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High-Resolution Nuclear Magnetic Resonance Studies of the Structure of Transfer Ribonucleic Acid and Other Polynucleotides in Solution

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During protein synthesis in the living cell, each amino acid unit is escorted to the ribosome by a transfer ribonucleic molecule (tRNA). A different tRNA molecule is required for each amino acid; that for phenylalanine is symbolized $tRNA^{Phe}$. It is at the ribosome that the actual synthesis of protein molecules occurs.[1-3]

More than forty different tRNAs have been purified and their base sequences reported since the original determination of the sequence of yeast $tRNA^{Ala}$ by Holley, *et al.*[4] Typically, the purified tRNAs contain between 75 and 80 bases in a linear chain with molecular weights of ~30,000.

From the nucleotide sequence Holley, *et al.*, proposed a cloverleaf model for tRNA in which some sections were in the form of helices with Watson–Crick base pairs, while other parts of the molecule were not paired.[4] Sequences of subsequent tRNAs were homologous with the original cloverleaf model which is shown for the particular case of yeast $tRNA^{Phe}$ in Figure 1, and this homology has been one of the strongest pieces of evidence supporting the cloverleaf model of the secondary structure of tRNA.[5]

Besides secondary structure, tRNA molecules have also been assumed to have well-defined tertiary structures, and several different specific models have been proposed.[6,7] Recently the crystal structure for $tRNA^{Phe}$ was solved at moderate resolutions (4.0 Å).[8] It is clear, however, from a variety of solution studies that a single tRNA molecule can exist in more than one conformation depending upon conditions such as temperature, ionic strength, and magnesium ion concentration and that there are differences in structure among the different purified tRNA molecules.[9-13] Thus, even when high-resolution tRNA crystal structures become available, the crucial problem of determining the structure of these molecules *in solution* will still remain.

Although the determination of the structure of a protein in solution is quite formidable, the analogous problem is much simpler in the case of tRNA molecules because the substantial amount of Watson–Crick base pairing introduces more or less well-ordered helical sections.[6] This reduces the number of structural alternatives so that with additional information of the relative orientations of the helices, on the stacking of bases in the single-stranded regions and on the additional intramolecular bonds, the "structure" of the molecule in solution is effectively determined.

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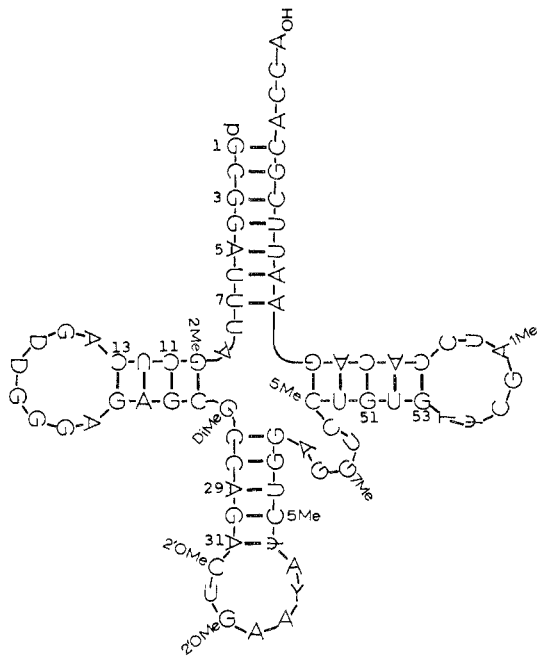

Figure 1. The cloverleaf model for yeast $tRNA^{Phe}$.[5]

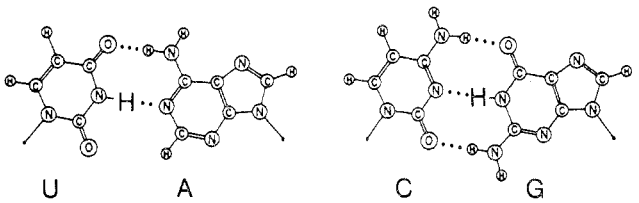

Figure 2. The standard Watson-Crick base pairing of AU and GC bases. The hydrogen-bonded ring NH protons are shown in bold print.

