1975

Studies on the Secondary and Tertiary Structure of Yeast Denaturable Leucine Transfer Ribonucleic Acid.

Erin Raschke Hawkins
Louisiana State University and Agricultural & Mechanical College

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STUDIES ON THE SECONDARY AND TERTIARY STRUCTURE
OF YEAST DENATURABLE LEUCINE TRANSFER
RIBONUCLEIC ACID.

The Louisiana State University and Agricultural
and Mechanical College, Ph.D., 1975
Chemistry, biological

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STUDIES ON THE SECONDARY AND TERTIARY STRUCTURE OF YEAST DENATURABLE LEUCINE TRANSFER RIBONUCLEIC ACID

A DISSERTATION

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biochemistry

by

Erin Raschke Hawkins
B.S., University of Texas, Austin, 1970 M.A., University of California, Berkeley, 1972
May, 1975
ACKNOWLEDGEMENTS

The author wishes to express her appreciation to all those who have made completion of this work possible. Sincere thanks go especially to Dr. Simon Hsin Chang for his assistance and guidance throughout the course of the research. The extremely helpful discussions of Dr. Wayne L. Mattice concerning the circular dichroism studies are also gratefully acknowledged. The author would further like to thank Drs. Kurt and Erika Randerath and Li-Li Chia at Baylor College of Medicine for their kind assistance with the base composition analysis technique, Charles K. Brum for his assistance in obtaining the UV absorbance melting curves and the tRNA\textsubscript{Leu\_CUA} CD spectrum, Joann T. Parker for her invaluable advice and assistance in preparing the figures, and Claire W. Harmon for her cheerful assistance whenever and wherever needed. Appreciation is also expressed to Dr. David R. Kearns, Dr. Dieter Söll, and the members of the dissertation committee for their supportive discussions.

The author would further like to express her deep appreciation to her parents, Mr. and Mrs. W. H. Raschke, her brother, Bill, and her sister, Lesa, without whose continued assistance, encouragement, and moral support over the years this degree would not have been possible.
Special thanks go also to her husband, Kerry, for his patience and willing assistance in so many ways throughout these graduate studies.

The author was the recipient of a National Science Foundation predoctoral fellowship. The research was supported in part by National Science Foundation Grant GB-40607 awarded to Dr. Simon H. Chang.
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LIST OF ABBREVIATIONS

**Nucleosides:**

- $m^5C$ 5-methylcytidine
- $ac^4C$ $N^4$-acetylcytidine
- $m^1G$ 1-methylguanosine
- $m^2G$ $N^2$-methylguanosine
- $m^2G$ $N^2,N^2$-dimethylguanosine
- $G^m$ 2'-O-methylguanosine
- *G* kethoxalated guanosine
- D 5,6-dihydouridine
- T ribothymidine
- ψ pseudouridine
- $ms^2i^6A$ 2-thiomethyl-$N^6$-($\Delta^2$-isopentenyl)adenosine
- $i^6A$ $N^6$-($\Delta^2$-isopentenyl)adenosine
- $t^6A$ $N$-[9-($\beta$-D-ribofuranosyl)purin-6-ylcarbamoyl]threone

**Methods:**

One $A_{260}$ unit that amount of material which produces an absorbance of 1 at 25° in a 1 cm light path at 260 nm

**tRNA: Modification Nomenclature and Solvent Conditions**

$N_S$ tRNA subjected to short-term modification with kethoxal while in the native conformation
LIST OF ABBREVIATIONS (cont'd)

\(N_L\)  tRNA subjected to long-term modification with kethoxal while in the native conformation

\(D_S\)  tRNA subjected to short-term modification with kethoxal while in the denatured conformation

\(D_L\)  tRNA subjected to long-term modification with kethoxal while in the denatured conformation

\(D_{H_2O}\)  tRNA dialyzed extensively against distilled water and subsequently heated to 60° for 5 minutes and quick-cooled in the presence of 10 mM potassium cacodylate, pH 7.0, 10 mM borate

\(D_{EDTA}\)  tRNA dialyzed by either of the two procedures described in Chapter 2 and to which 1 mM EDTA has been added

\(D_{Mg^{++}}\)  tRNA to which 10 mM MgCl\(_2\) has been added at 25°

Enzyme Nomenclature:

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<th>E.C. Number</th>
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<td>Tl ribonuclease (Tl RNase)</td>
<td>2.7.7.26</td>
<td>Ribonucleate guanosine-nucleotide-2'-transferase</td>
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<td>Pancreatic ribonuclease</td>
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<td>3.1.4.1</td>
<td>Orthophosphoric diester phosphohydrolase</td>
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<td>Leucyl-tRNA synthetase</td>
<td>6.1.1.4</td>
<td>L-leucine: tRNA ligase (AMP)</td>
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ABSTRACT

The present investigation was undertaken to characterize further the structural differences between the native and denatured conformers of yeast denaturable leucine transfer ribonucleic acid \( \text{tRNA}_{3}^{\text{Leu}} \). This study was approached from three basic standpoints: chemical modification using kethoxal \((\beta\text{-ethoxy-}\alpha\text{-keto-butyraldehyde})\), circular dichroism measurements under varying conditions of temperature and magnesium concentration, and comparison with a similar but nondenaturable yeast \( \text{tRNA}_{3}^{\text{Leu}} \) species.

Kethoxal selectively modifies exposed, non-base-paired guanosine residues in a polynucleotide chain. Thus differences in base-pairing patterns in native and denatured \( \text{tRNA}_{3}^{\text{Leu}} \) were studied by a comparison of their kethoxal incorporation characteristics. Two moles of kethoxal were initially incorporated by the native molecule at positions 17 and 18 in the dihydrouridine loop. On the other hand, nine guanosine residues were rapidly modified in the denatured conformer, including the two residues exposed in the native structure as well as residues located in the dihydrouridine stem and anticodon stem regions. These results are in agreement with studies of other denaturable \( \text{tRNA}_{3}^{\text{Leu}} \) species and provide evidence in support of the model proposed by Kearns et al. for denatured \( \text{tRNA}_{3}^{\text{Leu}} \). In this model the dihydrouridine stem and anticodon stem are no longer base paired, while additional base pairing is
established between portions of the anticodon loop and the TψC loop to form a new helix. Thus the importance of the dihydrouridine stem and anticodon stem in the biological function of the molecule is implicated.

Circular dichroism studies of nonmodified tRNA\textsuperscript{Leu} and its kethoxalated derivatives were carried out to investigate further the changes which occur during the renaturation process and which result in the loss of acceptor activity after kethoxalation. Comparison of the denatured tRNA\textsuperscript{Leu} \textsubscript{3} spectra in the presence and absence of magnesium provided evidence of a two-step renaturation process. The first step occurring on addition of magnesium to the denatured conformer consisted of a rapid transition from the "open" denatured structure to a more compact conformation, perhaps lacking the new helix of the Kearns model. The second, slower step involved a rearrangement to the native structure and was required for biological activity. Magnesium was tightly bound during the renaturation process, and the presence of a chelating agent was required for its complete removal prior to conversion to the denatured conformation. Kethoxalation of guanosine residues in the dihydrouridine stem and anticodon stem regions, although resulting in 70% loss of acceptor activity, did not appear to prevent re-formation of a native-like conformation. The ability of the tRNA structure to tolerate the bulky kethoxal modifications provided further evidence for flexibility in the dihydrouridine stem and anticodon stem
regions of the molecule.

Comparison of the tRNA\textsuperscript{Leu\textsubscript{3}} sequence with that of non-denaturable tRNA\textsuperscript{Leu\textsubscript{CUA}} revealed differences in the anticodon and TΨC loops which confirmed the importance of these regions in forming the stable denatured conformation. Circular dichroism spectra also indicated possible structural differences between the native conformers of the two tRNA species. Although the existence of denatured tRNA structures has not yet been demonstrated \textit{in vivo}, their existence \textit{in vitro} provides further evidence in support of a conformational mobility among tRNA molecules, which could conceivably play a significant role in their function and control.
1. INTRODUCTION

1.1 Historical Background

"Granted that the RNA of the microsomal particles, regularly arranged, is the template, how does it direct the amino acids into the correct order? ....RNA presents mainly a sequence of sites where hydrogen bonding could occur. One would expect, therefore, that whatever went on to the template in a specific way did so by forming hydrogen bonds. It is therefore a natural hypothesis that the amino acid is carried to the template by an 'adaptor' molecule, and that the adaptor is the part which actually fits on to the RNA."(1)

With these far-sighted and far-reaching words, Francis Crick first postulated the existence and role of the class of biological macromolecules now known as transfer ribonucleic acid (tRNA). Presented in 1958 along with the Sequence Hypothesis (the concept of nucleic acid sequence coding for protein sequence) and the famed Central Dogma (the principle of information transfer from DNA → RNA → protein), the Adaptor Hypothesis soon became the object of intense investigation. In the context of a scientific community rapidly uncovering the basic secrets of protein synthesis, Crick's proposals were set forth on the basis of several noteworthy findings. A requirement had been demonstrated for the ATP-dependent enzymatic activation of
amino acids as a first step in protein synthesis, followed by the transfer of these precursors to microsomal particles for polymerization (2). Preliminary reports by Hultin and Beskow (3) further indicated that during the first step of this process $^{14}$C-labeled amino acids became bound in some fashion which prevented their dilution by the later addition of unlabeled precursors. At about the same time the discovery of the stringent-relaxed response in bacteria provided indications of a possible link between the control mechanisms for RNA synthesis and protein synthesis (4,5). In the course of investigating this possibility, Zamecnik and coworkers inquired whether a cell-free system capable of incorporating amino acids into protein could also incorporate nucleotide precursors into RNA. They found, in fact, that $^{14}$C-ATP served quite well as an RNA precursor in their crude protein-synthesizing system from rat liver cells. As a control, $^{14}$C-leucine was added instead of $^{14}$C-ATP, and much to their surprise, radioactivity was again found to be associated with the RNA component. Fractionation of the cellular RNA led to the discovery in 1957 of a "soluble" RNA species in the pH 5 precipitable portion of the 105,000 X g supernatant which became covalently linked to the radioactive amino acid (6). Addition of this labeled aminoacyl-RNA to the microsomal fraction resulted in the transfer of the label into protein.
Crick quickly saw in Zamecnik's work a possible candidate for his nucleic acid "adaptor" molecule. It seemed unlikely, however, from a physical-chemical point of view that the RNA would assume a configuration capable of forming twenty different cavities, one for recognizing each amino acid. Crick therefore proposed that this specificity would reside in a set of enzyme molecules responsible for joining each adaptor to its own amino acid. Thus, in its simplest form his hypothesis required the existence of at least twenty RNA adaptors and twenty corresponding enzymes.

Crick's proposals were soon given further support by work in two laboratories. Zamecnik and coworkers demonstrated that addition of previously isolated soluble RNA produced a marked stimulation of protein synthesis. Fractionation studies of the enzymes involved were begun and four steps were proposed for amino acid incorporation into protein: (i) activation, (ii) bonding with soluble RNA, (iii) peptide bonding, and (iv) cross linking and patternization (7). Simultaneously Berg and Ofengand reported the results of studies regarding the enzymatic system employed for linking amino acids to the RNA (8). Their approach to the problem was based on a long-time interest in the general problem of acyl group activation, beginning with the enzymatic mechanism of acetyl-CoA formation. The widespread occurrence of such activating
reactions in biological systems led them to study the analogous reaction occurring with amino acids (9). From their earlier work with acetate activation, they postulated that the set of enzymes catalyzing the formation of adenylation amino acids might also be responsible for the transfer of the amino acids to RNA acceptors. They were able to characterize the transfer reaction and to show that it did appear to involve the amino acid activating enzyme as well as ATP, Mg\(^{++}\), the amino acid, and a specific RNA fraction. A fixed number of sites was found to be available for each amino acid per unit of RNA, and these sites appeared to function independently. From their data they proposed an overall reaction analogous to previously studied systems, which involved an activation step (ATP + amino acid \( \rightarrow \) adenylation amino acid + PP\(_i\)) and a transfer step (adenylation amino acid + RNA \( \rightarrow \) RNA-amino acid + AMP).

