Reid, B. R., Ribeiro, N. S., Gould, G., Robillard, G., Hilbers, C. W., & Shulman, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2049.
Reid, B. R., Ribeiro, N. S., McCollum, L., Abbate, J., & Hurd, R. E. (1977) *Biochemistry 16*, 2086.
Robertus, J. D., Ladner, J. E., Finch, J. T., Rhodes, D., Brown, R. S., Clark, B. F. C., & Klug, A. (1974) *Nature (London) 250*, 546.
Robillard, G. T., Tarr, C. E., Vosman, F., & Berendsen, H. J. C. (1976) *Nature (London) 262*, 363.
Simsek, M., Petrissant, G., & RajBhandary, U. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2600.
Shulman, R. G., Hilbers, C. W., Kearns, D. R., Reid, B. R., & Wong, Y. P. (1973) *J. Mol. Biol. 78*, 57.
Stout, C. D., Mizuno, H., Rao, S. T., Swaminathan, P., Rubin, J., Brennan, T., & Sundaralingam, M. (1978) *Acta Crystallogr., Sect. B 34*, 1529.
Sussman, J. L., & Kim, S. H. (1976) *Science 192*, 853.
Wong, K. L., Kearns, D. R., Wintermeyer, W., & Zachau, H. G. (1975a) *Biochim. Biophys. Acta 395*, 1.
Wong, K. L., Wong, Y. P., & Kearns, D. R. (1975b) *Biopolymers 14*, 749.
Wong, K. L., Bolton, P. H., & Kearns, D. R. (1975c) *Biochim. Biophys. Acta 383*, 446.

Nuclear Magnetic Resonance Studies on Transfer Ribonucleic Acid: Assignment of AU Tertiary Resonances†

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ABSTRACT: The hydrogen-bonded ring NH nuclear magnetic resonance (NMR) spectra of several transfer ribonucleic acid (tRNA) species have been examined with particular emphasis on the extreme low-field portion. Between −13.8 and −15 ppm there are two extra resonances which are not derived from cloverleaf base pairs. A combined approach involving undermodified tRNAs, chemical modification, and hairpin fragment studies has assigned the T54–A58 resonance at −14.3 ppm in yeast $tRNA^{Phe}$ and *Escherichia coli* $tRNA_1^{Val}$; the U8–A14 resonance has been assigned at −14.3 ppm, and the s^4U8–A14 resonance in bacterial tRNAs has been assigned at −14.9 ppm. The T54–A58 resonance shifts between −14.3 and −13.8 ppm depending on the surrounding nucleotide sequence in the ribothymidine loop.

In an earlier paper we showed that several different class 1 D4V5 tRNA[1] species contain six to seven extra resonances derived from tertiary base pairing in their low-field (−11 to −15 ppm) NMR spectra (Reid et al., 1977). In an accompanying paper in this series we have used several independent methods to identify which resonances in the 26- to 27-proton spectra were derived from tertiary base pairs [see Reid et al. (1979)]. An interesting result from these studies was that several tRNAs with the same tertiary folding potential (Kim et al., 1974; Klug et al., 1974) as yeast $tRNA^{Phe}$ exhibited tertiary NMR resonances with chemical shifts similar to those observed for the yeast $tRNA^{Phe}$ tertiary resonances. Candidates for these extra low-field NMR resonances are the hydrogen-bonded ring NH protons of the following tertiary base pairs observed in the three-dimensional folding of yeast $tRNA^{Phe}$: 8–14, 54–58, 46–22, 19–56, 15–48, and possibly 26–44 and/or 18–55 (Sussman & Kim, 1976; Quigley & Rich, 1976; Jack et al., 1976). Two of these interactions involve AU-type base pairing, namely, U8 (s^4U8)–A14 and rT54–A58; the remaining tertiary interactions involving ring NH protons are GC-type interactions in that they involve hydrogen bonding of the G N1H proton. We have previously shown that AU base pairs resonate to lower field than GC base pairs because of the greater inherent deshielding of U N3H compared to G N1H [see Reid et al. (1979)]. This observation suggested that the U8 (s^4U8)–A14 interaction and the rT54–A58 interaction should each generate a resonance in the extreme low-field region of the low-field spectrum.

In this paper we have used a combination of helical RNA fragment analysis, chemical modification, and comparative spectroscopy to assign the resonances from U8 (s^4U8)–A14 and rT54–A58. In subsequent papers (Hurd & Reid, 1979; Hurd et al., 1979) we will assign the guanosine-type tertiary interaction in the low-field NMR spectrum.

Materials and Methods

Isolation of tRNA. *E. coli* $tRNA_1^{Val}$ (Reid et al., 1979) and *E. coli* $tRNA^{fMet}$ (Reid et al., 1977) were purified to homogeneity as described previously. The final preparations accepted over 1800 pmol of valine and methionine/A_{260} unit, respectively, when aminoacylated with the corresponding pure aminoacyl-tRNA synthetase enzymes. *E. coli* $tRNA_1^{Gly}$ and $tRNA_3^{Gly}$ were initially separated by BD-cellulose chromatography according to the procedure of Gillam et al. (1967). *E. coli* $tRNA_1^{Gly}$ eluted extremely early in the gradient followed closely by $tRNA_2^{Gly}$; the major glycine species, $tRNA_3^{Gly}$, eluted much later in the gradient at ~0.82 M NaCl. The $tRNA_1^{Gly}$ fraction was purified to homogeneity by DEAE-Sephadex chromatography (Nishimura, 1971); the $tRNA_3^{Gly}$ fraction was purified to homogeneity by DEAE-Sephadex chromatography, Sepharose 4B chromatography (Holmes et al., 1975), and RPC5 chromatography (Pearson

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[1] Abbreviations used: tRNA, transfer ribonucleic acid; DHU, dihydrouridine; rT, ribothymidine; m^7G, N^7-methylguanosine; Ψ, pseudouridine; s^4U, 4-thiouridine; EDTA, ethylenediaminetetracetic acid; NMR, nuclear magnetic resonance; BD-cellulose, benzoylated DEAE-cellulose.

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et al., 1971). The final preparations accepted 1700 pmol of glycine and 1580 pmol of glycine/A_{260} unit, respectively, when aminoacylated with partially purified glycyl-tRNA synthetase. *E. coli* $tRNA^{His}$ was purified to homogeneity by a combination of BD-cellulose chromatography at pH 5.0, DEAE-Sephadex chromatography at pH 7.5, BD-cellulose chromatography at pH 7.0, RPC5 chromatography at pH 4.5, and finally Sepharose 4B chromatography at pH 4.5. The final material accepted 1650 pmol of histidine/A_{260} unit when aminoacylated with partially purified histidyl-tRNA synthetase.