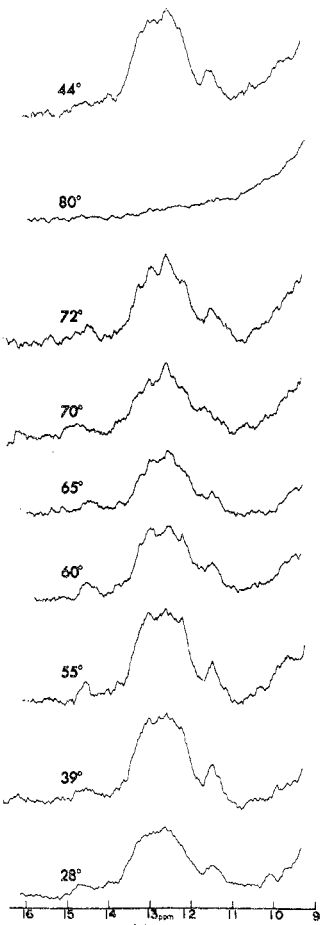

Figure 3. Temperature dependence of the 220-MHz nuclear magnetic resonance spectrum of $tRNA^{fMet}$ (*E. coli*) at a concentration of 40 mg/ml at pH 7, 3 m*M* $MgCl_2$, and 0.1 *M* NaCl.

Following this line of reasoning, the first problem is to determine which bases in the primary sequence are hydrogen bonded together in the standard Watson–Crick fashion depicted in Figure 2 for GC and AU pairs. Since the ring nitrogen protons and the NH_2 protons of the bases are the ones most affected by the base pairing, these protons give the most information about this aspect of tRNA structure.

In early high-resolution proton nmr studies of tRNA, D_2O was used as the solvent in order to avoid the great experimental difficulties due to the huge peak from H_2O and to simplify the spectrum by removing resonances from the exchangeable protons.[14-16] The spectra in D_2O were rather uniformative even at 220 MHz because, in contrast to the variety of residues in proteins, the proton containing residues of tRNA are limited to the identical ribose sugars and the four major bases adenine (A), uracil (U), guanine (G), and cytosine (C), plus a few modified bases.

High-resolution nmr studies of proteins had shown that in H_2O it was possible to observe resonances from hydrogen bonded protons at low fields where they were well resolved from other resonances of the molecule.[17,18] Hence, to see the analogous resonances from the hydrogen-bonded protons of base pairs, we dissolved tRNA in H_2O and looked for resonances of hydrogen-bonded protons which would not be observed in D_2O.[19,20] A collection of resonances exhibiting the property were first observed in the low-field region extending from 11 to 15 ppm (resonance positions are indicated in parts per million (ppm) downfield from the usual standard DSS (2,2-dimethyl-2-silapentane-5-sulfonate); on this scale the water resonance is at about 4.8 ppm) and the spectra shown in Figure 3 show an example of some of the early results obtained using a 220-MHz spectrometer.

Assignment of the Resonances in the 11- to 15-ppm Region of $TRNA^{Phe}$ (Yeast)

The resonances observed in the 11- to 15-ppm region are ascribed to the hydrogen-bonded ring NH protons on the basis of model system studies, comparisons of the spectra of different tRNA, and the fact that no other resonances are expected in this region.[19-25] Since each Watson–Crick base pair con-

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tains just one ring NH proton, *i.e.*, U_3H or G_1H (see Figure 2), *each proton detected corresponds to one base pair in the molecule.* The spectra shown in Figure 3 were obtained at 220 MHz.

Additional spectral resolution and information about the low-field region are obtained at 300 MHz, as the spectrum presented in Figure 4 illustrates. With the improvement in sensitivity and resolution resulting from the higher frequency, it is possible to determine accurately the number of low-field resonances by internal comparison. In the case of $tRNA^{Phe}$, this is based upon assigning the slightly split resonance at ~13.7 ppm in Figure 4, an intensity corresponding to three protons, and this yields a value of 19 protons in the region from 11 to 15 ppm. As we shall see, this number is consistent with the number expected from the cloverleaf model,[5] and the simplest interpretation of this agreement is that no additional tertiary structure Watson–Crick bonds exist in these molecules. We now want to show how the individual resonances in the spectrum of Figure 4 were assigned to individual base pairs in $tRNA^{Phe}$ and discuss how the factors responsible for their shifts were determined.