Studies of the soluble RNA fraction continued with attempts to obtain some degree of purity with respect to amino acid acceptor activity. A variety of fractionation procedures were developed based on differences in the electrophoretic mobility, the strength of binding to ion-exchange materials, the critical salt concentration required for precipitation by inorganic and organic cations, the extent of reaction with individual amino acids, and the partition coefficient in two-phase solvent systems (10). This final approach was employed in the countercurrent distribution system of
Holley and coworkers with which they achieved the first complete separation of two soluble RNA species, the alanine- and tyrosine-specific acceptor species from yeast (11). Although early studies with mixtures of soluble RNA molecules had indicated A/U and G/C ratios near unity, purification of individual acceptor species revealed significant deviations from this value (10). These results provided a strong indication that not all nucleotide residues in these small nucleic acid molecules were involved in base-pairing interactions. On the other hand, the presence of a relatively large number of modified nucleotides which had been observed in bulk preparations was confirmed in studies of purified species (10), as was the average chain length of 75-85 nucleotides, the molecular weight of around 25,000, and the sedimentation constant value of approximately 4S (12).

The isolation of individual soluble RNA species was followed by Matthaei and Nirenberg's development of a cell-free amino acid incorporation system which could utilize synthetic polynucleotides as messengers (13). Together these advancements made possible the conclusive demonstration of the adaptor role of the small RNA molecules. In a cell-free system, poly (U,G) had been shown to stimulate the incorporation of cysteine from its respective aminoacyl-RNA complex into a polypeptide chain, while the incorporation of alanine remained unaffected. By treatment
with Raney nickel, however, Chapeville and coworkers (14) were able to convert preformed cysteine-sRNA\textsuperscript{Cys} to alanine-sRNA\textsuperscript{Cys}, a procedure which was shown to leave the RNA component unaffected. After this modification, alanine incorporation was stimulated by alanine-sRNA\textsuperscript{Cys} in response to poly (U,G) in the same manner in which cysteine had originally been incorporated. Thus, once the aminoacyl-RNA complex was formed, its specificity in the subsequent reactions of protein synthesis was shown to reside solely in the structure of the RNA species.

Confirmation of these results was reported later the same year by Weisblum et al. in a study of the physical basis for degeneracy in the genetic code (15). The previous work of Jones and Nirenberg had demonstrated that most amino acids were represented by more than one coding sequence (16). In the case of two leucine acceptors from \textit{E. coli}, this degeneracy was found to be based on the different coding properties of each acceptor. Thus the coding specificity was again demonstrated to be carried by the adaptor RNA molecule.

Based on its method of preparation, the low molecular weight RNA component of the soluble fraction of cytoplasmic extracts was originally referred to as soluble RNA (sRNA). Allen and his collaborators, however, found that only a part of the sRNA they isolated from guinea pig liver was active in binding amino acids. As a result they
proposed that the active component be called "amino acid-transfer RNA" (tRNA) due to its apparent role as a transfer agent in protein synthesis (17). Nonetheless, this nomenclature was not universally adopted until used by Holley and his associates in their epic publication of the first sequence of a tRNA molecule (18).

This significant accomplishment opened the door to a wide field of experimental approaches to the study of tRNA structure-function relationships. Before delving into the current status of such studies, it would perhaps be advisable to briefly review what is known about the role of tRNA in the cell.

1.2 Functions of tRNA

The function of the tRNA molecule in cellular processes is indeed multifaceted. Its important role in protein synthesis which led to its discovery has perhaps been the most widely studied, but much about the process remains to be understood (19). Thus far it has been established that a particular tRNA molecule coding for methionine and recognizing the codon A-U-G serves as the initiator in the translation process in both prokaryotic systems (including those of eukaryotic organelles such as mitochondria and chloroplasts) and the cytoplasmic systems of eukaryotic cells. Exhibiting several differences in structure from the majority of tRNA species (see Section
1.4), the initiator tRNA (designated tRNA^Met_f or tRNA^fmet in prokaryotic systems and tRNA^Met_m or tRNA^met_i in eukaryotic systems) does not insert methionine into internal positions of the protein chain. This latter function is fulfilled by a second methionine-specific tRNA (tRNA^Met_m for prokaryotic systems and tRNA^Met_M for eukaryotic systems). Both species are recognized and aminoacylated by the methionine-specific aminoacyl-tRNA synthetase. In addition, however, the initiator tRNA in prokaryotic cells is recognized by a specific formylase which adds a formyl group to the methionyl moiety of the charged molecule. Thus all prokaryotic proteins initially possess an amino-terminal N-formyl-methionine residue, although in some cases this group is removed in the active protein. In eukaryotic systems no formylase activity is observed, although the initiating methionyl-tRNA^Met_F is in most cases recognized and formylated in vitro by the prokaryotic enzyme (20).

Recent studies have indicated that the translation process in both prokaryotic and eukaryotic systems begins with the binding of charged initiator tRNA to the small ribosomal subunit (30S and 40S, respectively) (21-25). In prokaryotic systems this process appears to be mediated by protein initiation factor IF-2 in the presence of GTP. Although the nomenclature of eukaryotic initiation factors has not yet been formalized, a similar activity has been
attributed to a protein characterized by various laboratories as M\textsubscript{1}, EIF1, or IF-I (20). The bound initiator tRNA, in conjunction with initiation factor IF-3 (M\textsubscript{3}, EIF3, IF-II), then serves to direct the subsequent binding of a natural messenger RNA (mRNA) to the initiation complex at an initiating (rather than an internal) A-U-G sequence. As the large subunit (50S or 60S) binds to the complex, the initiator tRNA is positioned at the peptidyl (P) site, possibly due to the blocking of the A site by IF-2 (M\textsubscript{1}). Unique features of the initiator tRNA, such as the blocking of its methionyl amino group which mimics peptidyl-tRNA (prokaryotic systems) or the alteration in sequence of its proposed ribosomal binding site (eukaryotic systems), have also been suggested to play a role in the specific binding and positioning of the initiator molecule. It is interesting to note in this regard that eukaryotic systems appear to require an additional initiation factor, M\textsubscript{2}, for binding initiator tRNA to the 40S ribosomal subunit. This requirement, as well as the GTP requirement, however, may be removed in cell-free systems by in vitro formylation of the methionyl-tRNA\textsubscript{F}\textsuperscript{Met} (26). Other significant differences in prokaryotic and eukaryotic initiation factor requirements have also been noted (20), and the full extent of the correlation between factors exhibiting similar functions in the two systems remains to be determined.

Following binding of the large ribosomal subunit, GTP hydrolysis and the subsequent removal of IF-2 (M\textsubscript{1}) allow
codon-directed binding of a charged tRNA at the aminoacyl (A) site. This process has been most thoroughly investigated in prokaryotic systems (27) where it is mediated by a protein elongation factor T (EF-T) composed of two subunits (Tu and Ts). Prior to forming a complex with aminoacyl-tRNA, the Tu subunit is activated by the binding of GTP and the release of the Ts subunit. The aminoacyl-tRNA-Tu-GTP complex thus formed then binds to the ribosomal A site in response to the proper codon, provided a peptidyl-tRNA (or a charged initiator tRNA) is present at the P site. The Tu carrier is subsequently removed through ribosome-catalyzed GTP hydrolysis, and the Tu-Ts complex is regenerated with the concomitant release of GDP.

At this point the peptidyl transferase, an integral part of the ribosomal 50S subunit, transfers the peptidyl group from the tRNA bound at the P site to the incoming aminoacyl-tRNA at the A site. Subsequent binding of elongation factor G (EF-G) in the presence of GTP allows the release of the uncharged tRNA from the ribosome as well as the translocation of the new peptidyl-tRNA from the A site to the P site. EF-G is released after GTP hydrolysis, and the elongation cycle continues. Protein factors functionally similar to EF-T and EF-G have been isolated in eukaryotic systems (EF-1 and EF-2 respectively), although less is known about their modes of action (27).
Termination of the translation process is brought about by the appearance in the messenger of one (or more) of the "termination" or "nonsense" codons U-A-G (amber), U-A-A (ochre), or U-G-A. These codons which are not normally recognized by a specific tRNA, direct the interaction of a release factor (RF) with ribosomal peptidyl transferase, resulting in peptidyl-tRNA hydrolysis. As of yet, the requirements for the release of deacylated tRNA from the ribosomal complex have not been clarified (28).

The importance of tRNA in assuring the fidelity of the translation process is apparent considering the amount of precision required. The tRNA must be charged with the proper amino acid and must recognize the proper codon in order to correctly insert that amino acid into a growing polypeptide chain. Alterations in this expected behavior, however, have been noted to occur and at times, in fact, play a significant role in providing for continued cell viability. For example, early in the 1960s the analysis of a number of nonsense suppressor mutations led to the conclusion that the insertion of a compatible amino acid in response to a nonsense codon was often the result of a mutation in a specific tRNA species (for a review, see reference 29). Direct chemical evidence of a change in the nucleotide sequence of a suppressor tRNA was obtained in 1968 by Goodman et al. (30). Their sequence analysis
of tRNA\textsuperscript{Tyr}\textsubscript{su3+} from \textit{E. coli} demonstrated that the mutation involved a simple G-C base substitution at the anticodon site which enabled the tRNA to respond to the U-A-G amber codon rather than the normal tyrosine codons U-A-U and U-A-C. It would follow from the wobble hypothesis (31) that tRNA suppressors of the ochre codon U-A-A would also be able to suppress the amber codon U-A-G, a fact which had been observed earlier in suppressor studies (29).

The problem arises, however, as to how a cell tolerates an efficient bypass of the normal chain termination process. Since ochre suppressors in general appear to work with relatively low efficiency, the U-A-A codon was initially favored as the natural termination signal (32). More recent evidence has indicated the possible use by the cell of two or more nonsense codons in tandem to ensure proper release of the chain from the ribosome (33). In addition, since nonsense suppressor mutations result in the loss of ability to recognize the sense codons, it would be expected that only a dispensable tRNA could be converted to the suppressor function. This prediction has been substantiated by the isolation of recessive-lethal suppressor mutants by Soll and Berg. Such mutants cannot exist in the haploid state, but require the presence of the normal tRNA gene as well (34).

It is necessary to note at this point that the phenomenon of suppression may not always be attributed to
a simple anticodon base change permitting the tRNA involved to insert its specific amino acid in response to a nonsense codon. To begin with, sequence studies have revealed that alterations are not limited to the anticodon region of the tRNA molecule. In fact, the nucleotide sequence of tRNA$^{Trp}_{su^7+UGA}$ has been found to differ from its normal counterpart only in residue 24 located in the dihydrouridine stem (35). Furthermore, alterations in the anticodon which permit nonsense codon recognition may also alter the amino acid specificity of the tRNA. For example, an anticodon change from C-C-A to C-U-A permits tRNA$^{Trp}_{su^7+}$ to be slowly acylated with glutamine but abolishes its tryptophan acceptor activity (36). In addition, double mutants of suppressor tRNAs have been isolated which possess the ability to insert a different amino acid and thus to suppress mutations which their parental suppressor tRNA cannot suppress. These alterations in specificity have been traced to single base changes in the nucleotide sequence of the amino acid acceptor stem (37).

In addition to their ability to convert nonsense to sense in the genetic code, tRNA alterations have also been shown to be agents of missense suppression. A particularly well-studied example involves the missense substitution of various amino acids for an essential glycine residue in the A protein of E. coli tryptophan synthetase, resulting in the formation of an inactive
enzyme (38). Suppressors of these mutations have been found which involve changes in each of the three different structural genes for tRNA\(^{\text{Gly}}\). In all cases studied thus far where an abnormal codon is recognized, however, the rate of glycyl acylation is greatly reduced (36). Although in some instances an adenosine residue adjacent to the anticodon becomes modified in these suppressor tRNAs, recent evidence appears to indicate that the reduced rate of aminoacylation is due to a base change in the anticodon itself rather than to the nearby modification (39).