Fragments. *E. coli* $tRNA_1^{Val}$ was cleaved at m^7G46 as described in the preceding paper (Reid et al., 1979); the fragments 1–45 and 47–76 were isolated on hot Sephadex G100 columns as described previously (Reid et al., 1972). Fragment 47–76 was dialyzed against distilled water and lyophilized prior to being dissolved in buffer for NMR analysis.

NMR Spectroscopy. NMR spectra were obtained with 1.0–1.4 mM tRNA samples in 0.19 mL of the appropriate buffer on a Bruker HXS-360 spectrometer (Stanford Magnetic Resonance Laboratory), under fast-sweep correlation spectroscopy conditions described earlier (Reid et al., 1979). The NMR spectra of the monomeric nucleosides 4-thiouridine and uridine in dry Me_2SO were obtained by FT NMR as described in an earlier paper in this series.

Results

Our previous observation that U N3H resonates at lower field than G N1H in the NMR spectra of these monomers led to our first hypothesis that uridine-type tertiary base pairs should be found at the low-field end of the ring NH spectrum of tRNAs. Analysis of several hairpin fragment spectra and intact tRNA spectra led us to the conclusion that Watson–Crick AU base pairs have an inherent unshifted resonance position at ca. −14.3 ppm; the stacking of neighboring bases produces sequence-dependent upfield shifts of varying amounts so that normal AU base pairs can only be found at −14.2 ppm or higher. Despite these arguments we noted that several tRNA spectra, e.g., yeast $tRNA^{Phe}$, *E. coli* $tRNA_1^{Val}$, etc., contain two protons between −14.3 and −15 ppm and we immediately focused our attention on these two extremely low-field protons as possible uridine tertiary base pairs.

A further interesting observation is that, while yeast tRNAs contain U8, the majority of *E. coli* tRNAs contain s^4U8, and this correlates with our observation that yeast tRNAs do not contain resonances below −14.4 ppm whereas the majority of *E. coli* tRNAs contain a resonance at −14.8 or −14.9 ppm. We therefore suggested that the s^4U8 N3H hydrogen bond to A14 was responsible for the −14.9-ppm resonance in bacterial tRNA spectra, and this hypothesis was supported by subsequent experiments by us (Reid et al., 1975) and by others (Wong et al., 1975a–c; Daniel & Cohn, 1975, 1976). In these experiments chemical dethiolation of s^4U8 to U8 was found to shift the 8–14 resonance from −14.9 to −14.3 ppm and the amino acid acceptor activity was maintained in these tRNA samples (Reid et al., 1975). A further example which unequivocally assigns to s^4U8–A14 the extremely deshielded single proton often found between −14.7 and −14.9 ppm in bacterial tRNA is shown in Figure 1. During our chromatographic isolation sequence of several tRNA species from *E. coli* B, by a fortunate accident the resulting $tRNA_3^{Gly}$ was found, by UV spectroscopy at 340 nm, to naturally contain only 0.3 mol of s^4U8/mol of tRNA. The NMR spectrum revealed a peak of 0.3 proton intensity at −14.75 ppm with the remaining intensity located at −14.25 ppm. In the specific case of *E. coli* valine tRNA we were fortunate enough to obtain from one particular crude tRNA extract a pure sample of $tRNA_1^{Val}$ containing

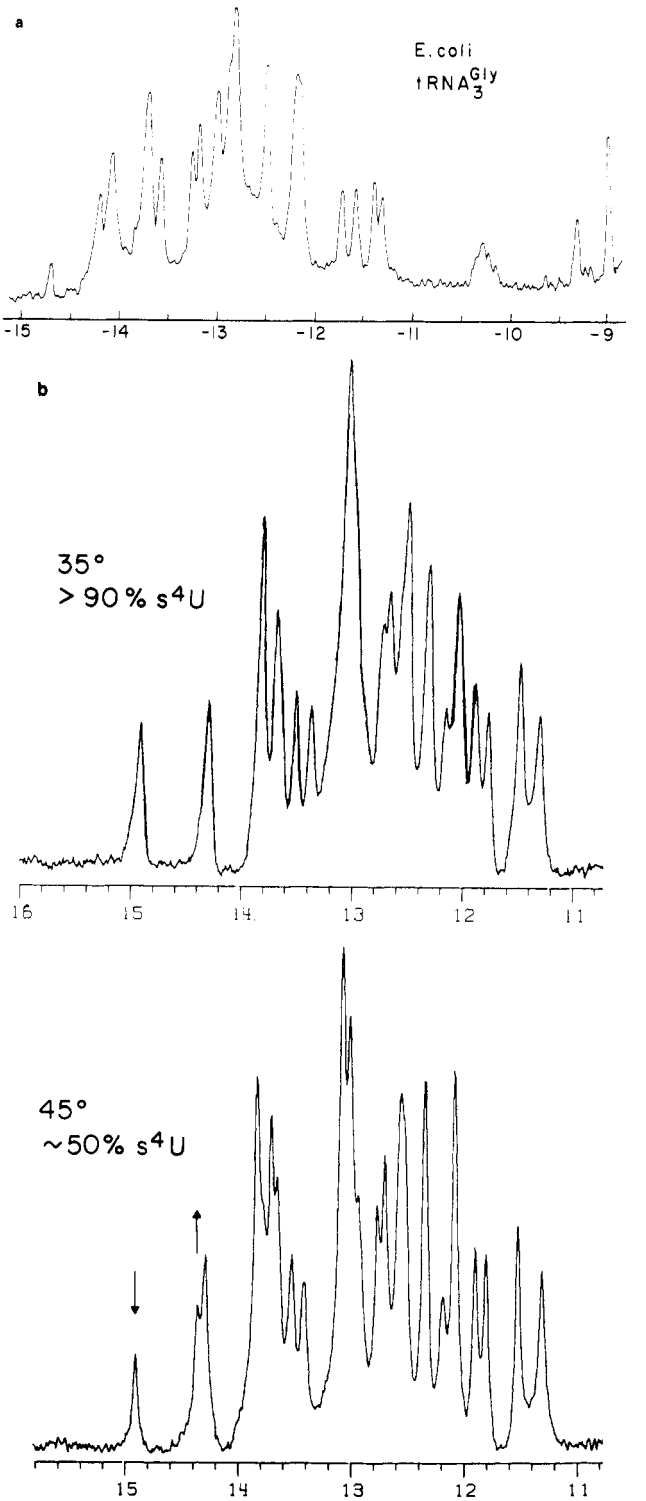

FIGURE 1: (a) The 360-MHz NMR spectrum of *E. coli* $tRNA_3^{Gly}$ in the presence of magnesium. This sample contained ~30% s^4U and revealed an ~0.3-proton resonance at −14.75 ppm with the rest of the intensity at −14.25 ppm. (b) NMR spectrum of *E. coli* $tRNA_1^{Val}$ containing more than 90% s^4U at position 8 (upper) compared to that of a sample containing 50% s^4U and 50% U at position 8 (lower). Note that half of the proton resonance at −14.9 ppm has moved upfield to −14.35 ppm.