Assignment of Low-Field Resonances (11 to 15 ppm) of tRNA to Specific Base Pairs. *Ring Current Shifts.* The low-field AU and GC resonances are displaced upfield from their standard positions by ring current fields from their nearest neighbors.[26-28] Thus, given the secondary structure of a polynucleotide (assumed to be A′RNA in the helical region),[6] all that is needed in order to predict spectra are the locations of the resonances for standard (unshifted) AU and GC base pairs and the magnitudes of the ring current shifts exerted by the four different bases on a neighbor base pair.

From studies of various model systems, as well as tRNAs, we have concluded that the unshifted positions are $(AU)^0 = 14.7 \pm 0.1$ ppm, $(GC)^0 = 13.6 \pm 0.1$ ppm, and $(A\Psi)^0 = 13.5$ ppm (Ψ = pseudouridine) while the ring current shifts were taken to be 20% larger than those calculated by Giessner-Prettre and Pullman.[28] An equally good fit to the various data could be obtained by using $(AU)^0 = 14.6$ and $(GC)^0 = 13.6$ in conjunction with the ring currents as calculated.[28] For the particular case of $tRNA^{Phe}$ (yeast) we have calculated the positions of the hydrogen-bonded protons in the cloverleaf shown in Figure 1 using the original set of parameters, and these are compared with the observations in Table II (example: position of GC-2 = 13.7 − 0.2 − 0.7 = 12.8 ppm). In Figure 4 we present the positions calculated on the basis of the second set of parameters. It can be seen that both sets give a good but not perfect fit to the data. At the present these two sets represent the limit of uncertainties of the parameters.

To resolve some of the uncertainties in the assignments, the nmr spectra of fragments of $tRNA^{Phe}$ were measured and their resonances were assigned by the ring current calculations[22,23] and then compared with the spectrum of the intact molecule. The results of this work are summarized below.

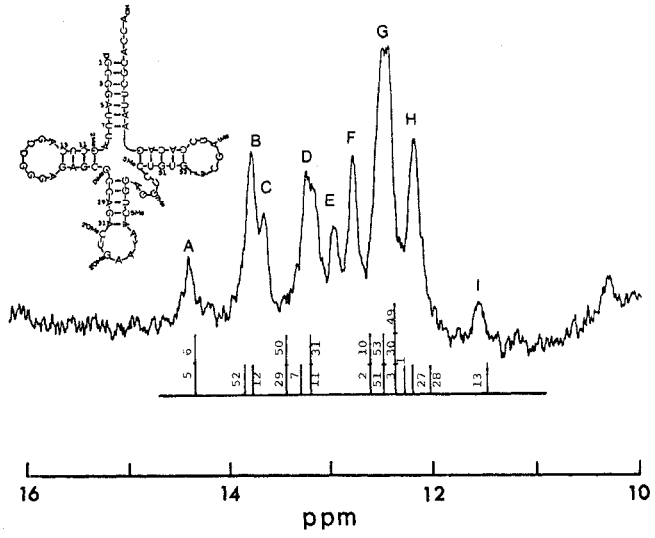

Figure 4. Proton nmr spectrum at 300 MHz of yeast $tRNA^{Phe}$ shown along with the predicted location of resonances associated with all base pairs present in the cloverleaf model except for the mismatched GU-4 pair and AU-5 which is assumed to be destabilized by it.

$tRNA^{Phe}$ Fragment Spectra. *5′ Half.* On initial inspection the spectrum of the 5′ half of $tRNA^{Phe}$ (shown in Figure 5a) appears to have only three resonances, whereas four are expected from its primary sequence. The locations of the three sharp resonances are well accounted for by the ring current shift calculations, but the highest field resonance from GC-13 appears to be absent. Closer examination of this spectrum, as well as others, reveals that this resonance is actually present as a very broad band extending from about 12.5 to 11.5 ppm. The predicted location of this resonance is 11.5 ppm, provided A-14 is stacked as if it were in a helical region and is exerting the ring current shift on GC-13 given in Table I.