Frameshift mutations have been shown to respond to a different type of alteration in the anticodon region of tRNA. Analysis of the sequence of tRNA\(^{\text{Gly}}\)\(_{\text{suf D}}\), which suppresses a frameshift mutation in the histidine operon of *Salmonella typhimurium*, has revealed the presence of an eight-membered anticodon loop, and therefore a quadruplet C-C-C-C anticodon instead of the wild-type triplet C-C-C (40). The normal seven nucleotides in the tRNA anticodon loop thus appear to play a role in maintaining the proper reading frame during translation.

In addition to playing a role in determining the nature of proteins synthesized in the translation process, tRNA molecules have been shown to possess regulatory properties with regard to the transcription process as well. Extensive studies on the regulation of histidine biosynthesis in *S. typhimurium* by Ames and coworkers have
resulted in the characterization of the gene responsible for histidyl-tRNA synthetase (his S), the structural gene for tRNA^{His} (his R), and possible genes involved in tRNA modification (his U and his W) (41). Mutations in these genes which result in derepression of the histidine biosynthetic enzymes have shown that the normal repression of the histidine operon is directly related to the concentration of histidyl-tRNA^{His} within the cell. An additional mutant, his T, also results in derepression, apparently as a result of the lack of two enzymatic U→Ψ modifications in the anticodon loop of tRNA^{His}. This modification appears to be required only for the corepressor activity of the tRNA, and the enzyme involved appears specific for the anticodon region of tRNA molecules in general (42). Other tRNAs altered by the his T mutation also appear to be involved in cellular control mechanisms (43), and it has been suggested that the modified residues in the anticodon region serve to maintain the structure required for interaction with regulatory elements. In fact, Ames and his colleagues have noted that the increase in the frequency of modified bases in tRNA from mycoplasm to mammals might reflect an increasing role for tRNA as a regulatory molecule (43).

In addition to its long-range repression effects, tRNA^{His} has been shown to bind the phosphoribosyltransferase enzyme encoded by the first gene of the histidine
operon. Thus the tRNA might also play a role in a positive control mechanism independent of the operator-repressor system (44).

A further regulatory role has been attributed to tRNA in the bacterial stringent response to essential amino acid deprivation. Curtailment of the synthesis of stable RNA and protein (ribosomal protein in particular) under such conditions (45) is observed to occur along with other alterations in cellular activity in "stringent" (rel+) but not in mutant "relaxed" (rel-) strains of *E. coli* (46). This shut-down response has been found to be triggered by the depletion of one or more of the aminoacyl-tRNA pools rather than the lack of the amino acids themselves (47). Accompanying this depletion is the accumulation of the compounds guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (48) which are synthesized by a ribosomal stringent factor (rel gene product) through pyrophosphate transfer from ATP to GDP or GTP (49). This reaction, occurring only in the absence of active protein biosynthesis (50), has been shown to require the codon-directed binding of an uncharged tRNA to the A site of the mRNA-ribosome complex (51, 52). Such binding appears to be mediated by EF-Tu in a reaction which is not competitive with aminoacyl-tRNA binding (51). It is the first known process to demonstrate a biological function for the interaction of uncharged tRNA with the protein-synthetic machinery. Further studies of tRNA-ribosome interaction
have recently shown that the tetranucleotide T-ψ-C-G alone, which appears to be common to all tRNAs binding at the ribosomal A site (see Section 1.4), is able to perform the role of the uncharged tRNA molecule in promoting this stringent response (53). The nature of the proposed structural changes in the ribosome accompanying or leading to stringent factor activation, however, remains to be elucidated.

Additional indications of an important regulatory role for tRNA have come from the numerous differences observed to occur in overall tRNA populations as well as among individual isoaccepting tRNA species accompanying alterations in cell physiology. Such differences have been documented in comparisons of the tRNA populations in embryonic tissues versus adult tissues, in vegetative bacterial cells versus sporulating cultures, in cells under hormonal influence versus cells lacking such stimulation, and in normal cells versus malignant ones (36). Specialization of tRNAs with regard to the synthesis of specific proteins has been observed in cells dedicated primarily to the synthesis of a single protein, such as reticulocytes and mammary gland cells, and in phage-infected cells committed to the production of phage proteins. The altered aminoacyl-tRNA chromatographic profiles accompanying such changes have implied that possible differences in synthetase specificity and modification enzyme activity as well as alterations in tRNA nucleo-
tide sequence may be of significance in cellular regulation processes.

An additional possibility for regulatory potential resides in tRNA conformation. The discovery in 1966 of a tRNA species which undergoes reversible changes between a stable native and a stable denatured form lends added emphasis to this alternative (54). A further discussion of this latter aspect of tRNA regulation will be presented in Section 1.5.

Although the majority of its roles appear to be related to ribosomal protein synthesis, tRNA functions in other areas of cellular metabolism as well. For example, an amino acid donor is required in the biosynthesis of glycylic lipopolysaccharide (55) and aminoacyl phosphatidylglycerol (56), in the regulation of enzyme activity through post-translational addition of terminal amino acid residues (57, 58), and in the cell wall biosynthesis of most Gram-positive bacteria (36). Of these roles, the formation of cell wall glycopeptides has perhaps been most thoroughly investigated. In this process aminoacyl-tRNA has been demonstrated to donate amino acid residues which form the cross-linking bridge between two pentapeptide side chains attached to the polysaccharide backbone. The length and amino acid composition of this bridge varies from one species to another (59). Although it appears as though any of the isoaccepting tRNAs for the required amino acid may
function in peptidoglycan synthesis, specific tRNAs have been isolated which function in this capacity alone and are inactive in ribosomal protein synthesis (60). Sequence analysis of such tRNA species has revealed a lack of the G-T-ψ-C sequence which is common to most tRNAs and which has therefore been proposed to play a role in ribosomal recognition (61). Striking similarities, however, have been found among the sequences of cell wall-specific tRNAs from one species to another (36).

Finally, recent evidence has implicated tRNA as a possible primer for reverse transcriptase activity in transformed cells (62). In a study of Rous sarcoma virus replication, James Dahlberg and his collaborators have characterized a polynucleotide possessing template primer activity which appears to be a tryptophan-specific tRNA molecule. Whether or not the \textit{in vitro} work carried out thus far, however, can be substantiated by \textit{in vivo} studies remains to be seen. Nonetheless, the role of tRNA remains complex, and its study promises to reveal much about cellular mechanisms and their control.

1.3 Rationale for Studying tRNA Structure

In light of its many roles within the cell, the importance of the tRNA molecule becomes more clearly understood. Not only is its correct sequence and conformation required to ensure the fidelity of ribosomal protein
synthesis, but numerous regulatory features and non-ribosomal reaction sequences also depend on tRNA for their proper function. In fact, not only the significance of the role played by tRNA, but its complexity as well draws interest to the molecule as a subject for investigation. It is not at all clear how this set of relatively small nucleotide species which exhibit many common structural features (see Section 1.4) can at the same time possess such widely varying and yet extremely specific protein recognition sites. Much effort has been and is being devoted to finding an answer to this question.

In addition to these justifications for studying tRNA structure are a number of practical aspects which make the molecule a relatively convenient and advantageous, as well as significant, subject for laboratory investigation. To begin with, the average chain length of approximately 80 nucleotide residues makes tRNA a small molecule in relation to other polynucleotide and protein molecules. Furthermore, it is stable to many laboratory procedures and does not require the precise temperature controls, buffering systems, etc. which are often necessary in protein research. Partially as a result of this small size and stability, tRNA molecules are relatively easily purified and sequenced. In addition, the availability of nucleases which preferentially cleave the molecule at specific residues further serves to facilitate the
sequencing process. Numerous modified nucleotides act as markers for the final process of aligning digestion products to obtain the overall sequence. Molecules of known primary structure can then be modified, crystallized, etc. in attempts to elucidate secondary and tertiary configurations.

Studies of tRNA molecules are applicable to a wide variety of other investigations as well. For example, tRNA has been used in a number of laboratories as a probe in the study of transcription. Not only has it played a role in the analysis of genetic control mechanisms such as suppression and repression (36, 63), but it has also been useful in procedures for isolating (64-66), sequencing (67, 68), and even synthesizing (69) a gene. Needless to say, the involvement of tRNA in protein synthesis has also led to its extensive and well-documented use in the elucidation of the translation process (19). Recently, its application to this study has been extended to such uses as the affinity labeling of aminoacyl-tRNA synthetases (70) and ribosomal proteins (71-73).

Due to these characteristics of the tRNA molecule both in the laboratory and in the cell, it is able to serve as an excellent tool in the study of protein-nucleic acid interaction. Being a discrete, crystallizable entity, it may be investigated with much greater facility in this regard than other nucleic acid species within the cell. Furthermore many of the proteins interacting with tRNA are
soluble and do not require the presence of a macromolecular complex for biological activity. Thus solution studies are possible with purified protein and nucleic acid species, and different types of interactions may be compared under similar controlled conditions. In addition, the ability of the tRNA molecule to participate in one or more of these interactions may be assessed with regard to known alterations in the tRNA structure. As a result, tRNA has been found to be particularly useful as a probe into structure versus function relationships. Obtaining an understanding of such interrelationships comprises one of the major thrusts of the field of biochemistry as a whole as it attempts to elucidate the intricacies of the living cell.

1.4 Structure versus Function of tRNA

The means by which tRNA structure enables the molecule to perform its many functions has been of interest since the discovery of tRNA in 1957. Direct information regarding structure-function relationships, however, did not appear until 1965 when Holley and coworkers published the first nucleotide sequence of a tRNA molecule, alanine-specific tRNA from yeast (18). As a result of physico-chemical studies indicating the presence of a considerable amount of base pairing in tRNA, Holley arranged the sequence so as to permit maximum hydrogen bonding. All tRNA molecules
sequenced thus far may be represented in this fashion which Holley termed the "cloverleaf" structure. To date no evidence has been found contrary to the existence of this structure in the native conformation of tRNA molecules.

Soon after Holley's work was published, other tRNA sequences became known. Today over sixty such sequences have been determined, and the number is growing rapidly. As data accumulates, a number of similarities in structure among the tRNA molecules have become apparent. In addition to the cloverleaf secondary structure and the anticodon and aminoacyl acceptor regions, a loop containing the modified nucleotide dihydrouridine (D) is found to be common to all tRNAs, as well as a region containing the sequence G-T-Ψ-C-G(A). On the whole, tRNA molecules possess a relatively large proportion of modified nucleotides, many of which are found to be characteristic of specific regions of the molecule. Furthermore, the region between the anticodon stem and the TΨC stem has been found to vary in length from 4 to 21 bases and thus to account for most of the differences observed in tRNA chain lengths. A summary of these characteristic features of the cloverleaf structure is shown in Figure 1. Positions in the nucleotide chain are numbered according to a standard system recently proposed by Ofengand for the designation of nucleotides in all tRNA molecules (74).
Figure 1. Proposed standard numbering system for tRNA. Cloverleaf stems are designated by small letters, and loops by Roman numerals. Nucleotide residues are numbered from the 5' end, with the bases in a variable region designated a, b, ... consecutively. Residues conserved among non-initiator tRNA molecules participating in ribosomal protein synthesis are designated by standard abbreviations. Pu indicates a purine nucleotide, Py a pyrimidine nucleotide, and * a modified nucleotide. This numbering system, proposed recently by Ofengand (74), will be used throughout the remainder of the text for all tRNA molecules discussed.
It is apparent from a number of studies that a certain amount of secondary and possibly tertiary structure is characteristic of the tRNA molecule soon after its transcription. The roles of such structural features have been objects of extensive study, and although the final answers remain elusive, a number of generalizations appear justified. Since the secondary structure proposed by Holley has been found to be common to all native tRNAs studied thus far, these interrelationships of tRNA structure and function will be discussed from the viewpoint of the cloverleaf.