50% s^4U8 and 50% U8 which could be directly compared with a control sample containing over 90% s^4U at position 8. The resulting spectra are far less ambiguous than those resulting from chemical dethiolation of tRNA (Reid et al., 1975; Wong et al., 1975a–c) and are shown in Figure 1b; it is obvious that half a resonance remains at −14.9 ppm (s^4U8) and that the

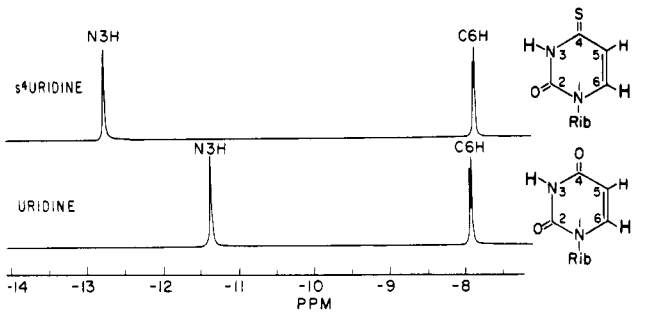

FIGURE 2: NMR spectra of 4-thiouridine and uridine in dry Me_2SO-d_6. Note the extra deshielding of N3H in 4-thiouridine.

other half appears at −14.35 ppm (U8). The double proton peaks at −12.3 and −12.1 ppm appear sharper in the sulfur-deficient spectra, but this is probably due to temperature-dependent effects rather than to thiolation. Parenthetically we should add that these two spectra were obtained by Redfield 214 FT NMR methods rather than by frequency sweep methods, and a further (unintended) variation was that instead of using freshly prepared buffer a magnesium-containing buffer which had been stored for 2 months at 2 °C was used. This may have reduced the free magnesium ion below the stated 15 mM concentration, but, apart from the downfield shift to −12.7 ppm of one of the four protons at −12.6 ppm, the spectra are the expected normal magnesium spectra of *E. coli* $tRNA_1^{Val}$. Thus, without any of the associated complications due to side reactions of dethiolating reagents such as cyanogen bromide, we could assign the s^4U8–A14 resonance at −14.8 ± 0.1 ppm (and the U8–A14 resonance at −14.3 ± 0.1 ppm).

A possible reason for the extremely low-field position of this resonance in bacterial tRNAs containing s^4U8 is the deshielding effect on the N3H of uridine of sulfur instead of oxygen at carbon 4. To investigate this possibility further, we carried out NMR studies on the nucleoside monomers s^4U and U in Me_2SO. As shown in Figure 2, the N3H of s^4U is indeed deshielded by the sulfur atom and is observed ~1.4 ppm to lower field than the N3H of uridine. Hence, s^4U8–A14 resonances could theoretically resonate as low as −15.7 ppm. While this is the principal factor responsible for the exceptionally low-resonance position of the s^4U–A14 hydrogen-bond resonance in bacterial tRNAs, even the U8–A14 ring NH proton (in yeast tRNAs and dethiolated bacterial tRNAs) has a somewhat atypically large chemical shift compared to normal AU resonances since it is observed at −14.3 ppm even after being shifted upfield by its stacking neighbors. The reason for this slightly abnormal position for U8–A14 can be seen in the crystal structure of tRNA which reveals that, at least in yeast $tRNA^{Phe}$, the U8–A14 tertiary interaction (see Figure 6) is not a normal Watson–Crick AU pair but a reversed Hoogsteen base pair in which N3H is hydrogen bonded to N7 of A14 (Sussman & Kim, 1976; Quigley & Rich, 1976; Jack et al., 1976); the electronic reasons for the extra deshielding in Hoogsteen hydrogen bonding have been discussed by Robillard et al. (1976). Thus, comparative spectroscopy, chemical modification, and analysis of undermodified tRNA species all unequivocally assign the s^4U8–A14 hydrogen bond to the ca. −14.8-ppm tertiary resonance and the U8–A14 hydrogen bond to the ca. −14.3-ppm tertiary resonance; nonstandard base pairing and the extra deshielding due to the sulfur in s^4U8 satisfactorily explain the exceptionally low-field chemical shifts of these resonances.

The only other crystallographically observed tertiary base pair involving a uridine hydrogen-bonded ring NH proton is

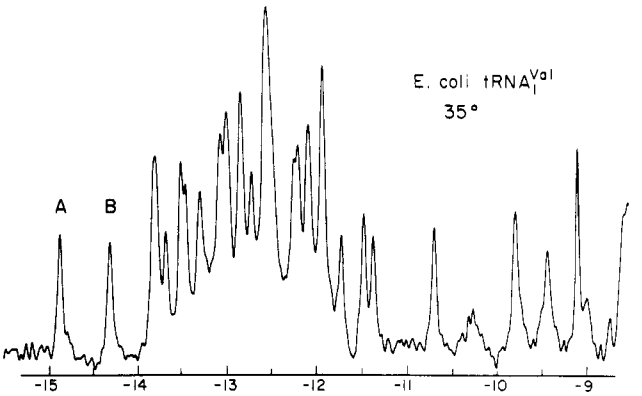

FIGURE 3: The 360-MHz NMR spectrum of *E. coli* $tRNA_1^{Val}$ at 35 °C in 10 mM sodium cacodylate, 10 mM EDTA, 9 mM $MgCl_2$, and 50 mM NaCl, pH 7.0. The resonances designated A and B cannot be accounted for by cloverleaf base pairs.

the interaction between rT54 and A58 in the rT loop of the majority of tRNAs sequenced to date. In the crystal structure of yeast $tRNA^{Phe}$ this interaction involves a hydrogen bond from N3H of rT; we have previously shown that the N3H protons of thymidine and uridine have virtually identical chemical shifts (Hurd & Reid, 1977), and hence the rT54–A58 interaction is also expected to generate a resonance at lower field than the −13.4- to −11.7-ppm region in which GC resonances are normally observed.

