooling the 5.8S rRNA. Because the observed structural changes are reversible, I can confidently analyze the thermal melting of yeast 5.8S rRNA in terms of chain unfolding rather than chain breaking. Similar results have been seen for 5S RNA's in the absence of Mg²⁺ (Li & Marshall, 1985; Chang & Marshall, 1986b).

Although 5.8S rRNA from rat or chicken liver is known to dimerize at ≥ 67.5°C at RNA concentrations as low as 0.8 mg/ml from gel electrophoresis (Sitz et al., 1978) and hydrodynamic (Behlke et al., 1985) experiments, yeast 5.8S rRNA was found to dimerize only to a small extent (ca. 5%) independent of incubation temperature. Consistent with those results, the present NMR results find no NMR evidence for the formation of multimers of yeast 5.8S rRNA at elevated temperature at the RNA concentrations used for NMR experiments.

3. Calculated thermal stabilities of 5.8S rRNA base-paired helical segments

In order to identify particular proton NMR resonances from the temperature-dependence of their linewidths and intensities, I must first estimate the relative thermal stabilities of various base-paired arms of the 5.8S rRNA secondary structure. Free energies calculated from Tinoco rules (Gralla & Crothers, 1973) are listed in Table 6 for each of helical arms of the three secondary structures shown in
Figure 44. Reversibility of yeast 5.8S rRNA heat-induced unfolding. Top: Initial spectrum at 30°C. Middle: Spectrum for sample which had been heated to 91°C for ca. 30 min and then allowed to cool to 31°C. Bottom: Spectrum for sample which had been maintained at 61°C for 48 hr and then allowed to cool to 30°C.
Figures 39, 40, 41. The entries in Table 6 should not be taken too literally, because (a) the stability for tertiary structural elements (currently unknown) cannot be estimated, (b) fraying at the ends of the short helical segments may affect the helical stability, and (c) cooperative folding can result in well-resolved melting transitions that do not correspond to independent melting of individual isolated helical segments, (d) stability contribution of G-U pair is unknown.

Nevertheless, an important prediction from Table 6 is that the most stable helix (ca. 20 kcal/mole stability free energy) is the GC-rich arm which is common to all three of the secondary base-pairing schemes. By comparison, the "stem" helix (labeled as "I" or "a" in the models) is predicted to be weakly paired (< 5 kcal/mole stability free energy), in accord with limited pancreatic and T1 ribonuclease digestion (Wildeman & Nazar, 1981) and evidence for intermolecular contacts involving the 3'- and 5'-termini of 5.8S RNA in the 5.8S/28S complex from mouse (Walker & Pace, 1983; Pace et al., 1977; Walker et al., 1983; Walker et al., 1982).

4 Identification of GC-rich arm base pairs from high-temperature proton NMR

Figure 43 shows 500 MHz proton FT/NMR spectra of yeast 5.8S rRNA as a function of temperature. As the temperature increases, each of the base pair hydrogen-bonded imino proton resonances begins to lose intensity and broaden due to increased rate of chemical exchange with water. Loss of intensity or "melting" in the NMR spectrum occurs when the lifetime of a particular base-pair with respect to opening drops
Table 6. Free energies of stability for individual helices computed from Tinoco rules (Gralla & Crothers, 1973) for three proposed secondary structures of yeast 5.8S ribosomal RNA.

<table>
<thead>
<tr>
<th>Helix</th>
<th>$\Delta G$, kcal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cloverleaf model (Luoma &amp; Marshall, 1978)</td>
</tr>
<tr>
<td>I</td>
<td>-2.0 (including bulges &amp; interior loop)</td>
</tr>
<tr>
<td>II</td>
<td>-8.4 (including hairpin and bulge)</td>
</tr>
<tr>
<td>III</td>
<td>-5.1 (including hairpin, bulges and interior loops)</td>
</tr>
<tr>
<td>IV (GC-rich arm)</td>
<td>-20.6 (including hairpin loop)</td>
</tr>
<tr>
<td></td>
<td>Burp-gun model (Nazar et al., 1975)</td>
</tr>
<tr>
<td>a</td>
<td>-3.1 (including bulges &amp; interior loop)</td>
</tr>
<tr>
<td>b</td>
<td>-15.4 (including 16-base bulge)</td>
</tr>
<tr>
<td>c</td>
<td>-3.4 (including hairpin loop)</td>
</tr>
<tr>
<td>d</td>
<td>-1.1 (including hairpin loop &amp; bulge)</td>
</tr>
<tr>
<td>e (GC-rich arm)</td>
<td>-20.6 (including hairpin loop)</td>
</tr>
<tr>
<td></td>
<td>Rubin model (Rubin, 1973)</td>
</tr>
<tr>
<td>I</td>
<td>(bases not paired)</td>
</tr>
<tr>
<td>II</td>
<td>-5.3 (including hairpin loop)</td>
</tr>
<tr>
<td>III</td>
<td>-5.1 (including hairpin, bulges and interior loops)</td>
</tr>
<tr>
<td>IV (GC-rich arm)</td>
<td>-20.6 (including hairpin loop)</td>
</tr>
</tbody>
</table>
to 1 to 5 msec (Reid, 1979; Kearns, 1976; Crothers et al., 1974).

The "melting-out" of a particular base pair proton resonance therefore reflects both an equilibrium effect (i.e., fraction of the time that a base-pair proton spends in its base pair rather than on an H$_2$O molecule) and a kinetic effect (increased line-broadening with increasing off rate constant for chemical exchange). DSC and optical melting curves, on the other hand, measure the equilibrium fraction of bases that are unpaired or unstacked, respectively.

Since the GC-rich arm is predicted to be highest in thermodynamic stability (Table 6), it is reasonable to expect that it should unfold at a higher temperature than other secondary structural segments. Moreover, NMR peaks arising from adjacent secondary base-pairs in the same helical segment are expected to melt at similar temperature. My strategy is therefore to pick out proton resonances which persist at the highest temperatures as belonging to G·C-rich arm base-pairs, and then use proton homonuclear Overhauser enhancement experiments (at lower temperature) to identify (e.g., A·U, G·C, or G·U) those peaks and assign them to particular primary nucleotide sequence positions.