The 3' end of the tRNA molecule terminates with a single-stranded region four nucleotides in length. The final three of these, C-C-A\textsuperscript{OH}, appear to be common to all tRNAs, and the ribose moiety of the terminal adenosine serves as the point of aminoacyl attachment. Recent studies using tRNAs with modified, nonisomerizable terminal adenosine residues have demonstrated that the aminoacyl-tRNA synthetase forms an ester bond specifically between the 2'-OH group of the ribose and the carboxyl group of the amino acid (75, 76). The slight decrease in activity observed in the presence of a 3'-H has been attributed to a concomitant loss in the nucleophilicity of the 2'-OH group. Replacement of the 3'-OH by a 3'-O-methyl group, however, has been shown to virtually eliminate synthetase activity, perhaps due to steric hindrance (77). Periodate
oxidation of the terminal ribose produces a tRNA species (tRNA$_{ox}$) which in some cases binds the cognate synthetase and stimulates ATP-PP$_i$ exchange (78), but has never been observed to accept an amino acid (79). Subsequent reduction by borohydride (tRNA$_{ox-red}$) generally restores acceptor activity to some degree. The extent and velocity of this "open-chain" charging reaction have been found to depend on the tRNA, the synthetase, and the stability of the aminoacyl bond formed (79).

While both 2' and 3' aminoacyl-tRNA isomers have been observed to bind to the ribosomal A site with equal affinity (80), conflicting evidence has been reported regarding the species required for acceptor activity in the peptidyl transferase reaction. Early studies using puromycin and its inactive 2' analog (81), as well as tRNA with a 3'-amino-3'-deoxyadenosine residue incorporated at the 3' terminus ("puromycin-tRNA") (82) implied that the 3'-O-aminoacyl tRNA isomer was the active species. Recent use of chemically synthesized non-isomerizable 2' and 3'-O-aminoacyl mono- and dinucleoside phosphates has also indicated that the peptidyl transferase exclusively utilizes the 3'-O-aminoacyl isomer as an acceptor substrate (83). The study of 3'-O-aminoacyl-tRNA, synthesized by the addition of a derivatized adenosine to abbreviated tRNA (tRNA-C-C$_{OH}$) using polynucleotide phosphorylase, has added further evidence in support of the specificity for
the 3' isomer (84). In contrast, the results of Cramer and his associates have indicated that both the 2'-O-aminoacyl tRNA isomer and to a lesser extent aminoacyl-tRNA \textsubscript{ox-red} possess the ability to accept a peptidyl group (85). To explain these results they have proposed a model in which a slight distortion in the ribose ring or the aminoacyl group places the amino groups of both isomers in an identical spatial position.

Additional information regarding the position of the aminoacyl group during translation was obtained in a study of 2' (3') deoxy-3'(2') -- N-acetyl-aminoacyl-tRNA binding to the ribosomal P site (84). Both isomers were bound with equal but quite low affinity. Furthermore, neither was active as a donor in the peptidyl transferase reaction. These results, along with the reduced acceptor activity of both 2' and 3' deoxy analogs, have indicated an important role for the vicinal hydroxyl group in functional aminoacyl- and peptidyl-tRNAs. Clarification of this role, however, awaits further study.

The terminal C-C-A\textsubscript{OH} residues are readily removed and replaced both \textit{in vivo} and \textit{in vitro} by the soluble enzyme nucleotidyl transferase. As indicated by the sequence of promotor molecules, this removable portion of the molecule is encoded by the \textit{E. coli} chromosome but not by T4 DNA (86). Since this region is required for aminoacylation, its removal provides a possible control mechanism for
determining the amount of active tRNA within the cell. The enzyme does not recognize tRNA molecules which lack more than 3 terminal residues and does not add more than this number of nucleotides. As a result of its ability to incorporate modified nucleotides, nucleotidyl transferase has been used to produce modified tRNA molecules such as the deoxyadenosine derivatives used in the above study. It has also been used to demonstrate that bulky modifications of this portion of the molecule do not affect the tRNA crystal cell content. Thus, as expected, the C-C-A\(^{\text{OH}}\) region extends into to the solvent and is not involved in extensive secondary and tertiary interactions affecting overall tRNA structure (87). The specific orientation of this portion of the molecule has been demonstrated to be important, however, in recognition of aminoacyl-tRNA by elongation factor T. Disruption of the stacking interactions required may be brought about by cleavage of the terminal ribose ring (88-90) or by bisulfate conversion of C-C-A\(^{\text{OH}}\) to U-C-A\(^{\text{OH}}\) (91). Uridine has a reduced tendency to form a single-stranded, base-stacked structure and thus reduces the binding affinity of the aminoacyl-tRNA. A tRNA species possessing a tetranucleotide C-C-C-A\(^{\text{OH}}\) terminus also binds poorly to EF-Tu and GTP (92). N-acyl aminoacyl-tRNAs, on the other hand, do not bind the elongation factor at all (93-95).
The fourth unpaired nucleotide from the 3' end has also been implicated as playing an important role in the function of the molecule. Roe et al. found this nucleotide to be constant in all tRNAs charged heterologously by yeast phenylalanine tRNA synthetase (96). In a further comparison of tRNA sequences, Söll and coworkers subsequently observed a striking consistency in the fourth nucleotide of tRNAs specific for any given amino acid as well as for chemically related amino acids (97). Furthermore, a single change in the fourth nucleotide from A to G in tRNA^{Tyr} of E. coli was found to change its specificity from tyrosine (A class) to glutamate (G class) (98). It thus appeared to be possible to group tRNAs on the basis of their fourth nucleotide and their amino acid specificity. From this data Söll's group postulated the existence of a universal "discriminator site" at the fourth position from the 3' end of the tRNA molecule which would serve to divide all tRNAs into classes for recognition purposes. These groups could then be further subdivided with regard to secondary discriminator sites or recognition mechanisms which might differ from class to class. This proposed hierarchy of recognition steps would account for the conflicting data from various studies regarding the regions of the tRNA molecule involved in the recognition process, and yet would maintain some degree of universality in the recognition code. The basis for such discriminator site
function has not yet been clarified. Chemically similar amino acids could respond to the same discriminator nucleotide because they evolved from a set of related amino acids which were indistinguishable in a primitive system. On the other hand, the fourth nucleotide from the 3' end might interact with the amino acid directly during the enzyme-mediated charging process, and similar amino acids could thus naturally select the same nucleotide. Further experimentation is needed to verify the significance and the origin of this observed correlation.

In most tRNA molecules the base-pairing of the acceptor stem begins between 5' terminal nucleotide and the fifth residue from the 3' end. The E. coli initiator tRNA
$_{Met}^f$, however, is unique in having a non-hydrogen-bonding base at the 5' terminus. Recent studies have indicated that this feature is important in the inability of this molecule to form a complex with bacterial EF-Tu and GTP (91). Bisulfite-catalyzed conversion of the unpaired cytidine to uridine allows base-pairing and subsequent formation of an EF-Tu-GTP-Met-tRNA
$_{Met}^f$ complex. This complex has properties similar to those formed with non-initiator tRNAs although it is somewhat less stable. The requirement for base-pairing at the 5' terminus is apparently due to the need for a specific spatial orientation of the 5'-terminal phosphate which has been shown to have a strong positive role in stabilizing such complexes (91). Stabilization
could occur through the direct binding of the phosphate to the protein or through proper orientation of another part of the aminoacyl-tRNA structure (e.g. the aminoacyl moiety) to allow binding. This interaction is not the only requirement for effective EF-Tu binding, however, as evidenced by the relatively weak binding of modified base-paired Met-tRNA\textsubscript{Met}\textsuperscript{f}. In addition, both yeast initiator tRNA (96) and tRNA\textsubscript{Gly}\textsubscript{IA} from \textit{S. epidermidis} (97), which is involved in cell wall biosynthesis but not ribosomal protein synthesis, possess a fully base-paired acceptor stem and yet exhibit weak interactions with their homologous T factors. The unpaired base in \textit{E. coli} tRNA\textsubscript{Met} may play a further, perhaps even more significant role in vivo. Unmodified fMet-tRNA\textsubscript{Met}\textsuperscript{f} is resistant to peptidyl-tRNA hydrolase activity (98, 99) whereas its bisulfite treated, fully base-paired analog is not (100). Thus the unpaired base may be critical to bacterial viability as a result of its capacity to prevent enzymatic removal of the formyl-methionine from the initiator tRNA (91).

The remainder of the acceptor stem (normally seven base pairs in length) has also been implicated as an important feature in tRNA structure-function relationships (101). Enzymatic dissection and fragment reconstitution studies have shown that this region of the molecule is required for recognition by the cognate synthetase and that the combined first and fourth quarters of the
molecule alone may exhibit a slight degree of acceptor activity (102). Photochemical modification has also implicated the acceptor stem as an inactivation target in synthetase recognition (103, 104). Based on these data, Schulman and Chambers proposed the Acceptor Stem Hypothesis in 1968, stating that the first three base pairs in the acceptor stem comprised the specific synthetase recognition site (103). Studies with isoacceptor tRNAs from various sources, however, soon discounted the universality of this theory. Differences in sequences in this region were found among tRNAs recognized by the same enzyme (105-107), and identical sequences have been demonstrated to occur in tRNAs of different specificities (108). The major conclusion which may be derived from these studies, therefore, is that the ordered structure of the acceptor stem is a strong requirement for synthetase recognition and more than likely for other interactions as well.

The residue following the base-paired acceptor stem (number 8 from the 5' end) has been found to be constant in all tRNAs sequenced thus far. In E. coli tRNAs it has been identified as 4-thiouridine while other sources exhibit an unmodified uridine. Furthermore, the following nucleotide is consistently a purine. These residues have recently been postulated to play a role in important tertiary hydrogen-bonding interactions which stabilize the three-dimensional structure of the tRNA molecule
(109). The proposed locations of such tertiary interactions in yeast tRNA\textsubscript{Phe}, as determined by X-ray crystallographic studies, are illustrated in Figure 2.

The dihydrouridine (D) stem consists of only three or four base pairs and is therefore the least stable helix in the tRNA structure. Yet it also appears to be of extreme importance in biological function. By analysis of the primary structures of eleven tRNAs aminoacylated by yeast phenylalanyl-tRNA synthetase, Dudock and coworkers demonstrated that not only the fourth nucleotide from the 3' end, but also the residues in the D stem region, including the non-base-paired purine at the 5' end of the stem, were common to all eleven (110, 111). Thus the stem was concluded to be a part of the recognition site required by this particular enzyme. Additional support for this hypothesis was found through the use of the method of dissected molecules and fragment recombination, since the loss of this region of the molecule prevented enzyme recognition (112, 113). That this relationship again does not appear to be universal, however, has been shown by differing sequences in this region among isoaccepting tRNAs (107) and identical sequences among tRNAs of differing specificity (101). The major role of the D stem residues may in fact lie in their involvement in tRNA tertiary interactions (109).
Figure 2. Secondary and tertiary hydrogen-bonding interactions in yeast tRNA\textsubscript{Phe}. Ofengand's standard numbering system (74) is used for the tRNA\textsubscript{Phe} nucleotide sequence (331). Constant nucleotides as well as constant purine or pyrimidine positions for all non-initiator tRNAs involved in ribosomal protein synthesis are shown. Variable regions in the dihydrouridine loop are designated α and β. Solid lines connecting nucleotides indicate tertiary hydrogen-bonding interactions consisting of one, two, or three hydrogen bonds as determined by the X-ray crystallographic studies of Kim et al. (109).
It is interesting to note that the number of base pairs in the D stem may be coupled with the number of nucleotides in the variable loop to serve in a classification scheme for tRNA molecules \((109, 114)\). For example the major class, composed of almost half of the approximately 60 tRNAs sequenced, has four base pairs in the D stem and five nucleotides in the variable loop, and thus is designated D4V5. The other three classes are D3V5, D(3 or 4)V4, and D3VN, where \(N\) varies from 13 to 21 nucleotides \((109)\).