In Figure 3 is shown the low-field spectrum of *E. coli* $tRNA_1^{Val}$ in which we have shown, by several methods, that neither of the two protons between −14 and −15 ppm (denoted A and B) are derived from secondary base pairs. The five secondary AU pairs have similar neighboring upfield shifts and are expected in a closely spaced cluster (near −13.8 ppm from hairpin fragment studies). Since resonance A at −14.9 ppm has been experimentally assigned to s^4U8–A14 and GC resonances cannot resonate at such low field, the most likely candidate for resonance B at −14.3 ppm is the rT54–A58 tertiary base pair. Additional evidence comes from the fact that, under native conditions in the presence of excess magnesium, this resonance is one of the most labile protons in the spectrum as shown in Figure 4. As the temperature is increased, resonance B is one of the earliest protons to exchange out of the spectrum via solvent access and it is closely followed by tertiary resonance A (s^4U8–A14) at slightly higher temperature (see Figure 1 of the preceding paper). The observed thermal lability of resonance B does not unequivocally prove that it is tertiary base pair; however, the fact that this base pair begins to come apart slightly before the unfolding of the known tertiary base pair s^4U8–A14, and before major changes are seen in the rest of the spectrum, can be taken as circumstantial evidence that peak B at −14.3 ppm does indeed reflect a tertiary interaction. From its chemical shift it must be derived from a uridine or thymidine N3H hydrogen bond. If we assume that the folding of *E. coli* $tRNA_1^{Val}$ in solution is similar to the crystal structure of yeast $tRNA^{Phe}$, then the only remaining interpretation is that peak B is derived from the rT54–A58 base pair.

Despite the persuasive nature of this deductive assignment, we also felt the need for a positive method of assigning the rT54–A58 resonance. Unfortunately, ribothymidine does not exhibit any selective reactivity which might have led to an assignment by chemical modification. Neither did we have available any tRNA species with sequences indicating that the rT54–A58 interaction might be absent. Our only hope lay in the fact that, unlike all other tertiary base pairs which involve interactions between distal loops or helixes, the rT54–A58 base

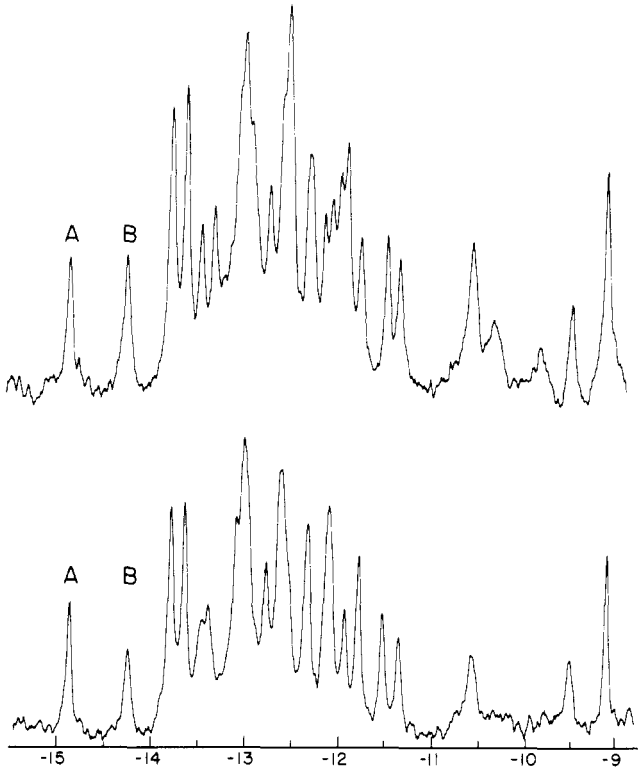

FIGURE 4: The 360-MHz NMR spectra of *E. coli* $tRNA_1^{Val}$ in 10 mM sodium cacodylate, 15 mM $MgCl_2$, and 100 mM NaCl, pH 7.0, at 41 (upper) and 55 °C (lower). Resonance B (−14.3 ppm) begins to water-exchange before resonance A (−14.9 ppm).

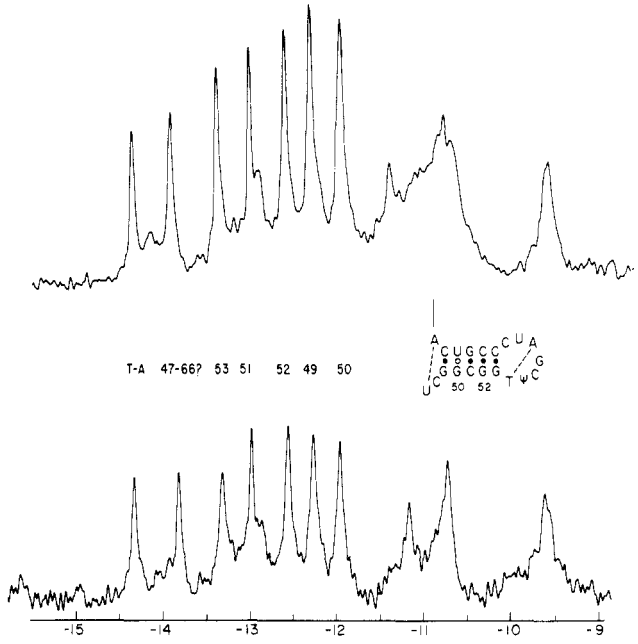

FIGURE 5: NMR spectra of the rT helix of *E. coli* $tRNA_1^{Val}$ in 10 mM sodium cacodylate and 10 mM EDTA, pH 7.0, at 24 °C (upper) and in 5 mM sodium phosphate and 100 mM NaCl, pH 7.0, at 27 °C (lower).

pair involved two nucleotides located within the same loop in the cloverleaf structure. Consequently, we reasoned that this tertiary base pair might form in a fragment containing the rT helix and rT loop if the temperature were lowered sufficiently. As shown in Figure 5, the helical fragment of *E. coli* $tRNA_1^{Val}$ containing residues 47–76, which previously contained an additional broadened resonance at −14 ppm at intermediate temperatures (see Figure 6 in the preceding paper), reveals

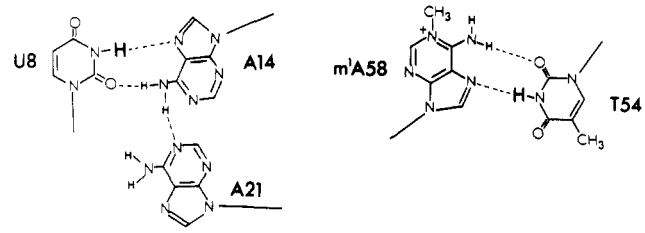

FIGURE 6: Hydrogen bonding in the 8–14 and 54–58 tertiary interactions in yeast $tRNA^{Phe}$.