In contrast to 5S rRNA's, whose base-pair hydrogen-bond imino proton resonances typically melt out completely at ca. less or equal 60°C, yeast 5.8S rRNA exhibits at least seven well-resolved imino proton resonances which persist at 61°C (peaks C, E, E$_1$, F, H, J, and L in Figure 43f). Peaks B and D are partly melted but still visible at 61°C while peaks C, E, E$_1$ and F do not broaden until 81°C.

The G·C-rich arm with a net stability of -21 kcal/mole should be the last secondary feature to melt at high temperature. Integration
of the 61°C spectrum (Figure 43f) yields approximately 8 base-pair protons, in reasonable agreement with the 10 base-pair protons (the G·U pair contributes 2 NMR-observable base-pair protons) expected for the G·C-rich arm. The absence of 1-2 NMR-observable base-pair protons could be due to end-fraying (Boyle et al., 1980; Heus et al., 1983) of terminal G·C base pairs, G\textsubscript{116}·C\textsubscript{137} and/or G\textsubscript{124}·C\textsubscript{129}.

5. Assignment of G·C-rich arm base pairs from temperature-dependence proton NMR spectra

Based upon their exceptional thermal stability, the four well-resolved resonances remaining at 71°C (peaks C, E, E\textsubscript{1}, and F in Figure 43g) almost certainly correspond to the four consecutive G·C base pairs, C\textsubscript{117}·G\textsubscript{136} to C\textsubscript{120}·G\textsubscript{133} of the G·C-rich arm. Based upon its relatively large downfield chemical shift (13.45 ppm) and somewhat lower melting temperature, peak B can tentatively be assigned to U\textsubscript{122}·A\textsubscript{131}. Similarly, peak D might be a terminal G\textsubscript{116}·C\textsubscript{137} or G\textsubscript{124}·C\textsubscript{129}, since terminal base pairs are much more susceptible to opening than internal base pairs.

The simultaneous broadening at 71°C of the three resonances J, L, and H (Figure 43g) suggests that they may lie close together in the next most stable segment of the G·C-rich arm (see Figure 41). Peaks J/L can be assigned to U\textsubscript{121}·G\textsubscript{132}, based on their common melting temperature and their chemical shifts which fall in the 10.0-12.5 ppm range which is typical for G·U imino protons (Johnston & Redfield, 1978; Chang & Marshall, 1986a). Peak H would then most likely be G\textsubscript{123}·C\textsubscript{130} because of its common melting with the adjacent U\textsubscript{122}·A\textsubscript{131}.
base pair (peak B) and next-nearest U_{121}·G_{132} base pair (peaks J/L).

The difference spectrum between 61°C and 71°C (Figure 45, middle), clearly shows that peaks C and E_1 begin to melt before peaks E and F. It might therefore anticipate that peaks C and E_1 represent the two end-most remaining base pairs (C_{117}·G_{136} and C_{120}·G_{133}) of the four consecutive G·C's in the G·C-rich arm. The above assignments can next be confirmed via proton NOE experiments (see below). Finally, scrutiny of the spectra from lower temperatures (Figure 43a-e) reveals a multi-stage (rather than totally cooperative) melting process. However, in the absence of more complete identification and assignment of the base-pair protons involved, it is not in a position to analyze those changes in terms of the unfolding of particular secondary helical segments confidently.

When the temperature increases to 30°C the hump at K disappears (its chemical shift, 11.2 to 10.8 ppm is indicative of G·U basepairs), and peak A splits into 2 peaks, and peaks A1 and A4 sharpen. It is speculative that the disappearance of peak K at such low temperature is due the melting of the unstable molecular stalk, helix (I) which contains 3 GU base-pairs in Luoma & Marshall's model. At 35°C peak C is shifted and a new peak C1 starts to appear. At this point peaks D and G has just begun to broaden. Further temperature increases to 41°C results in peaks D and G been severely broadened (Figure 43d). At same time peak C and peak C1 becomes fully resolved. The improvement in resolution of some linewidths upon heating to 51°C is attributed to a more flexible structure at the higher temperature due to viscosity effects and hence increased molecular tumbling.
Figure 45. 500 MHz proton NMR difference spectra between yeast 5.8S rRNA spectra acquired at different temperatures. Top: room-temperature spectrum. Middle: Difference spectrum between 61°C and 71°C. Bottom: Difference spectrum between 71°C and 81°C. A negative peak in the difference spectrum corresponds to a resonance which melts away in proceeding from the lower to the higher temperature.
6. Base-pair sequencing of the GC-rich arm via proton NOE connectivity

NOE difference spectra provide for identification (A•U, G•C, or G•U) and next-nearest base-pair assignment for RNA base pairs. The identification as U_{121}•G_{132} from temperature-dependence (Figure 43f) affords an excellent point for base sequencing using NOE technique. In particular, a G•U pair affords a good starting point because of its relatively upfield chemical shift, and the strong NOE's between its two NMR-observable imino hydrogen-bond base-pair protons. The strong mutual NOEs observed via irradiation of peaks J and L (Figures 46b,c) clearly confirm their prior identification as U_{121}•G_{132} from temperature-dependence (Figure 43f). As usual for G•U pairs in tRNA's (Hurd & Reid, 1979; Johnston & Redfield, 1981; Hare & Reid, 1982a,b) and 5S rRNA's (Li & Marshall, 1986; Chang & Marshall, 1986a; Chen & Marshall, 1986), the enhancement from irradiation of the G imino proton (peak L in this case) is larger than that from irradiation of the U imino proton (peak J).

Figures 46b,c also show weak NOE connectivity from peaks J/L to peaks E/I (12.66 ppm) and B (13.47 ppm). Peak B is clearly an A•U base pair, based upon its narrow adenine-C2 NOE difference peak at 6.94 ppm (Figure 46d). Moreover, the A•U at peak B gives the expected weak NOE coupling back to peak J (11.73 ppm). Since there is only one A•U pair in the GC-rich arm, and since NOE shows that the A•U is next to U_{121}•G_{132}, peak B must be U_{122}•A_{131}.