The dihydrouridine loop, so named because of its high content of the modified base dihydrouridine, is characterized by both constant as well as variable regions \((74, 109)\). For comparison of these loop regions in tRNAs with only three base pairs in the D stem, the first two bases adjacent to the stem are often excluded from consideration. With this in mind, it has been found that the first base at the 5' end of the loop is consistently adenosine, which is normally followed by another purine residue. Located after these bases is the first of two variable regions \(\alpha\) which is separated by two constant guanosine residues from the second variable region \(\beta\) \((\text{Figure 2})\). Each of these regions may contain from one to three nucleotides, although the most common distribution appears to be two nucleotides in the \(\alpha\) region and one in the \(\beta\) region. There are a large number of tRNAs
with one nucleotide in each of the regions, but so far no examples have been found with three nucleotides in each location (109). The variable regions are comprised mostly of pyrimidines, with D accounting for over 70% of the bases. Recent work has suggested that the D loop might specify a synthetase recognition site with specificity residing in these variable areas (115, 116). Although this hypothesis could possibly play a role at some point in a hierarchical division of recognition features (97), it, too, does not appear to be a universal phenomenon since at least three _E. coli_ tRNAs have identical sequences in the variable regions. The β variable region is followed by a constant adenosine residue before the beginning of the D stem (considered as having four "base pairs"). A constant purine residue, often methylated or otherwise modified, follows the D stem and separates it from the anticodon stem.

The anticodon stem consists of five base pairs whose major role appears to be the proper alignment of the anticodon loop. This loop is generally comprised of seven nucleotides, two on either side of the triplet anticodon. Exceptions to this rule have been noted in tRNA suppressors of frameshift mutations which recognize a quadruplet codon (40). The two bases normally occurring on the 5' side of the anticodon are pyrimidines, with the one immediately adjacent to the anticodon being uridine. The nucleotide at the 5' position in the anticodon or the
wobble position (31), is often modified. While in some cases this modification is believed to aid in the formation of "wobble" base pairs, recent studies have also shown that other modifications are responsible for the inability of certain tRNA\textsubscript{s} to recognize wobble codons (108, 117). The nature of the base at the 3' end of the anticodon has been found to affect the extent of modification of the purine adjacent to it (3' side). For example, studies of missense suppressors in \textit{E. coli} have shown that a C to U base change at the 3' position of the anticodon in tRNA\textsubscript{Gly} or a C to A in tRNA\textsubscript{Gly} leads to modification of the adjacent adenosine even in the precursor molecule (39).

Numerous experiments have indicated that the anticodon sequence may also be involved in synthetase recognition. In \textit{vitro} and \textit{in vivo} modifications of bases in the anticodon have been shown in many cases to reduce greatly the rate of aminoacylation (118-120) or even to prevent it entirely (121, 122). Mutation of a single anticodon base (C-C-A to C-U-A) has even been observed to change the specificity of tRNA\textsubscript{Trp} such that it is enzymatically aminoacylated with glutamine (123). (It is interesting to note that both of these amino acids are found within the proposed G discriminator class (97).) Misaminoacylation of a chemically modified tRNA, however, has never been observed (124). Other studies have provided
evidence that the anticodon region interacts with the synthetase sufficiently to prevent oligonucleotide binding (125) and nuclease attack (126). On the other hand, reconstitution of tRNA fragments has shown that while the anticodon is necessary for the aminoacylation of some tRNAs, it is dispensable for others (102, 113). Further contradictions arise in the case of tRNAs with seemingly unrelated codons (e.g., serine tRNAs) which are nonetheless recognized by a single synthetase. Thus, although the anticodon sequence may play a more or less critical role in certain aminoacylation reactions, it, too, lacks the qualities required for a universal recognition site. Recent chemical modification (127) and oligonucleotide binding (128) studies have provided evidence that the presence or absence of certain structural features, appearing as a result of sequence changes, is the true determinant of synthetase recognition in this region.

The nucleotide adjacent to the anticodon on the 3' side is generally a purine which is often extensively modified. Available sequence data appears to indicate that the nature of this nucleotide is important in codon recognition (129). For example, all E. coli tRNAs sequenced thus far which recognize a codon starting with U possess either a 2-methylthio-6-(Δ^2-isopentenyl) adenosine (ms^2i^6A) in this position or its parent compound, 6-(Δ^2-isopentenyl) adenosine (i^6A) (130). The extent of
the modification of $i^6A$ to $ms^2i^6A$ appears to vary with the
growth conditions of the cell. The presence of these
compounds and similar derivatives presumably located at
the 3' side of the anticodon, appears to be a common
characteristic of tRNAs from a wide variety of organisms
(129).

An exception to this rule has been found in several
eukaryotic tRNAs specific for phenylalanine (codon
U-U-U). Determination of the structure of the Y base
adjacent to the anticodon in yeast tRNA$^{Phe}$ has shown it
to be quite different from $ms^2i^6A$, but also extremely
hydrophobic (131, 132). Similar bases have been identified
in tRNA$^{Phe}$ species from rat liver (133) and wheat germ
(134). The hydrophobic nature of these Y-type nucleo-
tides allows the tRNA$^{Phe}$ to be separated easily from the
majority of tRNA species using columns of benzoylated-
DEAE cellulose, and thus to be purified in large quanti-
ties. Since most physical studies of tRNA structure,
including X-ray crystallography, require large amounts of
a pure tRNA species, the Y base may be considered largely
responsible for yeast tRNA$^{Phe}$ being one of the most ex-
tensively characterized tRNA molecules (135).

Another exception is found in yeast tRNA$^{Leu}_3$ which
recognizes the U-U-G codon. This tRNA contains a 1-
methylguanosine adjacent to the anticodon (136). This
modified nucleotide is also found in _E. coli_ tRNA$^{Leu}$
species of the C-U series (107, 137) and yeast tRNA_{Leu}^{CUA} (138), as well as yeast tRNA_{Asp} (139) and tRNA_{Pro} (129).

The nucleotide N-[9-(β-D-ribofuranosyl) purin-6-ylcarbamoyl] threonine (t^6A) and its derivatives have been found to occur in the tRNAs of a wide range of organisms and appear to be specifically located adjacent to the anticodon of tRNAs which recognize codons beginning with A (129). An exception to this rule is \textit{E. coli} tRNA_{f}^{Met} which contains an unmodified A (140), although yeast initiator tRNA_{f}^{Met} possesses the expected t^6A derivative (141). Other nucleotide derivatives found in the position adjacent to the anticodon include 6-methyladenosine and 2-methyladenosine (129). The fact that the purine residue in this position is absent in the tRNA_{Gly}^{Gly} of \textit{Staphylococcus epidermidis} which functions only in cell wall biosynthesis (61) lends credence to the proposal that this position is important in codon recognition. Although ms^{2,16A} has been shown to be essential for the amino acid transfer function of tRNAs containing it (142), and a hydrophobic group is necessary in the Y-base position for the proper function of yeast tRNA_{Phe} (143), the general mode of action of these nucleotides remains to be elucidated.

The anticodon region has also been demonstrated to be important in the role of tRNA as a regulator of enzyme synthesis. As mentioned earlier, an extensive
study of the regulation of the histidine biosynthetic pathway by Ames and coworkers has shown that charged histidyl-tRNA$^{\text{His}}$ acts as a corepressor in the control of the histidine operon. The his-tRNA$^{\text{His}}$ is inactive in this capacity, however, if its two uridine residues located adjacent to the purine position at the 3' end of the anticodon are not converted to pseudouridine (42, 144). Thus specific residues in the anticodon, or the conformation they induce, are required for repressor recognition and proper control of the biosynthetic pathway.

The variable loop, consisting of either four to five or thirteen to twenty-one nucleotides, is the major source of variability in the size of tRNA molecules. The longer variable arms probably form a hydrogen bonded stem region consisting of three to seven base pairs with a small loop at the tip. While two sequences have been reported with only three nucleotides in the variable region, these data are questionable since it appears unlikely that tRNA tertiary structure could be conserved under such conditions (109). As mentioned above, the variable region may be used along with the number of base pairs in the D stem in a tRNA classification system. While tRNAs with shorter variable regions may exhibit either three or four base pairs in the D stem, it is interesting to note that all tRNAs with longer variable arms appear to possess only three D-stem base pairs, with
the position of the fourth pair containing a purine (5' side) and an adenosine (3' side). The entire role of the variable region in the tRNA molecule remains to be determined, but it has recently been shown to be involved in tertiary interactions which stabilize the tRNA conformation (109, 145). For example, the constant pyrimidine located adjacent to the TΨC stem is always complementary to the constant purine immediately preceding the D loop. In addition, the nature of the variable region has been observed to affect the cation binding characteristics of the tRNA molecule (146) and to be involved in the formation of additional base-pairing structures during tRNA early melting transitions (147). Thus this region, apparently located on the surface of the crystal structure to accommodate its great degree of variability in length (109), could possibly play a role in modulating the activity of a flexible tRNA molecule in solution.

The TΨC stem consists of five base pairs, terminating with a constant G(5')-C(3') pair adjacent to the TΨC loop. This loop, so named because of its relatively constant content of the modified nucleotides ribothymidine (T) and pseudouridine (ψ), possesses many features which are conserved among most tRNAs involved in protein synthesis. Of the seven nucleotides in the loop region (or nine if the adjacent G·C base pair is included) only one is not either a constant purine or pyrimidine or totally
conserved in character. Thus the general sequence of these nine residues may be written as G-T-ψ-C-purine-A-(purine or pyrimidine)-pyrimidine-C. Until recently the only exception to this rule was found in the tRNA\textsuperscript{Gly} of \textit{Staphylococcus epidermidis} which is used for cell wall biosynthesis and is inactive in protein synthesis (61). Thus, the remarkable consistency among tRNA sequences in this region provided a strong indication that this portion of the molecule was involved in a function common to all tRNAs participating in ribosomal protein biosynthesis. Speculation along these lines was given support by experiments in several laboratories indicating interaction of the TψC region with a complementary sequence in the 5S RNA of the ribosome (148, 149).

Recent results, however, reported independently by RajBhandary and coworkers (150) and by Petrissant (151), have revealed that all the eukaryotic cytoplasmic initiator tRNA\textsubscript{Met} species examined lack the G-T-ψ-C-G(A) sequence. Instead this sequence is replaced by G-A-U-C-G(A) or G-A-U*-C-G(A) where U* is believed to be a derivative of U other than ψ. This difference is not due to the mere lack or alteration of a modifying enzyme since the ribothymidine is replaced by an adenosine residue. In addition the complete sequence of yeast tRNA\textsubscript{Met} indicates the presence of an adenosine rather than a pyrimidine at the final 3' position of the loop. These differences may play a role
in enabling the eukaryotic initiator tRNAs to function in the initiation of protein synthesis without the prerequisite of formylation. In fact, eukaryotic ribosomes and initiation factors could necessitate this change as opposed to the formylation required by prokaryotic and organelle systems. Alternately, the absence of the G-T-ψ-C-G(A) sequence could act in vivo to prevent binding of the initiator to the A site of the ribosome and thus prohibit participation by this tRNA species in polypeptide chain elongation. This possibility, however, has not been supported by in vitro studies (152, 153).