Depending on the conformation of the DHU loop, however, A-14 may not exert its full effect. Since this broad band did not narrow significantly at lower temperatures, we suggest that different, slowly interconverting, conformations of the DHU loop are responsible for producing a set of different environments for GC-13 and broadening its resonance.

Anticodon Half. At about 50° (see Figure 5b) the spectrum of the anticodon half of the molecule contains only three peaks, located at 13.2, 12.5, and 12.0 ppm, with areas 1:2:1. This corresponds to a total of only four resonances, whereas five were expected on the basis of the primary sequence of this fragment (three GC, one AU, and one AΨ).

On cooling below 40°, however, the missing resonance appeared around 13.3 ppm, thus giving the five resonances expected from this fragment. Since AU and AΨ base pairs are weaker than GC pairs, it is likely that the temperature-dependent resonance is due to an AU or an AΨ base pair. Because of its location at the terminus of a helix we conclude that AΨ-31 is responsible for the temperature-dependent resonance at 13.3 ppm. Since we had not previously studied model systems containing AΨ base pairs the

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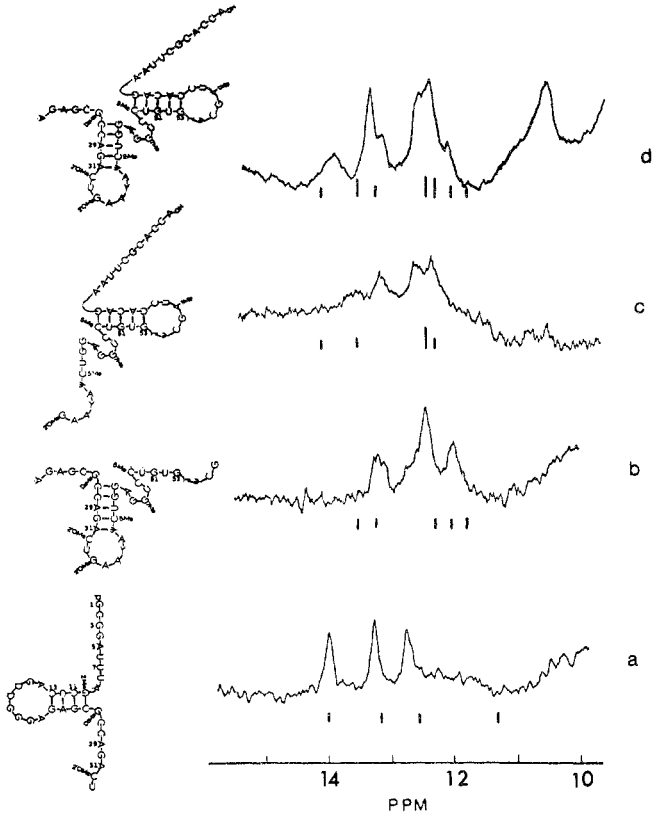

Figure 5. High-resolution 300-MHz nuclear magnetic resonance spectra of four different fragments of $tRNA^{Phe}$. The lines give the predicted location of resonances associated with specific base pairs.

position of the unshifted AΨ resonance was unknown, so that this information was used to infer that $(A\Psi)^0$ = 13.5 ppm. The calculated positions of the four other resonances in the anticodon stem are also shown in Figure 5b, and the agreement between experiment and theory is good.

3′ Half. According to the primary structure of the 3′ half of $tRNA^{Phe}$ the nmr spectrum of this fragment is expected to contain five resonances corresponding to two AU and three GC base pairs. The spectrum of this fragment is shown in Figure 5c, and while it is not well resolved, internal and external intensity comparisons indicate that, to within an experimental error of ±25%, there are five protons in this spectrum. The ring current shifts calculated for the five resonances agree only moderately well with the observed spectrum.

3′ Three-quarter Molecule. The nmr spectrum of the 3′ three-quarter molecule is shown in Figure 5d and, allowing for small shifts (less than 0.2 ppm), it is the sum of the nmr spectra of its components, *i.e.*, the anticodon hairpin and the 3′ half which are also shown in this figure. The spectrum of the 3′ three-quarter fragment should have an intensity corresponding to ten protons, and this is confirmed by integration of the spectrum assuming that the resonances between 13.1 and 13.3 ppm correspond to three protons.