Peaks E/E_1 include several overlapping hydrogen-bond proton resonances. Nevertheless, Figures 47b,c show that irradiation at
Figure 46. Proton 500 MHz FT/NMR spectrum (a) and proton homonuclear Overhauser difference spectra (b-f) resulting from irradiation of peaks J, L, B, H, and G at 22°C. In each case, the irradiated peak is denoted by a star (*), and decoupler power is shown at the right.
peaks E and E\textsubscript{1} (35 Hz apart, with E\textsubscript{1} at 12.66 ppm) gives clear NOE's to peaks J/L (11.71/10.77 ppm) and C (13.24 ppm). The reverse experiments (Figures 46b,c and 48d) reveal that peaks J/L are NOE-coupled to peak E\textsubscript{1}, whereas peak C is NOE-coupled to peak E. Since peak E\textsubscript{1} shows primary NOE behavior characteristic of a G-C imino proton, I can now extend our base-pair sequence to C\textsubscript{120}\cdot G\textsubscript{133} (E\textsubscript{1}) - U\textsubscript{121}\cdot G\textsubscript{132} (J/L) - U\textsubscript{122}\cdot A\textsubscript{131} (B). It is interesting that irradiation of this G\cdot U elicits NOE's to base pairs on both sides of the G\cdot U, since NOE's have previously been observed only to the 3'-side of G in a G\cdot U pair (Heus et al., 1983; Chang & Marshall, 1986a).

Next, since peaks C and E are both G\cdot C's (Figures 47b,c and 48d,e), and since both are stable to 71°C (Figure 43g), they must correspond to two consecutive base pairs from the G\cdot C-rich arm. Furthermore, since peaks C and E are adjacent G\cdot C's; since peaks C, E, E\textsubscript{1}, and F are the most temperature-stable base pair protons in the G\cdot C-rich arm; and since peak E\textsubscript{1} is C\textsubscript{120}\cdot G\textsubscript{133}, it is reasonable to infer that peaks C, E, E\textsubscript{1} and F correspond to the tightly stacked segment of four consecutive G\cdot C pairs: C\textsubscript{117}\cdot G\textsubscript{136} - C\textsubscript{118}\cdot G\textsubscript{135} - C\textsubscript{119}\cdot G\textsubscript{134} - C\textsubscript{120}\cdot G\textsubscript{133}. In agreement with that assignment, Figures 49b,c show that the NOE connectivity between peaks C and E is conserved at higher temperature (55°C), even at low irradiation power.

It is next necessary to decide the order among G\cdot C pairs corresponding to peaks C, E, E\textsubscript{1}, and F. The possibilities are: C-E-F-E\textsubscript{1}, E-C-F-E\textsubscript{1}, F-C-E-E\textsubscript{1}, and F-E-C-E\textsubscript{1}. Since irradiation at peak C gives an NOE difference peak at E but not at peaks E\textsubscript{1} or F, all but...
Figure 47: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, produced as in Figure 46, for peaks E1 and F.
Figure 48 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, produced as in Figure 46, for peaks D and C.
the first arrangement are ruled out, leaving C₁₁₁₇•G₁₃₆ (C) - C₁₁₁₈•G₁₃₅ (E) - C₁₁₁₉•G₁₃₄ (F) - C₁₂₀•G₁₃₃ (E₁) - U₁₂₁•G₁₃₂ (J/L) - U₁₂₂•A₁₃₁ (B) as the only possible NOE-consistent assignment.

Another argument in favor of peak C as C₁₁₁₇•G₁₃₆ is its relatively downfield chemical shift (see Table 7); a G•C pair stacked in between G•C's on either side would be expected to lie more upfield than 13.24 ppm (Arter & Schmidt, 1976). It is not possible to confirm the E-F-E₁ connections directly via one-dimensional NOE, because of too-close overlap between those resonances (see Figures 47b-f and 49b,c).

There are 2-3 possible locations for peak H, the most stable (Figure 43f) remaining unassigned resonance attributed to the GC-rich arm: G₁₁₆•C₁₃₇ (adjacent to peak C), G₁₂₃•C₁₃₀ (adjacent to peak B), and G₁₂₄•C₁₂₉ (terminating into the hairpin loop (see Figure 41)). Direct irradiation at peak H (Figure 46e) is not especially informative, since H represents a superposition of at least two resonances. However, irradiation at peak B (Figure 46d) gives a difference peak at H and J. Although irradiation at peak C also gives a small difference peak at H (Figure 48d), the same spectrum shows a small difference peak at J, which is four base pairs distant from the base pair previously assigned to C. Therefore, it would appear that irradiation centered at peak C spills over partly to peak B, which is already known to be NOE-coupled to peak J (and L); moreover, at 55°C (a temperature at which peak B has virtually melted out) irradiation at peak C gives only an NOE difference peak at E, with no difference
Table 7. Assignments for imino proton resonances from the GC-rich arm of yeast 5.8S rRNA, and approximate (+ 5°C) temperature for onset of melting for that base pair.

<table>
<thead>
<tr>
<th>Resonance</th>
<th>Chemical Shift (ppm)</th>
<th>Base pair</th>
<th>Melting Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>-12.85</td>
<td>G116•C137</td>
<td>41</td>
</tr>
<tr>
<td>C</td>
<td>-13.24</td>
<td>C117•G136</td>
<td>71</td>
</tr>
<tr>
<td>E</td>
<td>-12.70</td>
<td>C118•G135</td>
<td>81</td>
</tr>
<tr>
<td>F</td>
<td>-12.57</td>
<td>C119•G134</td>
<td>81</td>
</tr>
<tr>
<td>El</td>
<td>-12.66</td>
<td>C120•G133</td>
<td>71</td>
</tr>
<tr>
<td>J/L</td>
<td>-11.70/-10.77</td>
<td>U121•G132</td>
<td>51</td>
</tr>
<tr>
<td>B</td>
<td>-13.47</td>
<td>U122•A131</td>
<td>51</td>
</tr>
<tr>
<td>H</td>
<td>-12.05</td>
<td>G123•C130</td>
<td>61</td>
</tr>
</tbody>
</table>
Figure 49: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5.8S rRNA, for peaks E, F, and C, produced as in Figure 46 but at 55°C.
peak at H and J (Figure 49d). Based upon these results, I assign peak H as G_{123} \cdot C_{130} (adjacent to peak B), consistent with the common melting temperature of peaks H and J/L (which is one base pair removed from peak H in the G-C-rich arm).