The role of the TψC region in ribosomal interaction has been further brought into question by the recent findings of Rich and coworkers. Their X-ray crystallographic data concerning the overall structure of the tRNA molecule have indicated that in the crystal structure portions of the TψC loop interact with residues in the D loop and are thus unavailable for ribosomal hydrogen bonding (109). Their results, however, do not preclude the opening up of the TψC portion of the molecule when it interacts with elongation factors and/or ribosomal components. Needless to say, further work is required before clarification of the role of this portion of the molecule as well as that of all other regions is possible.

The experimental results discussed thus far clearly indicate that the interaction of tRNA with proteins and
other nucleic acids is far from being a simple matter. No mere correlation of nucleotide sequences can explain the specificity of the molecule in its multitude of functions. In fact, the number and complexity of these interactions, as well as the "distant" regions of the tRNA cloverleaf structure taking part in any one of them, seem to imply that a more compact arrangement than the cloverleaf is required for cellular activity. Physical evidence specifically in support of such tertiary structure was found early in the study of tRNA and has been greatly expanded in recent years.

Numerous approaches have been employed in the attempts to elucidate further the nature of this tertiary structure which appears to be so significant in tRNA function. These approaches include (i) the study of naturally occurring differences or similarities in tRNA structure which affect tRNA functional ability, (ii) the characterization of effects produced by in vitro chemical modifications or interactions, and (iii) the measurement of tRNA physical properties in solution and in crystal form. Among the naturally-occurring probes used in this regard are the numerous mutant tRNAs which have been isolated (see Section 1.2). As mentioned earlier, studies of missense and nonsense suppressors have been used to indicate that both the acceptor stem (98) and the anticodon region (120) are involved in interactions with the cognate synthetase.
In addition, the absence of specific modified residues in other mutant tRNAs has revealed the importance of the anticodon region in interactions with regulatory proteins (42, 144). Homologous isoaccepting tRNAs, which possess different nucleotide sequences but are recognized by the same synthetase, have served to provide insight into aspects of the tRNA structure which appear to play a role in enzyme recognition (124). In addition, heterologous aminoacylation reactions involving purified synthetases and noncognate tRNA species of different cellular origin have proven to be very successful probes of tRNA-synthetase interactions (154). Data from these studies have demonstrated that the dihydrouridine stem and the fourth nucleotide from the 3' terminus are apparently oriented in the tertiary structure in a manner which enables them to play a role in the synthetase binding site (97, 110, 111). Similarly, studies of initiator tRNA species have led to a better understanding of the structural features required for EF-Tu recognition, protein elongation, and ribosomal interaction (91, 148, 149). The specific nature and location of various modified residues in the tRNA structure, as well as variations noted in the extent of modification with varying physiological states of the cell, have provided further indication of the importance of such modifications in maintaining the proper conformation for maximal tRNA activity (155). The ability of certain
tRNA molecules to form stable denatured conformers suggests another possibility for the involvement of structural features in cellular control mechanisms (54). Furthermore, the extensive compilation of nucleotide sequences now available has made possible the identification of numerous conserved nucleotide residues among tRNA molecules and the evaluation of their involvement in tertiary structural interactions as shown in Figure 2 (109). Thus much has been revealed about tRNA structure-function relationships merely from studies of naturally-occurring differences and similarities in sequence, modification, and structure.

In addition to these naturally-occurring probes, however, many methods involving chemical modifications or interactions have been used. Among these modification techniques already mentioned are the oxidation and reduction of the 3' terminal ribose and the bisulfite conversion of cytidine residues to uridine (91, 122). Other studies have made use of the photodimerization of pyrimidine residues after U.V. irradiation to study the role of inactivation targets in synthetase recognition and/or in maintaining the proper tertiary conformation of the molecule (103). Specific photochemically-induced cross-linking between the 4-thiouridine at position 8 and the cytidine at position 13 of E. coli tRNAs may be brought about by U.V. irradiation at 335 nm (156). This technique
has recently been employed to study the proximity of these residues in the tertiary structure (157) and to examine the effect of their linkage on biological activity (158). Although synthetase activities were found to be affected variably by such modifications, no large separation of these residues appeared to be required during the course of the aminoacylation reaction.

Additional modification techniques involving reactions with selective chemical reagents have been used to study the role of specific residues in tRNA function and to determine their extent of exposure in the native tRNA structure. For example, cyanogen bromide has been found to react with the 2-thiouridine derivatives located at the 5' terminal position in certain tRNA anticodon sequences. Modification with this reagent has provided evidence in favor of the involvement of the anticodon region in synthetase recognition as well as in ribosomal binding (120, 159, 160). On the other hand, the modification of 4-thiouridine at position 8 appeared to have little effect on tRNA activity (161). The reactivities of cytidine residues with methoxyamine and of uridine, pseudouridine, and guanosine residues with carbodiimide derivatives have recently revealed a diversity in the anticodon loop conformations of several tRNA molecules which could be attributed to changes in nucleotide sequence in the anticodon region (127). In the TΨC loop,
the failure of acrylonitrile to modify the pseudouridine residue was interpreted as evidence that the region was buried in the tRNA tertiary structure (168). Likewise the identification of exposed, non-base-paired guanosine residues in tRNA tertiary structures was made possible by differential reaction with such reagents as the chemical carcinogen N-acetoxy-2-acetylaminofluorine (163, 164) and kethoxal (β-ethoxy-α-ketobutyraldehyde) (165-170). These studies have implied the availability of certain guanosine residues in the D loop for chemical modification and have emphasized the importance of the ionic environment in the maintenance of correct tRNA tertiary structure. As a final example, the isotope labeling method recently reported by Gamble and Schimmel (171) appears to hold a great deal of promise as a probe of tRNA structure. This technique is based on differential rates of incorporation of tritium into the C-8 position of purine bases. These rates have been found to reflect the local environment of a purine residue and thus yield information regarding the tertiary conformation of the molecule. The main advantage of this approach lies in the fact that tritium replacement of hydrogen avoids perturbation of the tRNA structure by the introduction of a complex chemical probe.

The reagents mentioned here provide merely a sampling of the many compounds used to probe the aspects and
implications of tRNA tertiary structure. On the whole, the rates and extents of modifications observed have repeatedly demonstrated the existence and importance of tRNA tertiary interactions and promise to reveal more far reaching conclusions as the scope of these studies continues to broaden.

Among the many other approaches used in tRNA structure studies are those involving limited nuclease digestion. For example, it has been demonstrated that tertiary structure is able to protect regions of the tRNA molecule from immediate nuclease attack (172, 173). Furthermore, recombination of half and quarter tRNA molecules obtained in this fashion have been used in the study of the role of ordered structure in synthetase recognition (101). Difficulties involved in this approach include the possibility of contamination by fragments other than those being examined and the problem of complex stability and proper ordered structure formation among the fragments. In addition, nuclease digestion in the presence of the cognate synthetase has indicated synthetase interaction with the anticodon and dihydrouridine loop in some cases, and the 3' terminus in others (126).

The principle of oligonucleotide binding (174) has also been used in the study of tRNA tertiary structure. Through a comparison of the binding constants of tetramers and their constituent trimers to a tRNA, it is possible to
designate which portions of the sequence are available for binding and which are involved in hydrogen bonding, tertiary structural interactions, or other constraints which prevent such interaction (175, 176). Comparison of the hybridization pattern of free and enzyme-bound tRNA has indicated the protection of both the anticodon and the 3'-terminal region by the cognate synthetase (177). These studies have also indicated a variability in anticodon structures among different tRNA molecules in agreement with the chemical modification data mentioned above (128). In a similar manner the complex formation between oligodeoxyribonucleotide ethyl phosphotriesters and tRNA molecules has been studied (178). Due to the lack of charge of these triesters at neutral pH, their binding to tRNA may be studied at low salt concentrations when corresponding ribonucleotides are four to six-fold less effective. These studies have also implied the role of the anticodon in synthetase binding (179).

A further probe into the nature of tRNA structural features is found in the use of tritium exchange. In this technique hydrogen atoms bonded to atoms having unshared electrons (i.e. N and O in tRNA molecules) are replaced to a small extent by tritium. The exchange of those tritium atoms restrained by structural features (hydrogen bonding in particular) is retarded, and it is possible to follow their release by sampling techniques
(180, 181). In addition to its other applications, this method has been used to compare the secondary and tertiary hydrogen-bonding interactions of native and denatured tRNA_{Leu} and to study the mechanism of conversion between the two conformers (182) (see Section 1.6).

Numerous physical measurements of tRNA structural parameters have been carried out as well. Ultraviolet absorption measurements were initially used in observing the thermal melting curves of crude tRNA preparations, providing one of the first indications of a defined ordered structure (183). Later refinements in spectrophotometric techniques, purification of individual tRNA molecules, and the application of such methods as mixing calorimetry (184-186), differential heat capacity calorimetry (187), and temperature jump relaxation kinetics (186) have allowed a more detailed analysis of the thermal transition and its relationship to the ionic strength of the medium. The results of these studies, however, have not presented a consistent picture of the thermal denaturation process. For example, detailed investigation of the temperature and salt transitions of four _E. coli_ tRNAs led Cole and coworkers to postulate the existence of four structural forms for the tRNA molecule in solution (188, 189). These conformations include (i) the cloverleaf (or a close variant form lacking the D stem helix) which is present at relatively high salt concentrations and moderate
temperatures, (ii) a "native" form exhibiting added tertiary structure and appearing at high salt concentrations and low temperatures, (iii) an extended form possessing altered base pairing patterns which occurs in low salt solutions and low temperature ranges in order to reduce the charge density on the molecule, and (iv) an unfolded or coil form with possible local stacking interactions which is present at high temperatures. On the other hand, Levy and colleagues in a study of yeast tRNA<sup>Phe</sup> presented data consistent with only two conformations of the molecule, a single folded and a single unfolded state (184-186). In light of these studies, the possibility that different mechanisms could be involved in the melting transitions of different tRNAs was examined by Brandts et al. (187). Using differential heat capacity calorimetry, they found a distinct difference in the single phase transition properties of yeast tRNA<sup>Phe</sup> as compared to the biphasic nature of other tRNA transitions. The single observable calorimetric transition for tRNA<sup>Phe</sup>, however, was found to be more complex than the proposed all-or-none interconversion. The level of cooperativity observed was indicative of the unfolding of two semi-independent structural regions such as the two continuous helices proposed by Kim et al. (135). Additional differences among tRNA melting transitions have been documented with regard to the order of melting of the
four helical stems in the molecule. This order has been found to depend on such factors as the G•C content of the helices, the coupling between helix-coil transitions, the stabilizing effect of tertiary interactions, and the loop closure free energies (190-192). On the whole, these studies have implied that the interplay between secondary and tertiary structures at various temperatures and salt concentrations is more than likely an individual characteristic of each tRNA molecule. This conclusion would serve in part to explain the many differences which have been observed in the structural and functional characteristics of various tRNA molecules.

The use of fluorescence techniques has also proven rewarding in investigations of tRNA structure. A number of such studies have taken advantage of the naturally-occurring fluorescent nucleotides in tRNA such as 4-thiouridine (193), the Y base in yeast tRNA\textsuperscript{Phe} (194), or the fluorescent nucleotide in E. coli tRNA\textsuperscript{Glu} (195). Introduction of a second fluorescent probe with appropriate excitation and emission properties into tRNA\textsuperscript{Phe} has made possible the measurement of the distance between the introduced label and the Y base in the anticodon by fluorescence energy transfer (183, 196). In tRNAs without an internal fluorescent species, specifically directed double fluorescent labels can be introduced for this purpose. The recent studies of Yang and Söll have employed
a number of such site-specific probes in determining intramolecular distances in tRNA molecules in solution (197, 198). In general, the results they have found using this "spectroscopic ruler" approach closely parallel the dimensions reported from X-ray crystallographic data (135). The major difference in the results obtained using the two approaches is the slightly greater distance between the D and T\(\Psi\)C loop regions implied by the fluorescence studies. It is necessary to keep in mind with regard to studies involving fluorescent modifications, however, that the possibility of perturbing the native structure by the introduction of a modifying group is particularly great in the case of bulky fluorescent probes.