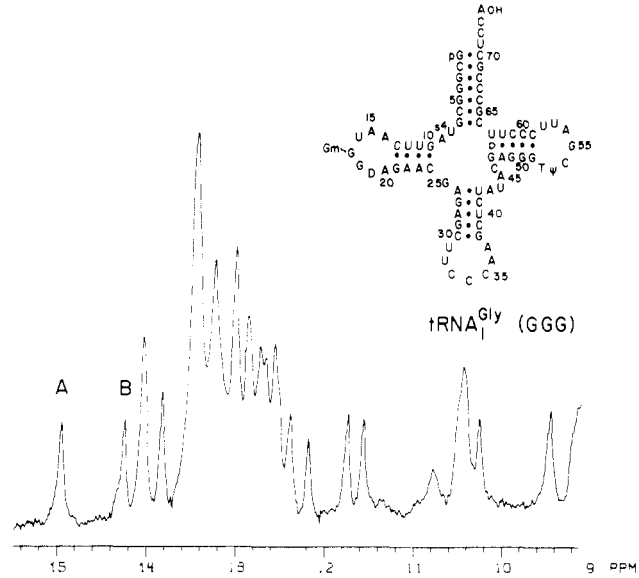

FIGURE 7: The 360-MHz NMR spectrum of *E. coli* $tRNA_1^{Gly}$ in the presence of magnesium at 45 °C. The three protons between −13.7 and −14 ppm are accounted for by cloverleaf base pairs (11, 25, and 48); the two resonances labeled A and B are not from secondary base pairs. This spectrum was accidentally misscaled on the *x* axis, and the true position of each peak should be moved upfield by 0.05–0.10 ppm in chemical shift.

discrete additional resonances at −14.3 and −13.9 ppm when the temperature is lowered. Below 27 °C, especially in phosphate and 100 mM NaCl, the new peak at −14.3 ppm approaches a full proton intensity. Thus, the expected resonance at −14.3 ppm from rT54–A58 is indeed observable in a helical fragment containing this loop; the second additional resonance at ca. −13.9 ppm was somewhat unexpected, and we tentatively attribute it to base pairing between the single-stranded "tails" of this fragment, probably U47 and A66.

A further point worth noting is that, even at −14.3 ppm, this tertiary resonance is still at lower field than one would expect for a normal Watson–Crick AU (or AT) resonance if one takes into account the upfield shifts expected from stacked neighbors. As shown in Figure 6, the reason for this is that, like the U8–A14 interaction, crystallographically the rT54–A58 interaction is not a normal Watson–Crick AU pair but is also a reversed Hoogsteen interaction involving hydrogen bonding to N7 of A58. As in the case of U8–A14, the extra downfield shift is presumably caused by this nonstandard hydrogen bonding.

Thus, several tRNAs, such as yeast $tRNA^{Phe}$, *E. coli* $tRNA_1^{Val}$, etc., contain a tertiary resonance at ca. −14.3 ppm which is most reasonably assigned to the pairing of the unique ribothymidine with the adenine residue following it by four residues in the sequence. A further example is shown in Figure 7. *E. coli* $tRNA_1^{Gly}$ contains five secondary AU base pairs in differing environments in the sequence. On the basis of the previously described ring current shift values of Arter &

Schmidt (1976), these secondary AU pairs account for the two protons at −14.0 ppm, the single proton at −13.8 ppm, and two of the four protons observed at −13.4 ppm. Again on the assumption of similar folding to the crystal structure, the extra resonances from tertiary base pairs at −14.9 and −14.25 ppm, designated A and B, can only be assigned to s^4U8–A14 and "rT54–A58", respectively (in $tRNA_1^{Gly}$ the rT is actually in position 52). However, it must also be mentioned that several tRNAs expected to contain the rT54–A58 interaction contain no resonances between −14.0 and −14.7 ppm; this suggested to us that certain tRNAs may contain rT54–A58 resonances which have been differentially shifted upfield by sequence-dependent environmental effects. In the crystal structure of yeast $tRNA^{Phe}$ the environment of the 54–58 pair is defined by stacking with the tertiary base pair G18–Ψ55 on one side and stacking with GC53 (the terminal base pair of the rT helix) on the other side; possible additional shifts might result from the adjacent residues 57 and 59 on either side of A58 (Sussman & Kim, 1976). The terminal GC53 base pair of the rT helix is common in all of the tRNA species we have studied and, since they all contain the corresponding G in the DHU loop and Ψ in the rT loop, we assume that the G18–Ψ55 interaction is formed as in yeast $tRNA^{Phe}$. Hence, the two adjacent stacks on the rT54–A58 base pair appear to be identical with yeast $tRNA^{Phe}$ in all of the tRNA species studied. We therefore turned out attention to residues 57 and 59 (or more correctly the residues preceding and following the adenine which accepts the rT hydrogen bond) as the possible cause of the upfield shift on the rT54–A58 hydrogen-bond resonance.