From here on, further assignments must be viewed as somewhat speculative. Irradiation of the remaining resonance which is stable at high temperature (peak D in Figures 43e,f) gives a difference peak at C which is likely due in part to power spillover (since the difference peak at C is much reduced when irradiation power is decreased in Figure 48c), but which suggests that Peak D could be G_{116} \cdot C_{137}. The remaining G-C-rich arm base pair, G_{124} \cdot C_{129} evidently has lower thermal stability and is not NMR-visible at elevated temperatures.

In summary, this work has combined 500 MHz proton NMR temperature-dependence and homonuclear Overhauser enhancements to identify and assign 8 of the 9 base pairs in the GC-rich arm of yeast 5.8S rRNA: G_{116} \cdot C_{137} (D) - C_{117} \cdot G_{136} (C) - C_{118} \cdot G_{135} (E) - G_{119} \cdot G_{134} (F) - C_{120} \cdot G_{133} (E_1) - U_{121} \cdot G_{132} (J/L) - U_{122} \cdot A_{131} (B) - G_{123} \cdot C_{130} (H). This work constitutes the most direct evidence to date for the existence of a G-C-rich arm which is common to most currently popular secondary structural models for 5.8S rRNA. Table 7 lists the assignments for each base pair, along with the melting onset temperature for each peak. As expected resonance D, assigned as terminal G_{116} \cdot C_{137}, exhibits the lowest melting temperature. The next most stable base pairs are peaks B and J/L, assigned as the adjacent
A-U and G-U pairs in the G-C-rich arm, consistent with the lower expected stability of A-U and G-U compared to G-C base pairs. The opening of the G-U and A-U pairs would be expected to weaken the adjacent G_{123}·C_{130}, which displays the next highest melting temperature. The four consecutive G·C pairs are all extremely temperature-stable, but the two interior pairs melt at a slightly higher temperature than the two exterior pairs of that segment. The overall conclusion is that the G·C-rich arm melts from either end, and interior A-U and G-U pairs melt before interior G·C pairs.

Future experiments on 5.8S rRNA's might be directed at comparing different biological species with (slightly) different primary nucleotide sequences and/or isolation of enzyme-cleaved fragments (Lightfoot, 1978) as aids in identifying other base-paired helical segments of the molecule.
CHAPTER VI

A NOVEL METHOD FOR BASE-PAIR ASSIGNMENTS VIA SPECIFIC PROTON NMR LINE BROADENING IN MORPHOLINO-SPINLABELLED RIBOSOMAL 5S RNA AND 500 MHZ PROTON HOMONUCLEAR OVERHAUSER ENHANCEMENTS

A. Introduction

Proton homonuclear Overhauser enhancements experiments have successfully identified almost all the secondary and tertiary base pairs in several transfer ribonucleic acids (Heerschap et al., 1982; Heerschap et al., 1983a,b; Hare & Reid, 1982a,b; Roy & Redfield, 1983; Johnston & Redfield, 1981; Reid, 1981; Schimmel & Redfield; 1980 Kearns, 1976). However the extension of this direct and powerful nmr technique (NOE) for determining base-paired sequences to 5S RNA presents several major problems: (a) the three-dimensional structure of tRNA has been determined crystallographically (Rich, 1977; Kim, 1976) whereas the secondary structure for 5S rRNA is still unknown. Although single crystals of 5S rRNA have been reported but the crystals were of poor quality to yield any useful structural information (Abdel-Meguid et al., 1983; Morikawa et al., 1982); (b) there are about 120 base pairs in 5S rRNA against 80 base pairs in a typical tRNA resulting in greater overlapping of base pair hydrogen bond imino proton resonances in the -9 ppm to -15 ppm region; (c) the
higher molecular weight of 5S rRNA leads to broader $^1$H nmr resonances and longer longitudinal magnetic relaxation, $T_1$.

Various strategies have been used for RNA nmr spectral assignments: ring current prediction (Hurd & Reid, 1979; Shulman et al., 1973; Kime et al., 1984); isolation of enzymatic cleavage fragments (Boyle et al., 1980; Kime and Moore, 1983a,b,c; Kime et al., 1984; Li and Marshall, 1986; Chen & Marshall, 1986); spectral comparisons between RNA's of similar primary sequence (Rordorf et al., 1976; Hurd and Reid, 1979; Chen & Marshall, 1986); NOE's; and change in salt concentration or temperature to induce differential shifts in selected resonances (Heerschap et al., 1982; Heerschap et al., 1983a,b; Kime and Moore, 1983a,b).

Although the semi-empirical ring current approach has proved quite helpful in predicting the chemical positions of base pair imino proton resonances in tRNA (for example tRNA$^{\text{Phe}}$; Johnston & Redfield, 1981) but not for 5S rRNA for three major reasons: (a) all RNA helices are assumed to have the same conformation (A') in the ring current calculations. This assumption works quite well for tRNA's since its 3-dimensional structure has been solved. The conformation of 5S rRNA has yet to be established; (b) tertiary interactions are not known in 5S rRNA; (c) G·U base pairs which are common in 5S rRNA, are rare in tRNA. The chemical shifts induced by a G·U base pair upon other base pair hydrogen-bond imino protons are not well understood. Hence previous workers treated the G·U pair as if it were a G·C pair (Reid et al., 1979; Johnston & Redfield, 1981). In most cases this
assumption is questionable since the three-dimensional structure of tRNA^Phe reveals that a G•U pair can distort the RNA from its basic A-helix conformation (Mizuno & Sundaralingam, 1978).

Proton NMR characterization of purified RNA fragments obtained via specific enzymatic cleavage (Kime & Moore, 1983a,b,c; Kime, 1984; Li & Marshall, 1986; Chen & Marshall, 1986) offers a promising approach, provided that the fragments retain the structure of the intact 5S rRNA molecule. Differential chemical shifts induced by temperature have been successfully exploited in the present work (see Chapter V) to demonstrate the existence of the exceptionally thermal stable, common G•C-rich arm in yeast 5.8S rRNA. The use of melting data to assign resonances in 5S rRNA is difficult due to the lack of any exceptional thermally stable helical segment.