Fluorescent labels introduced at the 3' terminus alone have been used in the study of conformational changes in the tRNA molecule. Cramer and colleagues have introduced formycin-monophosphate into the 3' terminus of yeast tRNA\(^{\text{Phe}}\) through the nucleotidyl transferase reaction. The fluorescence properties of the probe were shown to reflect stacking interactions in the C-C-F sequence which could be disrupted by periodate oxidation of the terminal ribose (199). In another recent study, Lynch and Schimmel have attached 2-napthoxyacetic acid to the amino group of isoleucine-tRNA\(^{\text{Ile}}\) in order to study the binding of Mg\(^{++}\) (200, 201). Using this probe they have demonstrated structural changes to occur with Mg\(^{++}\) binding which appear
to reflect the existence of aberrant base-pairing patterns
in Mg\(^{++}\)-free tRNA structures (202), perhaps similar to
those suggested by Cole et al. (189).

Further investigations of Mg\(^{++}\) binding have employed
the fluorescent lanthanide ions Tb(III) and Eu(III) to
probe the environment of the cationic binding sites.
These studies have implied that one or more cations bind
in the vicinity of 4-thiouridine (position 8) in E. coli
tRNA\(_{Ile}\) (203), a result in agreement with numerous other
approaches (157, 188, 202, 204).

Circular dichroism (CD) and optical rotatory dispersion
(ORD) measurements have also proven useful in the
study of tRNA structure. In polynucleotides, the ob-
served CD bands arise due to interactions between the
monomer components. Such interactions depend on the geo-
metry of the polynucleotide chain and thus provide informa-
tion regarding secondary and tertiary structure. In the
case of tRNA, all the observed CD bands are related to the
ordered structure of the molecule, although some are
apparently more sensitive to conformational change than
others (205). Tinoco and coworkers have attempted to
predict the CD spectra of tRNAs from their nucleotide
sequences and from secondary structure approximations
derived from studies of model compounds (206). Their
results have indicated that at 40° the experimental CD
spectra may be closely approximated by calculations
involving observed tRNA single-strand base-stacking interactions and model double-strand base-pairing spectra. For most tRNAs, two to four base pairs in addition to those derived from the cloverleaf structure were found to be required to improve the agreement between the calculated and observed spectra. These base pairs could possibly be attributed to tertiary interactions such as those described by Kim et al. (109).

An additional application of the CD technique has recently been reported by Willick et al. in the exploration of tRNA conformational changes induced by Mg$^{2+}$ binding (204). Their results indicate the presence of a set of specific coordination sites formed by the folded tRNA molecule, with the final conformation of the complex depending on the coordination property, rather than the ionic radius, of the ion occupying these sites. Further CD studies by Prinz et al. have shown that alterations in tRNA conformation are induced by low concentrations of the organic solvents often used in crystallization procedures (207).

Circular dichroism is often used in preference to optical rotatory dispersion due to the advantages of fewer technical difficulties, better resolution of neighboring optically active absorption bands, and the ability to compute rotational strength values directly from CD data (208). CD, however, is an absorptive property and thus
is encountered only in the region of absorption of the chromophores. ORD, on the other hand, is a dispersive property, and the signal at any wavelength is a sum of the contributions from all optically active absorption bands, however distant. The interdependence of CD and ORD can be evaluated using the Kronig-Kramers transform (209), and discrepancies between the ORD computed from CD measurements and the experimentally observed ORD indicate the existence of CD bands in regions where detection is not possible (e.g., below 180 nm). This method has led to the prediction of a CD band at 165 nm which is extremely conformation dependent (205), in addition to the observed bands near 185 nm (203), 236 nm, 260 nm (210) and 295 nm (211). The current development of the vacuum ultraviolet circular dichrometer which will provide data in the region between 140 and 180 nm (205) will greatly aid in the utilization of the 165 nm band as an additional probe of tRNA conformation.

The application of proton nmr investigations to tRNA research was increased greatly with the demonstration by Kearns et al. that aqueous solutions of tRNA exhibit a set of resonances in the very low field region between -11 to -15 parts per million downfield from the usual DSS standard (2,2-dimethyl-2-silapentane-5-sulfonate) (212). These resonances were assigned to the ring nitrogen protons of hydrogen-bonded bases (one per base pair). In addition,
data from model systems as well as from tRNAs with
different sequences demonstrated that G·C base pairs
exhibited resonances between -11 and -12.8 p.p.m. while
A·U base pairs were responsible for resonances between
-13.3 to -15 p.p.m.. Thus high-resolution nmr measure­
ments may be used in determining the total number of
hydrogen-bonded base pairs as well as their division into
A·U and G·C pairs. Recent coupling of nmr techniques with
sequential melting studies has even permitted the assign­
ment of observed resonances to specific cloverleaf base
pairs (213, 214). The use of paramagnetic rare earth ions
in conjunction with nmr has furthered the investigation of
tRNA metal binding characteristics and the elucidation of
tRNA tertiary structure (215). In addition, nmr has
proven useful in the study of the effects of aminoacyla­
tion on tRNA conformation (216), and has revealed the
absence of secondary structural changes in tRNA during its
interaction with the cognate synthetase and with elonga­
tion factor T (217, 218). Many of these experimental
observations have been made possible by the recent develop­
ment of the 300 MHz spectrometer which has greatly improved
the resolution obtained in nmr studies.

The approaches mentioned here represent only a portion
of the techniques currently employed in the study of tRNA
conformation in solution. Electron spin resonance (219,
220), laser-excited Raman spectroscopy (221, 222), small-
angle X-ray scattering (223, 224), and others would need to be included to make the list complete. Numerous alterations in structure resulting from the variety of treatments used have been catalogued, but as of yet the overall picture of tRNA structure-function relationships remains rather hazy.

Perhaps the most rewarding approach used thus far in the study of tRNA structure has been X-ray crystallography. Since 1968 several laboratories have succeeded in crystallizing yeast tRNA\textsuperscript{Phe} (225-229), and extensive analyses of these crystals have been carried out. In most cases, heavy atoms such as osmium, samarium, and platinum have been allowed to diffuse into the tRNA crystals to form the heavy atom derivatives required for interpretation of the electron density maps (230). Chemical modification prior to crystallization, however, has recently been carried out by nucleotidyl transferase insertion of 4-thiouridine instead of cytidine adjacent to the 3' terminal adenosine (287). It is hoped that mercury derivatives suitable for X-ray analysis will be obtained by treating the resulting sulfur-containing tRNAs with mercurials. This procedure would eliminate the assumptions currently involved in interpreting the location of the heavy atom (231). The results at 4 Å resolution of the yeast tRNA\textsuperscript{Phe} structure (135) indicated that the molecule consists of two segments of double helix, each about one
turn in length, which are oriented at right angles to each other in the form of an L. One arm of the molecule is composed of the acceptor stem connected to the TipC stem in a continuous double helix. This relationship positions the TipC loop at the corner of the "L", immediately adjacent to the D loop. The second essentially continuous double helix contains the D stem and the anticodon stem. Thus the anticodon is at one end of the L-shaped molecule and the 3' C-C-A terminus extends into the medium at the opposite end, 82 Å away. The length of the molecule from the anticodon loop to the TipC loop is 77 Å, and virtually the entire molecule is 20 Å thick, the thickness of an RNA double helix. Refinement of the X-ray analysis to 3 Å resolution for both the orthorhombic (109, 232, 233) and monoclinic (234-236) crystal forms of the molecule has confirmed and extended these results. The molecule was shown to consist of a horizontal and a vertical stacking unit, each containing not only the base stacking of the helical stem regions, but also stacking associated with tertiary interactions in the TipC, D, and variable loops. Only four bases in the entire molecule do not appear to be involved in some fashion in this extensive stacking arrangement. A comparison of 56 known tRNA sequences also revealed that a large number of the bases which appear to be common to all non-initiator tRNAs are involved in tertiary interactions which, together with
base stacking, appear to maintain the three-dimensional structure of the molecule (Figure 2) (109). Hydrogen-bonding schemes involving three nucleotide residues and often using non-Watson-Crick base-pairing patterns were demonstrated to be among those involved in such stabilizing interactions (109, 145). In addition, the few regions of the molecule which vary in length were shown to be on the surface of the molecule and could thus be accommodated by forming or enlarging protuberances without disrupting the overall structure.

The applicability of this crystallographic work to the actual structure of biologically active tRNA has been the subject of a number of investigations. For example, laser Raman scattering spectra of tRNAs in aqueous solution and in the solid state have indicated that crystallization does not significantly alter the tRNA conformation (222). In addition the observation that the tRNA acceptor activity of redissolved crystals is immediately restored favors the assumption that no major conformational changes occur on crystallization unless they are rapidly interconvertible (230). Furthermore, the important role played by constant residues in determining the yeast tRNA$^{Phe}$ structure and the easy accommodation of sequence variability in the crystal structure also appear to indicate that the findings obtained in the crystallographic study of this molecule
might be generally applicable to all tRNA molecules.

Some reservations as to the validity of these generalities, however, should be noted. To begin with, yeast tRNA_{Phe} has been demonstrated to behave differently than other tRNAs in a number of physical studies carried out in solution, including the determination of its thermal denaturation characteristics by differential heat capacity calorimetry (187) and CD (205). In addition, Cramer and coworkers have demonstrated that the organic solvents used in crystallization studies lead to partial denaturation of tRNA molecules (207). In particular, organic solvents were found to stabilize a highly ordered, nonnative conformation of yeast tRNA_{Phe}. These facts, in correlation with the observation that the tRNA_{Phe} molecule crystallizes in as many as twelve different crystal forms (235), emphasize the question as to whether the crystal structure being studied is a true, or a general, representation of a biologically active tRNA molecule.

Perhaps the most significant argument of all against relying too heavily on X-ray data, however, lies in the increasingly strong evidence from solution studies that the tRNA molecule is flexible in nature, possibly undergoing numerous conformational changes during the course of performing its tasks within the cell. Such flexibility would aid in the formation of the many recognition sites required and could be easily seen as a possible means of
modulating the molecule's biological activity. Clearly our understanding of tRNA structure-function relationships, although expanding rapidly, is still quite limited. No one technique will provide all the answers, and thus a concerted effort from many different approaches is called for. Only with such combined effort can we hope to obtain a sufficient number of clues to piece together the puzzle. The remainder of this dissertation will be dedicated to the addition of a few more such clues to the present compilation and to the description of their relevance in relation to the overall picture.

1.5 Rationale for Studying Yeast Denaturable tRNA\textsubscript{Leu}\textsuperscript{3}

In choosing a system for use in approaching the question of tRNA structure-function relationships, several primary considerations must be taken into account. First, the tRNA involved must be available in sufficient quantity to allow detailed physical and chemical analysis. In addition the nucleotide sequence must be known before possible reactive residues may be identified or the basis for physical changes can be postulated. The tRNA\textsubscript{Leu}\textsuperscript{3} species isolated from baker's yeast fulfills these requirements well. A method for its purification in large quantity has recently been reported by our laboratory (237), and its sequence has been obtained independently
by both our laboratory (136, 238) and that of J. R. Fresco (239).

The tRNA$^{\text{Leu}}$ species, however, has a number of distinct additional advantages which make its study particularly rewarding. Since it is of eukaryotic origin, a comparison of its physical and functional properties with those of tRNAs from higher organisms might possibly be more valid than that of a bacterial tRNA molecule. In addition, it is isolated from the same source as the most widely studied tRNA species, yeast tRNA$^{\text{Phe}}$. Structural differences between the two, including the longer variable arm and shorter D stem of tRNA$^{\text{Leu}}$, make a comparison of their tertiary structures extremely interesting as well as applicable to a wide range of tRNA molecules.