The large majority of tRNA species sequenced to date contain, as the first five residues in their seven-residue rT loop, the sequence TΨCGA; i.e., in most cases G57 precedes A58. However, there is much greater variability in residue 59 following A58. In yeast $tRNA^{Phe}$, *E. coli* $tRNA_1^{Val}$, and *E. coli* $tRNA_1^{Gly}$ the corresponding sequence is TΨCGAU, and in these cases we observe the T–A resonance close to −14.3 ppm as already described. In *E. coli* $tRNA_3^{Gly}$ (Figure 1) and *E. coli* $tRNA^{Trp}$ (not shown) the corresponding sequence is TΨCGAC; i.e., the U following A58 has been replaced by a G residue. In these cases the extra, noncloverleaf low-field resonance which we attribute to the T–A hydrogen bond is located at either −14.1 or −13.9 ppm. On continuation of this series, in $tRNA^{His}$, shown in Figure 8, $tRNA_m^{Met}$ [see Reid et al. (1979)], and $tRNA^{Arg}$ and $tRNA^{Lys}$ [see Hurd et al. (1979) and Hurd & Reid (1979)] the corresponding rT loop sequence is TΨCGAA; i.e., an adenine now follows A58 (or its equivalent). In the spectrum of *E. coli* $tRNA^{His}$ (and $tRNA_m^{Met}$, $tRNA_1^{Arg}$ and $tRNA^{Lys}$) the nearest observed resonance which can be assigned to the T–A base pair is at ca. −13.8 ppm. Thus, replacing U59 with G or with A appears to cause an upfield shift on the rT54–A58 resonance position. Since adenine has by far the strongest ring current (Giessner-Prettre & Pullman, 1970) and can, when optimally positioned, produce upfield shifts of 1.0 ppm or greater, we infer that A59 is somewhat removed, either in angle or distance, from direct juxtaposition with rT54. Thus, the magnitude of the effect of replacing U59 by G or A is probably attenuated by its distance from rT54.

There are relatively few examples in which G57 is replaced by other residues. However, one important example is *E. coli* $tRNA^{fMet}$, the spectrum of which is shown in Figure 9. In this tRNA the corresponding rT loop sequence is TΨCAAA; i.e., the positions preceding and following "A58" (actually A59 in $tRNA^{fMet}$ due to an extra residue in the DHU loop) are both

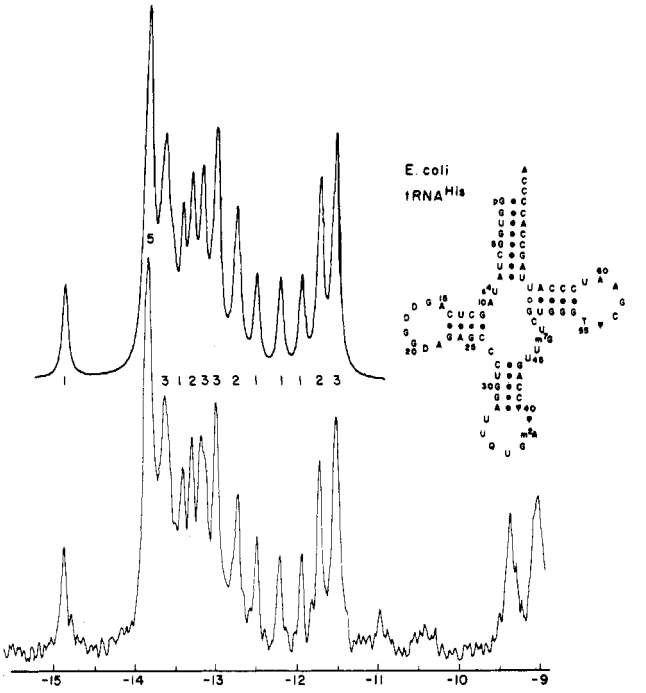

FIGURE 8: The 360-MHz NMR spectrum of *E. coli* $tRNA^{His}$ in the presence of magnesium at 45 °C. The upper trace is a computer simulation of the spectrum using Lorentzian lines of the experimental line width; simulation and integration reveal five protons in the peak at ca. −3.8 ppm. Four of the protons in this peak are derived from cloverleaf base pairs (3, 13, 29, and 51), and one of the five protons is a tertiary resonance.

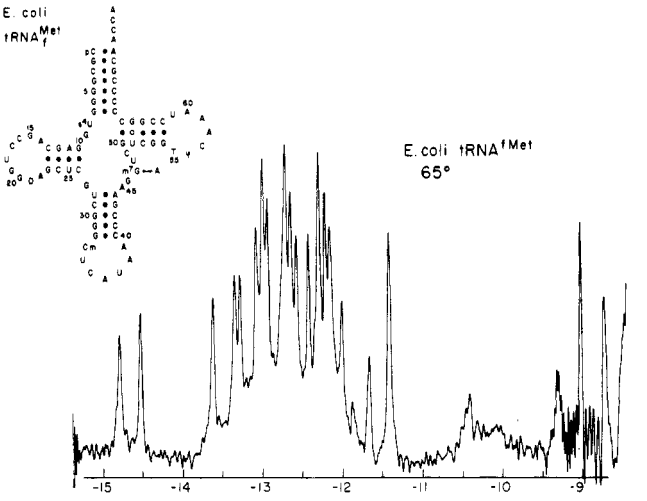

FIGURE 9: The 360-MHz NMR spectrum of *E. coli* $tRNA_f^{Met}$ in the presence of excess magnesium at 65 °C. The high GC content of this tRNA makes it extremely thermostable in magnesium and sodium chloride buffers; there are virtually no intensity losses between −12 and −15 ppm at 65 °C compared to the 30 °C spectrum. In addition to the inherently narrower line widths at higher temperatures, this spectrum has been resolution enhanced by −6 Hz (negative line broadening).

occupied by adenine residues which possess the strongest ring current. The spectrum of *E. coli* $tRNA^{fMet}$ contains no resonances between −13.8 and −14.5 ppm. Thus, the resonances at either −14.55 or −13.65 ppm become the nearest candidates for the T–A resonance. We have independently assigned the resonance at −14.55 ppm to the m^7G–CG13 interaction by chemical removal of m^7G from $tRNA^{fMet}$ [see Hurd & Reid (1979)]. The resonance at −13.65 ppm is very probably derived from the secondary base pair AU11, and the nearest unassigned noncloverleaf resonance is one of the two

protons at −13.25 ppm. Hence, our most reasonable assignment for the T–A resonance in *E. coli* $tRNA^{fMet}$ is ~1.0 ppm upfield from its counterpart in yeast $tRNA^{Phe}$. It appears that part of this upfield shift is derived from the residue preceding the A to which the ribothymidine is bonded, but most of the effect seems to come from the residue following this adenine in the sequence.