Because of the additivity of the fragment spectra, there is a 1:1 correlation between resonances seen in the smaller half molecule fragments and the larger 3′ three-quarter fragment, and hence the assignments of the 3′ half and anticodon half molecule spectra can be directly applied to the assignment of the 3′ three-quarter fragment. We note that in the 3′ three-quarter molecule's spectrum there is a peak with the intensity of *ca.* three protons in the region between 10 and 11 ppm which is not observed in the two component half molecules. This peak evidently arises because of some structure present in the 3′ three-quarter molecule, but not in the smaller fragment. Similar peaks are also observed in the spectrum of the intact molecule in the 9–11 ppm region. While these resonances are not assigned it seems, from these and other experiments, that they arise from ring NH protons which are protected from rapid exchange with water by the three-dimensional structure.

Table I
Summary of Ring-Current Shift Parameters Used in the Calculation of the Low-Field Proton Nmr Spectra of tRNA Molecules[a]

5′	3′	5′	3′
U = 0	A = 1.3	U = 0	A = 1.3
C = 0	G = 0.6	C = 0	G = 0.7
G = 0	C = 0.1	G = 0	C = 0.2
A = 0.1	U = 0	A = 0	U = 0.1
UA		**CG**	
∩∀		**ↃG**	
U = 0.1	A = 0	U = 0.1	A = 0
C = 0.2	G = 0	C = 0.25	G = 0
G = 0.6	C = 0	G = 0.7	C = 0
A = 0.7	U = 0	A = 1.2	U = 0
3′	5′	3′	5′

[a] The notation 5′ and 3′ refers to the sugar positions at the ends of chains containing the neighboring bases indicated. Shifts for the other two pairs of bases can be obtained by turning the table upside down. The values are upfield shifts in parts per million.

Intact Molecule Spectrum. The spectrum of the intact molecule should include all of the resonances present in the three different half-molecule fragments, plus six additional resonances from the three GC and three AU base pairs presumed to be present in the acceptor stem. We assume that the GU-4 base pair does not contribute a resonance in the low-field region since the previous evidence[29,30] supporting GU base pairing is incorrect[31] and there is no evidence for GU base-pair formation in poly GU.[32]

The high-resolution nmr spectrum of the intact molecule is shown in Figure 4. On the assumption that peaks B + C correspond in intensity to three protons, the intensities of all the peaks are listed in Table II. The intact molecule spectrum has been compared with the fragment's spectra and the calculations in order to assign the resonances, and these are also given in Table II. Allowing for small differences in the positions of resonances when comparing fragment and intact molecule spectra, resonable assignments can be made for most of the resonances.

Two of the resonances of peaks B + C must be assigned to the AU-52 and AU-12 base pairs, since these same resonances can be observed in this region in the spectra of the 3′ three-quarter and 5′ half mol-

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Table II
Summary of the Assignments of the Low-Field Resonances in tRNA^{Phe} (Yeast)

Line	Intensity	Position, ppm	Assignment	Calculated Position	Comments
A	1	14.4	AU-6	14.6	Present only in the intact molecule
B	3	13.8	AU-12	14.0	Observed at 14.0 ppm, in the 5′ half fragment
C		13.7	AU-52	14.1	Observed at 14.0 ppm in the 3′ three-quarter fragment and also at 13.7 ppm in the 3′ half fragment
			AU-7	13.5	Present only in the intact molecule
			AU-50	13.6	Observed at 13.4 in the 3′ three-quarter fragment and at 13.3 in the 3′ half fragment
D	3	13.3	AU-29	13.6	Observed at 13.4 in the 3′ three-quarter fragment and at 13.3 in the anticodon fragment
			Aψ-31	13.3	Observed at 13.3 in the 3′ three-quarter fragment and at 13.3 in the anticodon fragment Sensitive to removal of Y base
E	1	12.9	GC-11	13.2	Observed at 13.2 ppm in the 5′ half fragment
F	9	12.8	GC-1,2,3, 10,27,30,		In the range 12.6-11.5 ppm 10 GC pairs are calculated to occur
G		12.5	49,51,53		Upon removing -GCACCA two resonances at about 12.5 ppm are lost. The positions of these two are calculated to be 12.2 ppm for GC-1 and 12.7 ppm for GC-2.
H	1	12.2	GC-28	11.8	Observed at 12.0 in anticodon hairpin.
I	<1	11.5	GC-13	11.2–12.4	Broad line in the 5′ half fragment should correspond to this weak resonance. Sensitive to the conformation of the DHU loop.

ecules, respectively. The third resonance in this group is attributed to AU-7 which is predicted to give a resonance at 13.5 ppm.