This work reports a novel and a potential alternative approach to base pair sequencing by NMR. This new approach is based on site-specific spin-labelling that produces proton NMR line broadening according to the electron-proton separation and hence aids in identifying base pairs that are spatially close to the spin-label. The magnitude of the nmr line broadening varies as inverse sixth power of the interproton separation. Thus this broadening is short range, up to 15 Å. In this work a paramagnetic nitroxide spin-label, morpholino spin-label (4-amino-2,2,6,6-tetramethyl piperidine-1-oxyl) was directly attached at the 3'UCUA-end of the yeast 5S rRNA molecule. From the broadened nmr resonances it is able to identify and assign the terminal base pair, G1•C120 and its adjacent base pair, G2•U119 in
the terminal helix. These two identified base pairs provide a good reference point for further sequencing of the terminal helix in which eight of the nine base pairs that constitute the terminal helix were identified.

B. Materials & Methods

1. Isolation and purification of yeast 5.8S rRNA
   Refer to Chapter II for experimental details.

2. Spin-labelling of yeast 5S rRNA with TEMPO-NH2
   (Morpholino Spin Label)
   Yeast 5S rRNA was spin-labelled with 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO-NH2) according to the modified procedure described for tRNA by Caron Dugas (1976). TEMPO-NH2 was purchased from Eastman Kodak, Rochester, N.Y. The reaction scheme is shown in Figure 50. About 40 mg of pure yeast 5S rRNA was dissolved in 6 ml of 1.0 M sodium acetate buffer (pH 5.0) containing 20 mM sodium periodate to generate a terminal dialdehyde RNA species. This method was shown to be specific for the labelling 1',2'-diol end group in RNA (Caron & Dugas, 1976; Hanske, et al., 1974; Ofegand & Chen, 1972; RajBhandrany, 1968; Cramer et al., 1968). The solution was stirred at 4°C for 2 hours in dark, followed by neutralization by sodium hydroxide and precipitation with cold ethanol. The precipitated dialdehyde RNA species was washed with acetone and carefully dried with filtered nitrogen gas.
Figure 50: Reaction scheme for spin-labelling of yeast 5S rRNA with paramagnetic morpholino spin-label (TERMO-NH2).
The precipitate was dissolved in 5.5 ml of 0.2 M sodium bicarbonate buffer (pH 9.5) containing 10% dimethyl sulfoxide and 45 mg of TEMPO-NH2 was slowly added. The solution was stirred at 0°C in dark for 90 min. The amine functionality of TEMPO-NH2 reacts with the aldehyde groups via a schiff base reaction, thereby generating an imine. The imine was then reduced and ring-closed via the addition of 0.23 ml of freshly prepared 0.6 M sodium borohydride (NaBH4) solution. The solution was stirred for 30 min., after which another 0.23 ml of the NaBH4 solution was added and stirred for an additional 30 min. The mixture was then exhaustively dialyzed against 10 mM Tris buffer (pH 7.0) containing 100 mM sodium chloride with five buffer changes. The spin-labelled yeast 5S rRNA was next dialyzed against 10 mM Tris buffer (pH 7.0) containing 10 mM EDTA and 100 mM sodium chloride and finally against the same buffer but containing 1 mM EDTA. 

3. NMR Samples

The dialyzed nitroxide spin-labelled yeast 5S rRNA in 10 mm Tris buffer (pH 7.0) containing 100 mM NaCl and 1 mM EDTA was concentrated via AMICON "Centricon 10" microconcentrator (AMICON Corporation, Danvers, MA) to give a final concentration of 34 mg/ml. D2O was added to give a final 5% D2O concentration to provide a 2D field-frequency lock signal. Gel electrophoresis on the nitroxide-labelled yeast 5S rRNA showed that the conformational integrity of the 5S RNA was preserved.
4. EPR Spectroscopy

The EPR spectra were recorded on a Varian E-4 EPR spectrometer operating at 9.5 GHz. EPR spectra obtained from the yeast 5S rRNA spin-labelled with TEMPO-NH₂ (bottom) and free TEMPO-NH₂ (top) are depicted in Figure 51. The bottom spectrum is a typical representative of molecule carrying only one immobilized spin-label which confirms the position of TEMPO-NH₂ at the 3'-UCUA end of yeast 5S rRNA (Luoma et al., 1982; Caron & Dugas, 1975; Wong et al., 1975; Crothers et al., 1974; Yang & Soll, 1974). The bottom spectrum (Figure 51) shows that TEMPO-NH₂ was moderately immobilized which indicated that this region is quite flexible (Luoma et al., 1982).

5. NMR Spectroscopy

Refer to Chapter IV for experimental details.

C. RESULTS AND DISCUSSIONS

Figure 52 shows the three proposed 5S ribosomal RNA secondary base pairing schemes (Luoma & Marshall, 1978; Luehrsens & Fox, 1981; and Nishikawa & Takemura, 1974), adapted to the primary nucleotide sequence of S. Carlsbergensis. Helix I is the same for the Fox & Woese and Nishikawa & Takemura models. Luoma & Marshall model shares most of the common helix I. Five G-U's are found in Luoma & Marshall and three G-U's in Fox & Woese, and four G-U's in Nishikawa & Takemura. In principle identification of all the G-U base pairs is sufficient to discriminate between proposed 5S rRNA secondary structures.
ESR SPECTRA
FREE MORPHOLINO SPIN-LABEL

MORPHOLINO SPIN-LABELLED YEAST 5S rRNA

Figure 51: EPR spectra of free morpholino spin-label (Top) and the spin-labelled yeast 5S rRNA (bottom).
Figure 52: Three proposed secondary base-pairing schemes for 5S rRNA, each adapted to the primary sequence for *Saccharomyces carlsbergensis*. 
1. Identification of the Terminal Base-Pairs from Specific Proton Resonance Broadening on spin-labelled yeast 5S rRNA

Based upon a combination of homonuclear Overhauser enhancements and temperature-dependence of the proton 500 MHz spectrum, I was able to identify and assign eight of the nine base pairs in the most thermally stable helical arm (G•C-rich arm) of yeast 5.8S rRNA (see Chapter V). However it is obvious from Figure 52 that no particular arm in yeast 5S rRNA contains any segment of consecutive G•C base pairs as in the case of the 5.8S rRNA. Presented in Table 8, the free energy values were calculated for each of the helical arms of these three models shown in Figure 52. All of the values were calculated based on the stability rules of Tinoco (Gralla & Crothers, 1973). From Table 8 it is clear that helix I (i.e. terminal helix) is the most thermally stable helical arm in the three proposed base-pairing schemes. However, in Nishikawa & Takemura model, helix IV & V is of comparable stability to helix I. Although the overall stability of the terminal arm is high but no particular segment within the arm has unique thermal stability as in the case of the G•C-rich arm in 5.8S rRNA. As shown in Figure 53 all resonances disappear at temperatures greater or equal to 61°C. The temperature approach which works well for yeast 5.8S rRNA will not be applicable to yeast 5S rRNA. Hence a different strategy is required in identifying and assigning base pairs in 5S rRNA. The new approach developed in this work involves the detection of specific nmr line-broadening due to paramagnetic nitrooxide spin-label uniquely attached at the 3'-end of yeast 5S rRNA.
Table 8. Free energies of stability for individual helices computed from Tinoco rules (Gralla & Crothers, 1973) for three proposed secondary structures of yeast 5S ribosomal RNA.