Discussion

The history of misassignments for AU resonances in the low end of tRNA ring NH NMR spectra and the assumed presence or absence of tertiary AU resonances in this region has been a troublesome one. The effect of paramagnetic ions on the spectrum of yeast $tRNA^{Phe}$ has been interpreted on the assumption that no tertiary resonances were present in the spectrum (Jones & Kearns, 1974); the same assumption was made in a study of anticodon modifications (Wong et al., 1975b) and in a report on the binding of ethidium bromide to yeast $tRNA^{Phe}$ (Jones & Kearns, 1975) in which the two-proton peak at −14.4 ppm was assigned to secondary base pairs AU5 and UA6. The resulting perturbation of this peak was interpreted as a unique ethidium binding site at UA6; our present demonstration that both protons in this peak are tertiary resonances (U8–A14 and rT54–A58) casts serious doubt on this interpretation. Our evidence for assigning rT54–A58 at −14.3 to −14.4 ppm is undoubtedly weaker than our assignment of the 8–14 tertiary Hoogsteen pair at this position (in species containing U8 rather than s^4U8). Thus, from a consideration of only the yeast $tRNA^{Phe}$ spectrum it might remain feasible to attribute one of the two protons at ca. −14.35 ppm to a secondary AU pair. However, comparative analysis of an extended series of different tRNA species makes this an untenable assignment. For instance, UA4 in *E. coli* $tRNA_1^{Arg}$ and UA30 (as well as AU50) in *E. coli* $tRNA_m^{Met}$ are in minimally shifted nearest neighbor environments similar to AU5 and UA6 in yeast $tRNA^{Phe}$; however, neither of these tRNAs contains any resonances between −14.0 and −14.7 ppm in their low-field spectra. A further inconsistency within the yeast $tRNA^{Phe}$ data is that AU5 and UA6 are in very similar ring current environments and should resonate close to each other, yet one of the two protons at −14.35 ppm is most certainly the 8–14 tertiary base pair.

Despite what we consider to be overwhelming evidence that the low-field spectrum of yeast $tRNA^{Phe}$ and related tRNA species contains the crystallographically expected six to seven extra resonances from tertiary base pairing (Reid et al., 1975; Reid & Robillard, 1975; Reid et al., 1977), the existence of these tertiary resonances has been the subject of controversy over the last few years (Bolton et al., 1976; Kearns, 1976; Bolton & Kearns, 1976; Reid, 1976). Perhaps the least contentious area has been the assignment of the s^4U8–A14 resonance in bacterial tRNAs. Chemical modification of s^4U8 to U8 in several *E. coli* tRNAs has led to the unambiguous assignment of the s^4U8–A14 resonance at ca. −14.8 ppm (Reid et al., 1975; Wong et al., 1975a; Daniel & Cohn, 1975, 1976). An additional result from these studies, and those presented here, is that in dethiolated *E. coli* tRNAs, *E. coli* tRNAs, which naturally contain U8 instead of s^4U8, and yeast tRNAs, which always contain U instead of s^4U8 at position 8, the U8–A14 resonance is observed at ca. −14.3 ppm.

The assignment of the rT54–A58 resonance in previous literature reports has been much more problematical. Despite relatively recent reports that tertiary resonances were absent in tRNA spectra (Jones & Kearns, 1975; Wong et al., 1975b), Kearns and co-workers (Kearns, 1976; Bolton & Kearns, 1975) claim to be able to observe approximately two tertiary resonances in the NMR spectrum of unfractionated *E. coli* tRNA (a mixture of ~60 tRNA sequences). One of these "tertiary resonances" was observed at −13.8 ppm and was promptly "assigned" to the rT54–A58 resonance common to all tRNAs. While the T–A resonance is admittedly common to all tRNAs, its environment and hence chemical shift are different in different tRNA species; in some tRNAs it does resonate at −13.8 ppm.

The more recent acknowledgment by Kearns and co-workers that tertiary resonances do in fact exist in tRNA low-field spectra has not alleviated the problem of the integrated low-field spectrum intensity between −11 and −15 ppm. In a recent review (Kearns, 1976) it is still maintained that the spectrum of yeast $tRNA^{Phe}$ contains only 19.9 protons (base pairs), although the tertiary base pairs U8–A14 and T54–A58 (assigned at −13.75 ppm) are now acknowledged to exist in the spectrum. Since the tRNA already contains 20 secondary base pairs, this causes some awkward quantitative intensity problems; however, these problems have apparently been solved by ignoring the presence of base pair AU5 (Kearns, 1976).

Some of the most interesting results to emerge from our present series of investigations are the assignments for the base pairs of *E. coli* $tRNA^{fMet}$. We can now state that, in the $tRNA^{fMet}$ spectrum, the −14.8-ppm resonance is derived from s^4U8–A14 (this paper) and the −14.55-ppm resonance is derived from the hydrogen-bonded N1H of m^7G (Hurd & Reid, 1979); furthermore, the rT–A tertiary resonance in $tRNA^{fMet}$ occurs at either −13.65 or −13.3 ppm (most likely the latter). Thus, it appears that three of the four lowest field resonances in the spectrum of $tRNA^{fMet}$ are not derived from the cloverleaf structure at all. These observations are extremely relevant in terms of previous attempts to analyze the low-field NMR spectrum of *E. coli* $tRNA^{fMet}$ and to interpret the thermal unfolding sequence of this tRNA. In their analysis of temperature effects on $tRNA^{fMet}$, Crothers et al. (1974) claimed that no tertiary resonances were present in the spectrum which was assumed to contain 19 protons from 19 secondary base pairs. When the temperature was raised, intensity losses were first observed at −13.6, −13.2, −12.1, and −11.7 ppm and were attributed to loss of the (unobservable) tertiary structure and DHU stem resonances. Reexamination of their spectra reveals that, far from being unobservable, the tertiary resonances (e.g., −14.8 ppm, −14.55 ppm, etc.) are certainly present in their spectra (although misassigned to secondary base pairs). Similarly, Wong et al. (1975c) have also used NMR to determine the thermal unfolding sequence of *E. coli* $tRNA^{fMet}$. In their analysis of the spectrum they acknowledge one tertiary base pair resonance (s^4U8–A14) and a resonance due to "a protected U residue in the anticodon loop" (Wong et al., 1975c). In both of these studies the assumption that no tertiary base pairs (Crothers et al., 1974) or only one tertiary base pair (Wong et al., 1975c) can be observed in the spectrum casts serious doubt on the validity of the interpretations resulting from these studies. The data of Daniel & Cohn (1975, 1976), in which they estimate the intensity of the $tRNA^{fMet}$ low-field spectrum to correspond to 27 ± 1 ring NH hydrogen bonds, are in much closer agreement with our own estimate of the number of stable secondary and tertiary base pairs detectable in the spectrum of this tRNA.