According to ring current shift calculations, AU-5 and -6 should *both* appear at 14.6 ppm. Since there is only one peak at 14.4 ppm, it is clear that AU-5 (or -6) is either missing or is shifted upfield from its normal position. It seems unlikely that the resonance due to an interior AU base pair such as AU-6 should be missing. We therefore suggest that the resonance from AU-5 is missing because of its location adjacent to GU-4. This is consistent with our belief that G-4 is not hydrogen bonded with U-69 and that as a result of the mismatch the helix may be disrupted at this point.

The three resonances in peak D are assigned to AU-50 from the TΨ CG stem and AU-29 and AΨ-31 from the anticodon stem. The weak peak E at 12.95 ppm probably should be assigned to GC-11 since a sharp resonance is observed at about the same position in the 5′ half. At least seven of the ten resonances associated with peaks F, G, and H can be attributed to the GC base pairs no. 10, 27, 28, 30, 49, 51, and 53 which were observed in the 5′ half molecule or in the 3′ three-quarter molecule spectra. Peak H can be assigned to GC-28 since a resonance is observed at this same position in the anticodon hairpin. Peak F could be due to GC-53, based on its location in both the 3′ half and 3′ three-quarter spectra, but this is not certain.

The three additional GC base pairs (GC-1, -2, and -3) which are added by formation of the acceptor stem account for the remaining three resonances observed near 12.5 ppm for a total of ten. Peak I in the intact molecule is attributed to GC-13, and this assignment is consistent with its location (11.5) in the 5′ half spectrum and its variation in intensity and different appearance in spectra obtained under different conditions.

Conclusions Regarding the tRNA^{Phe} Study. The fact that we have been able to assign the low-field proton nmr spectrum of tRNA^{Phe} permits us to draw the following conclusions. To a good approximation (usually better than 0.2 ppm) a simple ring current shift theory accurately predicts the low-field nmr spectra of short helical tRNA fragments and intact tRNA. This agreement provides compelling support for the cloverleaf model as the correct description of the secondary structure of tRNA^{Phe} in solution. We found no evidence for additional tertiary-structure Watson–Crick type base pairs as are presumed to exist in a number of different models.[33-39] The observation that the fragment spectra are approximately additive indicates that the helical structure observed in the *fragments* is maintained in the intact molecule and that the most important factors responsible for stabilizing the secondary structure of *intact* tRNA are already present in the smaller fragments.

Although we do not observe additional resonances from tertiary-structure Watson–Crick type base pairs, this does not mean that the nmr spectra are not useful in determining certain aspects of the tertiary structure of tRNA. A number of resonances are sensitive to different aspects of the tertiary structure. GC-13, for example, is sensitive to the conformation of the dihydro-U loop, and the observation of this resonance at 11.5 ppm indicates that A-14 is in a more or less standard, stacked arrangement with respect to C-13. As discussed below, in other tRNA species base pairs located at the interior termini of helical regions are very useful in establishing tertiary structure in solution.[40]