<table>
<thead>
<tr>
<th>Helix</th>
<th>$\Delta G$, kcal/mole</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cloverleaf model (Luoma &amp; Marshall, 1978a,b)</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>-12.1 (including bulge)</td>
</tr>
<tr>
<td>II &amp; III</td>
<td>+5.5 (including hairpin loop, bulge and interior loop)</td>
</tr>
<tr>
<td>IV</td>
<td>-4.6 (including hairpin loop and bulge)</td>
</tr>
<tr>
<td>V</td>
<td>-1.1 (including hairpin loop and bulge)</td>
</tr>
<tr>
<td><strong>Fox &amp; Woese model (Luehresn &amp; Fox, 1981)</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>-11.9</td>
</tr>
<tr>
<td>II &amp; III</td>
<td>-5.2 (including hairpin loop, bulge and interior loop)</td>
</tr>
<tr>
<td>IV &amp; V</td>
<td>-4.0 (including hairpin loop and interior loop)</td>
</tr>
<tr>
<td><strong>Nishikawa &amp; Takemura model (1974a,b,c)</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>-11.9</td>
</tr>
<tr>
<td>II &amp; III</td>
<td>-5.4 (including hairpin loop, bulge and interior loops)</td>
</tr>
<tr>
<td>IV &amp; V</td>
<td>-12.7 (including hairpin, bulge and interior loop)</td>
</tr>
</tbody>
</table>
Figure 53: 500 MHz proton NMR spectra of yeast 5S rRNA at several temperatures.
The top spectrum (Figure 54) shows the 500 MHz proton FT-NMR spectrum of nitroxide spin-labelled yeast 5S rRNA (ca. 34 mg/ml) in 10 mM cacodylate, 100 mM sodium chloride, 1 mM EDTA, pH 7.0, in the absence of Mg\textsuperscript{2+}. The bottom spectrum however depicts the proton NMR spectrum of the unlabelled yeast 5S rRNA in the same buffer. A comparison of the two spectra manifests the reduction in peak intensity at only 2 positions, namely resonance F (12.74 ppm) and resonance L (11.61 ppm) which correspond to the respective regions where G\textbullet C and G\textbullet U base-pair hydrogen-bonded imino protons normally resonate.

From the proposed models in Figure 52, it clear that resonances at F (12.74 ppm) and L (11.61 ppm) can be assigned to G\textsubscript{1}\textbullet C\textsubscript{120} and G\textsubscript{2}\textbullet U\textsubscript{119} since only these two terminal base pairs are spatially close to the paramagnetic nitroxide spin-label chemically bonded to the unpaired U\textsubscript{121} to experience paramagnetic relaxation effect, and since also the spin-label can only be attached to the 3'-UCUA end of the RNA molecule. In addition the top spectrum shows that peak L is composed of 2 base pair hydrogen bonded imino protons, and peak F is comprised of at least 3 hydrogen-bond imino protons.

2. Confirmation of the assigned terminal base pairs G\textsubscript{1}\textbullet C\textsubscript{120} and G\textsubscript{2}\textbullet U\textsubscript{119} by \textsuperscript{1}H Nuclear Overhauser Enhancements

I proceed to confirm the above assignments using NOE connectivity experiments. The strong mutual NOEs observed via irradiation of peak L (11.61 ppm) and peak K (11.85 ppm) (Figure 55, spectra a,b) clearly confirm it is a G\textbullet U base pair. As has been noted previously
Figure 54: Proton 500 MHz FT/NMR spectra of yeast 5S rRNA in 10 mM Tris-HCl, pH 7.5 buffer containing 100 mM NaCl and 1 mM EDTA at room temperature. Top: Morpholino spin-labelled yeast 5S rRNA. Bottom: Unlabelled yeast 5S rRNA. The arrow indicates resonances where paramagnetic broadening occurred.
for tRNA's and prokaryotic 5S RNA (Chang & Marshall, 1986a), K/L (G•U pair) shows one-sided NOE connectivity to peak A. Peak A (14.25 ppm) is a A•U base pair as evident by the narrow peak at 7.77 ppm due to the C2-proton (spectrum e in Figure 55). This implies that peak A must be adjacent to peak K/L. Irradiation of peak F (12.74 ppm) shows weak NOE connectivity to K, which shows G•C NOE behavior characterized by the broad NOE in aromatic region (spectrum d, Figure 55). From the NOEs, the sequence identified so far is G•C (F) - G•U (K/L) - A•U (A) which can correspond either to C26G52 - G25U53 - A24U54 in helix III in Nishikawa & Takemura model and helix II in Luoma & Marshall model or to G1•C120 - G2•U119 - U3•A118 that is common to the terminal helix in all three models (Figure 52). The NOE results cannot distinguish between the two possible helical segments. However based on the specific nmr resonance-broadening results discussed earlier, F must be G1•C120 and K/L be G2•U119 as the result their proximity to the paramagnetic morpholino-label at U121. Hence the sequence identified via NOE must be belong to the common G1•C120 - G2•U119 - U3•A118 found in the terminal helix. The present assignment of G1•C120 to peak F (12.74 ppm) contradicts the previous conclusions by Chen & Marshall (1986) who however assigned G1•C120 to peak B (13.37 ppm) based solely upon low melting temperature of resonance B. The use of temperature criteria alone for base pair assignments requires great caution as demonstrated in the studies of tRNA. As demonstrated here, the present new technique involving the use of specific paramagnetic spin-labels in rRNA should be reliable and very helpful in aiding
Figure 55. Proton 500 MHz FT/NMR spectrum (a) and proton homonuclear Overhauser difference spectra (b-f) resulting from irradiation of peaks L, K, F, A, and C at 22°C. In each case, the irradiated peak is denoted by a star and decoupler power is shown at the right.
assignments of base-pairs via NOE. In addition base pairs already identify using spin-labelling experiments provide a good starting point for further identification and assignment of the base pairs via NOE connectivity experiments.