Our studies on the position of the rT54–A58 tertiary resonance in different tRNA species have revealed an interesting environmental effect of the residues on either side of A58. When this residue is preceded by G and followed by U, the

resonance is observed at ca. −14.3 ppm; when preceded by G and followed by G or A, the resonance moves upfield to ca. −14.0 and −13.8 ppm, respectively. When preceded by A and followed by A, the resonance moves dramatically upfield to ca. −13.3 ppm, reflecting the much stronger effect of the preceding nucleotide. Examination of the X-ray structure of yeast $tRNA^{Phe}$ indicates that these results are somewhat puzzling if one assumes that the static crystal structure is an accurate description of the solution structure. From the X-ray coordinates G57 is some 7 Å away from T54 N3H and is coplanar in a next-to-nearest neighbor relationship so that increased upfield shifts (of a second-order nature) would be expected upon replacement by adenine. However, U59 is over 10 Å away from T54 N3H and is almost perpendicular to the plane of T54–A58. U59 and C60 are in a crowded region at the corner of the T loop, and it would appear to be difficult to accommodate G59 or A59 at the U59 position in species containing a purine at this site. Thus, the experimental shifts observed when G57 and U59 are replaced by adenine may reflect local dynamic effects or alternatively an orientation of A59 which is different from that of U59 in yeast $tRNA^{Phe}$. The answer to this question must await solution of the X-ray structure of tRNA species with adenine in these positions. We should also add that an awkward exception to our assignment of the rT54–A58 tertiary resonance is the case of *E. coli* $tRNA^{Phe}$. Although the reported primary structure of this tRNA contains the sequence TΨCGAU, the spectrum does not contain a resonance at −14.3 ppm and we are at a loss to explain this discrepancy on the basis of the static X-ray structure.

Finally, completely independent NMR methods, based on the dynamics of base pair breathing, have recently been developed by Johnston & Redfield (1977) for determining which resonances in tRNA spectra are tertiary and which are secondary. They have shown that, under appropriate conditions, the major mode of saturation recovery of these resonances is exchange with water protons. In the absence of magnesium they observe six or seven resonances (presumably tertiary) which exhibit anomalously fast exchange with water. In yeast $tRNA^{Phe}$ both of the protons at −14.4 ppm exhibit the fast exchange characteristic of tertiary base pairs (Johnston & Redfield, 1977); furthermore, very recent preliminary data on *E. coli* $tRNA_1^{Val}$ reveal that the proton at −14.9 ppm and the proton at −14.3 ppm both exhibit the characteristic tertiary fast-exchange properties (P. D. Johnston and A. G. Redfield, personal communication).

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References

Arter, D. B., & Schmidt, P. G. (1976) *Nucleic Acids Res. 3*, 1437.

Bolton, P. H., & Kearns, D. R. (1975) *Nature (London) 255*, 347.

Bolton, P. H., & Kearns, D. R. (1976) *Nature (London) 262*, 423.

Bolton, P. H., Jones, C. R., Bastedo-Lemer, D., Wong, K. L., & Kearns, D. R. (1976) *Biochemistry 15*, 4370.

Crothers, D. M., Cole, P. E., Hilbers, C. W., & Shulman, R. G. (1974) *J. Mol. Biol. 87*, 63.

Daniel, W. E., & Cohn, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2582.

Daniel, W. E., & Cohn, M. (1976) *Biochemistry 15*, 3917.

Giessner-Prettre, C., & Pullman, B. (1970) *J. Theor. Biol. 27*, 87.

Gillam, I., Millward, S., Blew, D., Von Tigerstrom, M., Wimmer, E., & Tener, G. M. (1967) *Biochemistry 6*, 3043.

Holmes, W. M., Hurd, R. E., Reid, B. R., Rimerman, R. A., & Hatfield, G. W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1068.

Hurd, R. E., & Reid, B. R (1977) *Nucleic Acids Res. 4*, 2747.

Hurd, R. E., & Reid, B. R. (1979) *Biochemistry* (fourth of four papers in this issue).

Hurd, R. E., Azhderian, E., & Reid, B. R. (1979) *Biochemistry* (following paper in this issue).

Jack, A., Ladner, J. E., & Klug, A. (1976) *J. Mol. Biol. 108*, 619.

Johnston, P. D., & Redfield, A. G. (1977) *Nucleic Acids Res. 4*, 3599.

Jones, C. R., & Kearns, D. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4237.

Jones, C. R., & Kearns, D. R. (1975) *Biochemistry 14*, 2660.

Kearns, D. R. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 18*, 91.

Kim, S. H., Sussman, J. L., Suddath, F. L., Quigley, G. J., McPherson, A., Wang, A. J. H., Seeman, N. C., & Rich, A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4970.

Klug, A., Ladner, J., & Robertus, J. D. (1974) *J. Mol. Biol. 89*, 511.

Nishimura, S. (1971) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) Vol. 2, p 542, Harper and Row, New York.

Pearson, R. H., Weiss, J. F., & Kelmers, A. D. (1971) *Biochim. Biophys. Acta 228*, 770.

Quigley, G. J., & Rich, A. (1976) *Science 194*, 796.

Reid, B. R. (1976) *Nature (London) 262*, 424.

Reid, B. R., & Robilland, G. T. (1975) *Nature (London) 257*, 287.

Reid, B. R., Einarson, B., & Schmidt, J. (1972) *Biochimie 54*, 325.

Reid, B. R., Ribeiro, N. S., Gould, G., Robillard, G., Hilbers, C. W., & Shulm, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2049.

Reid, B. R., Ribeiro, N. S., McCollum, L., Abbate, J., & Hurd, R. E. (1977) *Biochemistry 16*, 2086.

Reid, B. R., McCollum, L., Ribeiro, N. S., Abbate, J., & Hurd, R. E. (1979) *Biochemistry* (preceding paper in this issue).

Robillard, G. T., Tarr, C. E., Vosman, F., & Berendsen, H. J. C. (1976) *Nature (London) 262*, 363.

Sussman, J. L., & Kim, S. H. (1976) *Science 192*, 853.

Wong, K. L., Bolton, P. H., & Kearns, D. R. (1975a) *Biochim. Biophys. Acta 382*, 446.

Wong, K. L., Kearns, D. R., Wintermeyer, W., & Zachau, H. G. (1975b) *Biochim. Biophys. Acta 395*, 1.

Wong, K. L., Wong, Y. P., & Kearns, D. R. (1975c) *Biopolymers 14*, 749.