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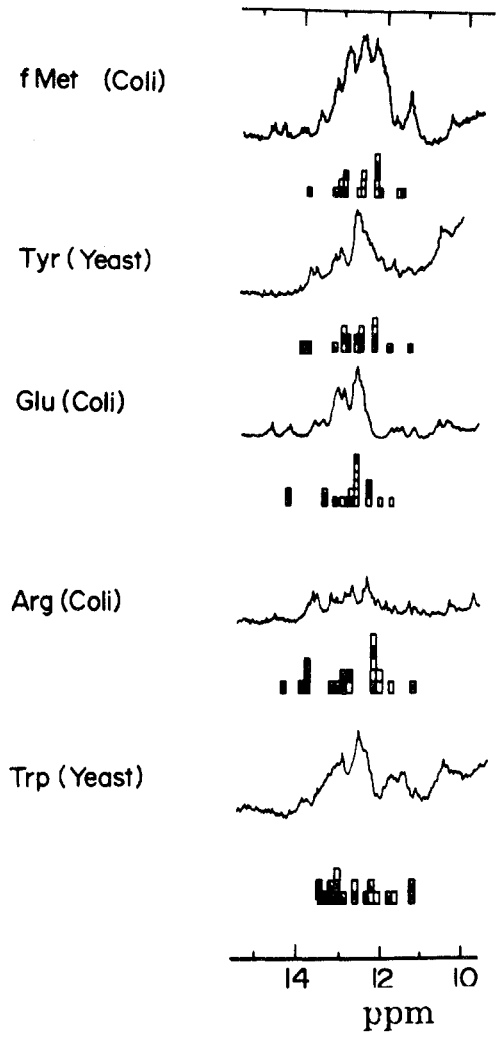

Figure 6. High-resolution 300-MHz nuclear magnetic resonance spectra of five purified tRNA species. All spectra were obtained in water containing 0.1 *M* NaCl and 5 m*M* Mg^{2+} at approximately 35°.

Nmr Investigations of Other tRNAs

Preliminary spectra of several other pure tRNA species are presented in Figure 6.[24] Calculations of the ring current shifts give a good first-order account of these spectra. Besides supporting our method of calculating shifts and the cloverleaf model of secondary structure, these other tRNA molecules also provide information about the tertiary structures in solution, as discussed below.

E. coli **tRNA^{Glu}**. According to the cloverleaf model, this molecule should have 4 AU and 16 GC base pairs. The predicted locations of the resonances associated with these various base pairs are shown in Figure 6. The four lowest field resonances can be assigned to base pairs AU-2, -7, -11 and -49 and the agreement between predicted and observed locations is quite good. Relative to the four low-field resonances, the integrated intensity of the resonances located in the GC region between 13 and 11 ppm is found to be 16, and this agrees very well with the number of base pairs expected on the basis of the cloverleaf model. Once again we see no evidence for tertiary structure Watson–Crick base pairs.

The resonances from the AU base pairs in this molecule are particularly valuable in providing information about certain tertiary structural features of the molecule. For example, AU-7 is predicted to occur at 13.5 ppm, provided it receives 0.7-ppm upfield shift from A-49 of the terminal base pair of the TΨC stem. Similarly, AU-49 is also predicted to occur at 13.5 ppm if it is *assumed that the acceptor stem and the TΨC stem form a continuous helix* in a manner such that A-66 is stacked on U-65. Without this stacking both the AU-7 and AU-65 resonances should be at 14.2 ppm. Since the resonances from AU-7 and AU-65 both occur within 0.1 ppm of 13.5 ppm, we conclude that the two helical arms of the acceptor stem and the TC stems are aligned to form a more or less continuous right-handed helix.[40] Thus, the nmr spectrum of this molecule has provided direct information about its tertiary structure in solution. It is interesting to note that this same structural feature appears in the crystalline samples of tRNA^{Phe} currently being studied by the MIT group.[8]

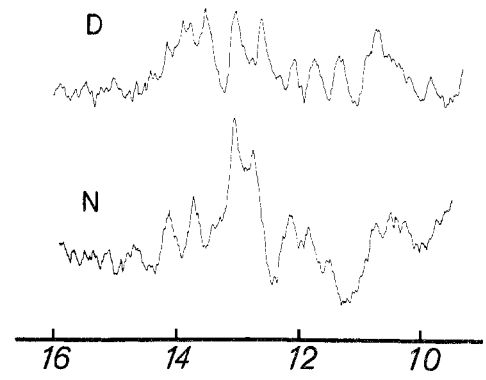

Figure 7. A comparison of the 220-MHz nmr spectra of the native and denatured conformers of tRNA$_3^{Leu}$.