3. Base-Pair Sequencing of the Terminal Helix I via Proton NOE Connectivity

As discussed in the previous sections, I already assigned the first two terminal base pairs, G₁·C₁₂₀ and G₂·U₁₁₉ to peaks F and K/L, now I proceed to extend assignments to the whole terminal helix.

Irradiation of peak K (11.85 ppm) produces strong mutual NOE's to peak L (11.61 ppm) and peak P (10.35 ppm) (spectra b,c in Figure 55 and spectra d,e,f in Figure 56) thus peak K must contains resonances from two different G·U base pairs (K/L and K/P). Highly resolution enhanced proton spectra in Figure 57 indeed shows that resonance K is actually composed of two peaks (11.85 ppm and 11.80 ppm). Peak K/L shows weak NOE connectivity to peak A (spectra b,c, Figure 55) which can be identified as an A·U pair from its chemical shift (14.25 ppm) and the sharp NOE difference signal at 7.75 ppm (spectrum e, Figure 55). As expected irradiation of peak A gives weak NOE's back to peak K/L. Therefore peak A must be U₃·A₁₁₈ adjacent to G₁·U₁₁₉ (K/L). Proceeding further, peaks A and C exhibit mutual NOE's (Figure 55, spectra e,f) indicating their adjacent relationship. Saturation of peak C (13.28 ppm) reveals that it contains three A·U's as evident by the three narrow NOE peaks at 7.80 ppm, 6.97 ppm and 6.75 ppm. One of the A·U pairs must lies between a A·U pair (peak A) and a G·C pair (H)
(Figure 55, spectra e,f and Figure 56, spectrum b) as irradiation of peak H (12.36 ppm) and A (14.25 ppm) give a strong NOE connectivity to peak C (13.28 ppm). Spectrum b (Figure 56) shows that peak H is a G·C pair from the broad NOE at the aromatic region. At this point I have identified 5 out of 9 nine base pairs in the sequence:

\[
\text{G·C (F) - G·U (K/L) - A·U (A) - A·U (C) - G·C (H)}
\]

which corresponds to the segment \(G_1·C_{120} - G_2·U_{119} - U_3·A_{18} - U_4·A_{117} - G_5·C_{116}\) in the terminal helix.

Saturation of peak H (12.36 ppm) produced NOE connectivities to peak F (12.74 ppm) and peak C (13.28 ppm). A weak reverse NOE was observed from F to H (spectrum d, Figure 55). Spectra d and e (Figure 56) shows that irradiation of P (10.35 ppm) produces a strong mutual NOE connectivity to K (11.85 ppm), and weak NOE's to B (10.40 ppm) and F (Figure 56d). K/P is a G·U base pair as confirmed by their mutual strong NOE's. Although peak F gives a weak NOE to peak K, however due to the multicomponent of F, it cannot be assigned with certainty that peak F is adjacent to peak K/P since one of the F's components is assigned to \(G_1·C_{120}\) which is adjacent to K/L. Because of NOE connectivites between peaks F and H (spectrum d, Figure 55), it is fairly confident that indeed F is adjacent to K/P. The sequence assignments of the terminal helix can now be further extended to

\[
\text{G·C (F) - G·U (K/L) - A·U (A) - A·U (C) - G·C (H) - G·C (F) - G·U (K/P) - G·C (B)}. 
\]

I have now identified eight of the nine base pairs terminal helix, \(G_1·C_{120} (F) - G_2·U_{119} (K/L) - U_3·A_{118} (A) - U_4·A_{117} (C) - G_5·C_{116} (H) - G_6·C_{115} (F) - G_7·U_{114} (K/P) - G_8·C_{113} (B)\). The
Figure 56: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5S rRNA, produced as in Figure 55, for peaks H, B, P, K, and M.
Figure 57: The effect of resolution enhancement by negative line-broadening (denoted by LB) on the proton 500 MHz NMR spectrum of yeast 5S rRNA. The same data set was used but enhanced by different values of LB. Top: Resonances B and K resolved into two components.
terminal ninth base pair of the helix remain unidentifiable probably due to its greater inter-base pair separation from B (the eighth base pair) to give any NOE signal since NOE effect can only be observed up to 4 to 5 Å in RNA. All three models do require distortion of the ninth base pair from the continuous terminal helix.

4. Identification of G•U Base Pairs

A close scrutiny of the proposed models (Figure 52) reveals that there are 5 G•U base pairs in the Luoma & Marshall structural secondary model, 4 G•U's for Fox & Woese model, and 4 G•U's for the Nishikawa & Takemura model. To discriminate among the proposed models, an attractive approach would be to identify the location of all the G•U base pairs in the secondary base pairing schemes. G•U imino resonances from an excellent starting point for two reasons. First, their chemical shifts typically fall in the otherwise uncrowded 10 to 12.5 ppm region of the 1H NMR spectrum. Second, saturation of either of the two imino protons of a G•U base pair gives a larger NOE (>15%) than for any other base pair.

Peaks K/L and K/P have prior been assigned to G2•U119 and G7•U114 in the terminal helix. Irradiation of M (11.47 ppm) produced large mutual NOE to N (10.91 ppm) and a weak NOE to B1 (13.51 ppm; Figure 58, spectra a-e). B1 exhibits characteristic broad NOE for G•C pair (spectrum e, Figure 58). At this point it is confident that M/N is the third G•U base pair and is next to a G•C (B1). From the three proposed models in Figure 52, it is reasonable to suggest that M/N cannot belong to helix III in Nishikawa & Takemura model and helix II
Figure 58: 500 MHz proton homonuclear Overhauser difference spectra of yeast 5S rRNA, produced as in Figure 55, for peaks M, N and B1.
in Luoma & Marshall model since M/N does not show NOE connectivity to a A•U pair. The only logical position for M/N is U81•G99 located at helix IV in these two models since this helix contains only one G•U pair and has no immediate adjacent A•U pair. This conclusion is consistent with the findings that peaks M and N are found in the RNase T2 fragment containing residues 42 to 121 suggesting peaks M/N is located in helix IV or helix V (Chen & Marshall, 1986). However it is unable to ascertain the exact location of M/N in Fox & Woese model because there are two possible G•U pairs (G67•U111 and U81•G99) in helix IV. The simultaneous melting of M/N and B1 at low temperature, about 40°C suggests that M/N and B1 are probably terminal base pairs (spectra c,d, Figure 53). It is speculative to assign M/N and B1 to G67•U111 (terminal base pair) and G68•G110 respectively based upon low melting temperature expected from end-fraying of a terminal base-pair (Boyle et al., 1980; Heus et al., 1983). Since U81•G99 is sandwiched between two G•C pairs (G79•C100 and G82•C98) and should therefore expect to melt at a higher temperature than the terminal G67•U111 pair. This NOE studies cannot discern among the three popular models for yeast 5S rRNA since the fourth/fifth G•U base pairs remain unidentified in intact 5S rRNA. However if the assignment of M/N to G67•U111 were correct then the present results would support a Fox & Woese secondary model for yeast 5S rRNA.

In each of the three pairs of G•U base-pair hydrogen-bond protons observed in intact yeast 5S rRNA, NOE connectivity to only one of the nearest-neighbor base-pairs is observed. The Overhauser connection is observed to the neighboring base-pair on the 3'-end but not the
5'-end of the G-residue of the G•U pair in accordance with previous findings reported by Hues et al (1983). This one-side NOE connectivity introduced serious complications to base-pair assignments via NOE. This work on 5.8S rRNA has observed that irradiation of a G•U pair can indeed elicit NOEs to base pairs on both sides of the G•U pair (see chapter V).

5. Heat-induced melting of yeast 5S ribosomal RNA

Figure 53 shows 500 MHz proton FT/NMR spectra of yeast 5S rRNA as a function temperature. Due to increased rate of chemical exchange with water as the temperature increases, each of the base-pair hydrogen-bond imino proton resonances begins to lose intensity and broaden. Hence the temperature-dependence of a particular base-pair proton resonance reflects its thermal stability. Moreover, nmr peaks arising from adjacent secondary base-pairs in the same helical segment are expected to melt at similar temperature.

From Figure 52, it is clear that the weakest junction in the terminal helix belongs to the G₁•C₁₂₀ - G₂•U₁₁₉ - U₃•A₁₁₈ - U₄•A₁₁₇ segment and this particular segment should be the most thermally labile in helix I. Resonances F, K/L, A, C are already assigned to the above segment. At 40°C, the simultaneous melting of peaks A and L, and concurrent decrease in intensity of peaks F, K and C (see Table 9) suggest that these 4 base pairs must be adjacent to each other. This melting behavior is consistent with the prior assignments resonances F, K/L, A and C respectively to the G₁•C₁₂₀ - G₂•U₁₁₉ - U₃•A₁₁₈ - U₄•A₁₁₇ segment. Figure 57 reveals that peak F contains
Table 9. Assignments for imino proton resonances from the terminal helix of yeast 5S rRNA, and approximate (+ 5°C) temperature for onset of melting for that base pair.

<table>
<thead>
<tr>
<th>Resonance</th>
<th>Chemical Shift (ppm)</th>
<th>Base pair</th>
<th>Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>-12.74</td>
<td>G₁·C₁₂₀</td>
<td>40</td>
</tr>
<tr>
<td>K/L</td>
<td>-11.85/11.61</td>
<td>G₂·U₁₉₉</td>
<td>40</td>
</tr>
<tr>
<td>A</td>
<td>-14.25</td>
<td>U₃·A₁₁₈</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>-13.28</td>
<td>U₄·A₁₁₇</td>
<td>40</td>
</tr>
<tr>
<td>H</td>
<td>-12.36</td>
<td>G₅·C₁₁₆</td>
<td>50</td>
</tr>
<tr>
<td>F</td>
<td>-12.74</td>
<td>C₆·G₁₁₅</td>
<td>50</td>
</tr>
<tr>
<td>K/P</td>
<td>-11.85/10.35</td>
<td>G₇·U₁₁₄</td>
<td>50</td>
</tr>
<tr>
<td>B</td>
<td>-13.38</td>
<td>G₈·C₁₁₃</td>
<td>-</td>
</tr>
</tbody>
</table>
at least 3 components, peak K contains at least 3 components, and peak L contains at least 2 components, one would only expect partial melting of those resonances. The melting of peak K/P at 50°C corroborates with its assignment to G7·U114 which is sandwiched between two G·C base pairs above and below it in the terminal helix.

D. Conclusions

Base-pair connectivities in 5S RNA are not readily extracted from proton homonuclear NOE experiments alone, because of a severe overlap of peaks and a shortage of resolvable A·U resonances. However, paramagnetic nitroxide probes offer another approach to spectral assignments. This work reports the first 1H NMR study on any paramagnetic nitroxide spin-labelled 5S rRNA.

The line-broadening effect of a nitroxide spin-label on a proton varies as the inverse power of their spatial separation. Because the electron magnetic moment is so much larger than the magnetic moment of the proton, detectable line-broadening in spin-labelled 5S RNA should be observable up to 15 Angstrom or so. Because the 3'-terminal ribose is the only sugar with two adjacent OH groups, periodate-oxidation followed by spin-labelling with 4-amino-2,2,6,6-tetramethylpiperidino-1-oxy (TEMPO-NH₂) and sodium borohydride-reduction produces 5S RNA specifically labelled with a paramagnetic nitroxide at the 3'-terminus. The high-field 1H NMR spectrum (500 MHz) of the nitroxide-labelled yeast 5S RNA in H₂O reveals terminal-stem base pair (G1·C120) and the adjacent base pair (G2·U119) from the selective
broadening resulting from its proximity to the 3'-terminus. The identified base pairs provide a good starting reference point from which I have identified eight out of the nine base pairs in the terminal helical arm by homonuclear Overhauser enhancements.
REFERENCES


Hare, D. R., & Reid, B. R. (1982b) Biochemistry 21, 5129-5131.


Hurd, R.E., & Reid, B. R. (1979) Biochemistry 18, 4017-4024


Smith, J.L. Ph.d dissertation (1980), University of British Columbia.