E. coli **tRNA^{fMet}**. The temperature dependence of the nmr spectrum of tRNA^{fMet} shown in Figure 3 illustrates some additional type of information that may be obtained. Under the particular salt conditions used in this experiment, there is virtually no change in the integrated intensity or shape of the spectrum as the temperature is raised from 28 to 72°. However, just below 80°, which is close to the melting point determined optically, the resonances disappear, and reappear upon cooling. These preliminary results indicate that nmr provides a unique method for following the thermodynamic behavior of each base pair in a molecule and the response of each base pair to various environmental effects such as salt, temperature, magnesium concentration, added components to the solution (organic solvents). Since an nmr "melting curve" provides a complete description of all base pairs in the molecule, it is clear that many long-standing problems connected with the thermodynamic properties of tRNA may be solved by this approach.

Other Problems Studied by High-Resolution Nmr

Structure of Native and Denatured tRNA^{Leu}. Although it is known[41] that certain tRNAs can be trapped in biologically inactive forms (denatured, D) and by appropriate heat treatment converted back to the native (N) form, detailed information on the differences between these two conformations has

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been lacking. From their low-field nmr spectra we have been able to determine the differences between the secondary structures of the N and D conformers,[42] as shown in Figure 7. If we take 13.2 ppm as the dividing line between AU and GC resonances, then on going from N to D there is a loss of 3–5 GC base pairs and a gain of perhaps 0–2 AU base pairs. While further work at higher frequency (300 MHz instead of 220 MHz) will be required to settle the matter, it appears that a loss of the base pairs in the dihydro-U stem, coupled with the formation of an AU and perhaps another GC base pair with other distant parts of the molecule, may account for the nmr changes.

Effect of Aminoacylation of the Structure of tRNA^{Phe}. There are suggestions in the literature that the amino-acylation of a tRNA may induce a change in its secondary and/or tertiary structure.[43-45] While there have been numerous attempts to investigate this problem, the results have been either conflicting or have left open the possibility that changes were occurring but going undetected. The low-field nmr spectra of charged and uncharged tRNA^{Phe} have now been examined and to within 0.05 ppm the two nmr spectra are identical.[46] This observation indicates that there are no changes in the secondary structure of this molecule as a result of aminoacylation, nor is there evidence of any sort of change in its tertiary structure.

5S RNA. 5S RNA is a polynucleotide containing 120 nucleotides which is found in ribosomes.[47] The sequences of 5S RNA from a number of different sources (*E. coli,* yeast) have been determined, but in contrast to RNA no compelling secondary structure is immediately suggested by the primary sequence. Because of this, there have been a number of different proposals regarding the secondary structure of 5S RNA from *E. coli,* with the number of base pairs per molecule ranging from a low of about 21 to a high of almost 50.[48-53] The nmr spectrum of yeast 5S RNA has been measured, and the results indicate that there are 25 ± 3 base pairs per molecule (~5 AU and ~20 GC pairs).[54] While no model for the structure of yeast 5S RNA has yet been suggested, these results provide a way of checking the validity of any proposed model.

Present Conclusions and Future Possibilities: Unsolved Problems

The nmr studies described in this Account provide an important step forward in the long-range goal of determining the structures of tRNA, 5S RNA, and other polynucleotides in solution. The tertiary structure problem has not been completely solved, but nmr has delineated the hydrogen bonds responsible for the secondary structure and provided answers about selected aspects of the tertiary structure. Once the complete structure in solution is determined, there remains the central question as to the relation between structure of these molecules and their function in biosynthesis. For example, we would like to know what portion(s) of the tRNA molecule is recognized by its cognate synthetase. In the actual act of translation which occurs on the ribosome, which parts of the tRNA are bound to the ribosome? There are various drugs which alter tRNA function in protein synthesis, and it would be interesting to see how these interact with or alter the structure of tRNA molecules in solution.

During the past 10 years there has been much interest in the study of the thermodynamic properties of polynucleotides with the anticipation that such data might be used to aid in the prediction of stable structures from primary sequences.[55,56] The acquisition of such data has, however, been difficult simply because there was previously no straightforward method for measuring the base-pairing structure of the polynucleotides in solution. High-resolution nmr, applied in the way described, has now provided an accurate means of measuring base pairing.

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