Furthermore, the nature and importance of the amino acid inserted by tRNA$^{\text{Leu}}$ makes this species a prime candidate for involvement in a cellular control mechanism. In addition to being one of the most abundant amino acids in proteins, leucine has been shown to play a central role in protein structure and function (240). Evidence of its importance is revealed by the fact that it was found to be the most invariant residue in insulin, hemoglobin, and cytochrome c homologs, as well as the most abundant residue in the region of the heme group in the five heme proteins studied. In the case of enzymes, a survey of fifteen proteins demonstrated that leucine and methionine
occur in the greatest frequency as neighbors to polar active site residues. Thus they serve to provide a rigid framework and a nonaqueous environment to aid the polar residues in substrate binding and enzymatic catalysis. In addition, leucine has been found to be the strongest overall structure-forming residue in proteins as well as the amino acid with the greatest tendency toward forming an α-helix (240). Being the most predominant amino acid residue in the inner helical regions of proteins, it could thus play a major role in forming nucleation centers which would serve to direct the folding of large protein molecules and perhaps influence the rate of their release from the ribosome. Control of the insertion of these residues by their specific tRNAs during protein synthesis could easily affect the conformational and synthetic properties of growing polypeptide chains.

In line with the abundance and importance of leucine residues in proteins, a total of six triplets have been demonstrated to code for this amino acid (equal to the maximum found for any residue). Correspondingly, in most systems studied, four to six different isoaccepting species of tRNA^{Leu} have been found (241, 242), and in several instances, the existence of multiple leucyl-tRNA synthetases of varying tRNA^{Leu} specificity has been demonstrated (243, 244). Thus ample opportunity is provided the cell for the use of leucyl-tRNA^{Leu} as a
regulatory factor in protein biosynthesis. Data thus far have indicated that in many instances the cell takes advantage of these opportunities (245).

One example of the involvement of tRNA_{Leu} in control is seen in the change in the tRNA_{Leu} complement of E. coli cells on infection with T-even phage (246, 247). After infection, two changes are seen to occur: (i) the destruction by endonucleolytic cleavage of an E. coli tRNA_{Leu} specific for C-U-G (248), and (ii) the production of seven or eight new tRNA species, including a leucine tRNA which differs from all host species of tRNA_{Leu} (249, 250). This tRNA species specifically hybridizes to phage DNA (251) and recognizes the codon U-U-A (252). None of the five E. coli tRNA_{Leu} species binds U-U-A in the ribosome binding assay (248), although tRNA_{Leu} 4 and/or 5 (totally comprising about 20% of the tRNA_{Leu}) are believed to be able to recognize this sequence in vivo (253). It is estimated that the amount of U-U-A-recognizing tRNA is doubled or tripled in phage-infected cells (254, 255). A correlation of burst size with ability to form phage-specific tRNA's has led Wilson to propose that the T-even tRNAs function to ensure optimum synthesis of phage proteins by supplementing the reading capacity for codons used more frequently by the phage than by the host (256), while simultaneously the capacity for reading host-specific codons is being reduced (248).
A second example of the possible use of tRNA\textsubscript{Leu} in control mechanisms has been found by Weisblum \textit{et al.} in their study of the codon degeneracy for leucine in hemoglobin biosynthesis (257). Using \textit{E. coli} tRNA\textsubscript{Leu}, they were able to demonstrate that different isoaccepting species inserted leucine into different positions in the \(\alpha\)-chain of hemoglobin. One of the tRNA's (U-U-G-specific) introduced leucine only at position 48. A possible control mechanism for the rate of \(\alpha\)-chain synthesis, based on the availability of this tRNA\textsubscript{Leu} species in an active form, may be visualized.

Numerous other reports have noted alterations in the tRNA\textsubscript{Leu} complement of cells (overall content as well as isoacceptor concentrations) which appear to indicate that these tRNA species are among those involved in cellular regulation. Such changes have been observed to accompany cell differentiation and specialization, hormonal induction, changes in nutrient conditions, and the onset of neoplasia.

One of the most fully documented examples of a possible tRNA\textsubscript{Leu}-mediated control mechanism, however, is found in the study of yeast denaturable tRNA\textsubscript{Leu}\textsubscript{3}. Isolating yeast tRNA under denaturing and non-denaturing conditions, Lindahl and coworkers discovered the existence of a tRNA\textsubscript{Leu} species which possessed two stable conformations, only one of which was active in protein synthesis (54).
Conversion of the denatured, inactive conformer to the native active state could be achieved by heating the tRNA for short periods of time in the presence of Mg$^{++}$. The true physiological significance of this reversible denaturation process remains unknown, but its role may be envisioned as a regulatory mechanism responsible for controlling the amount of active tRNA$^\text{Leu}_3$ present at any one time. A similar phenomenon has been noted to occur in several other tRNAs as well, including E. coli tRNA$^{\text{Trp}}$ (258), and yeast tRNA$^\text{Ser}$, tRNA$^\text{Phe}$ (259, 260), tRNA$^\text{Met}$, tRNA$^\text{Arg}_1$, and tRNA$^\text{Gln}$ (261).

The ability of tRNA$^\text{Leu}_3$ to form a stable, denatured, inactive conformer thus makes it an excellent probe for the study of tRNA structure-function relationships. The only difference between the active and inactive forms lies in their secondary and tertiary structures. An analysis of the alterations involved could therefore provide significant information regarding the importance and role of various structural features of the tRNA molecule.

An additional advantage to be found in the study of tRNA$^\text{Leu}_3$ arose during the latter stages of this work with the purification and sequence analysis of a non-denaturable tRNA$^\text{Leu}$ species from yeast (138). This tRNA, specific for the codon C-U-A, differs only slightly in sequence from tRNA$^\text{Leu}_3$, yet does not possess the ability
to attain a stable denatured conformation. A comparison of the differences between these two tRNAs holds further promise for providing information regarding the basis for their conformational properties and cellular roles.

1.6 Results of Previous tRNA<sub>3</sub> <sup>Leu</sup> Studies

In the mid-1960's a number of debates were in progress regarding the structural requirements for tRNA activity. One area of discrepancy concerned the participation of the divalent cation Mg<sup>++</sup> in the function of the molecule. The formation of the synthetase-aminoacyl-adenylate complex was known to be Mg<sup>++</sup>-dependent (262), but contradictory evidence had been reported concerning the role of Mg<sup>++</sup> in the transfer of the aminoacyl group to tRNA (263-265). A second controversy centered around the existence of a specific tRNA tertiary structure. Brahms and coworkers presented a hypothesis of unrestricted "conformational mobility" for the active tRNA molecule which involved complete freedom of conversion between energetically similar states as the molecule participated in its many functional interactions (266). On the other hand, Henley et al. proposed that the tRNA possessed a unique, stable, tertiary structure which was, in fact, a prerequisite for biological activity (183). In such a structure the unpaired residues would interact and serve to fix the helical segments in space. This proposal thus extended
the concept of functional dependence on molecular structure from the study of globular proteins to that of small nucleic acid molecules.

Evidence pertaining to both of these discussions was obtained by Lindahl and Adams in 1966 (267). Wishing to preserve the "native" structure of tRNA molecules as much as possible, they devised a method of purification which eliminated the usual denaturing steps such as phenol extraction, solution in distilled water, and extensive dialysis against EDTA. Their procedure revealed the existence of several tRNA species from yeast and E. coli which were inactivated by the conventional techniques. Early studies with unfractionated yeast tRNA demonstrated that the lower leucine acceptor activity in conventional preparations could be increased to the level of "native" preparations by prior incubation at 60° for five minutes in the presence of Mg²⁺ (268). Likewise the process could be reversed by exposing native tRNA to chelating agents such as EDTA or citrate. Elevated temperatures increased the rate of denaturation but were not required for the conversion. In order to relate the enhancement of leucine acceptor activity to a discrete tRNA species, countercurrent fractionation of a conventional preparation was carried out, and the leucine acceptor activity of the fractions was determined before and after heat treatment with Mg²⁺. Before treatment, two tRNA<sub>Leu</sub> components were
observed, while a third entity (hence designated tRNA\textsubscript{Leu}\textsuperscript{3}) became apparent after the activation procedure. Comparison of sedimentation coefficients revealed that the prior inactivity of the tRNA\textsubscript{Leu}\textsuperscript{3} species was not a result of aggregation effects.

The loss of activity observed as a result of Mg\textsuperscript{++} chelation was interpreted as an indication that Mg\textsuperscript{++} was directly incorporated into the tRNA and was thus an integral component of the biologically active form of the molecule. Lindahl et al. suggested that this requirement for a site-bound divalent cation might be a general phenomenon and that the examples uncovered might represent extreme cases, detected due to the difficulty with which the cation was reinserted (261). Thus most tRNAs in the absence of the cation would possess a conformation at moderate temperatures and ionic strength which would be favorable to cation binding. In contrast, tRNAs requiring renaturation at 60\degree assumed a structure at lower temperatures and in the absence of the cation which precluded binding. Higher temperatures served to disrupt this unfavorable conformation and to allow cation binding and formation of the native, active structure. The fact that tRNA could be trapped in a biologically inactive form demonstrated for the first time that a unique structure was required for biological activity. These results lent a great deal of weight to the earlier arguments in favor
of the existence of tRNA tertiary structure (266).

Additional indications of tertiary structure were obtained by Fresco and coworkers from studies of tRNA denaturation using methods sensitive to secondary structure (loss of hyperchromicity) and to overall conformation (changes in hydrodynamic properties including viscosity and sedimentation behavior) (268). Thermal denaturation profiles of unfractionated yeast tRNA in the absence of Mg$^{++}$ reflected a noncooperative denaturation process which occurred in two steps. The first step, occurring between 20° and 40°, involved a shape change accompanied by a relatively small loss of secondary structure. Above 50°, however, the molecule apparently became completely unfolded. In the presence of Mg$^{++}$, the tRNA structure was essentially stable up to approximately 60°. Above that temperature a cooperative transition in all parameters took place, indicative of the stabilization of secondary structure by tertiary interactions. The denaturing effects of raising temperature could also be brought about by lowering ionic strength. Below approximately 0.4M Na$^+$ at 30° (or 0.3M Na$^+$ at 20°) the tRNA molecules were observed to expand and denature. "Renaturation" could be brought about by charge neutralization with 1M Na$^+$, producing a tRNA structure with the same intrinsic viscosity as tRNA in 5mM Mg$^{++}$. The lack of cooperativity in the melting curve even at high Na$^+$ concentrations,
however, indicated again the importance of specific Mg\(^{++}\) binding in attaining the "correct" tertiary structure.

The study of the cation-dependent renaturation process was greatly aided by the purification to near homogeneity of a denaturable tRNA species. This was achieved through a combination of repeated countercurrent distribution steps and a new gel filtration procedure using Sephadex G-100 (261, 268). The latter process was based on the larger molecular volume of denatured tRNA\(^{\text{Leu}}\) with respect to the native conformation of other tRNAs present (268). Thus the denatured form was retarded less in the gel filtration column and could be effectively removed from contaminating non-denaturable tRNA species. This purification procedure was applied to other denaturable yeast tRNAs (tRNA\(^{\text{Met}}\), tRNA\(^{\text{Arg}}\), and tRNA\(^{\text{Gln}}\)), and the efficiency of the technique was observed to vary with the tRNA, depending on the volume difference between its two conformations and the relationship of its size to other tRNA components in the mixture. The conditions for purification were much more advantageous in the case of tRNA\(^{\text{Leu}}\) than for the other denaturable species, and thus this tRNA was chosen for further detailed studies.

The conditions required for the selective stabilization of native and denatured forms of tRNA\(^{\text{Leu}}\) were investigated by examining the specificity for divalent cations in the renaturation process at 60° (268). The
divalent cations Ca$^{++}$ and Mn$^{++}$, and to a lesser extent Co$^{++}$ and Zn$^{++}$, were found to be able to stabilize the tRNA structure against thermal denaturation and, to varying degrees, to change the sigmoidal denaturation profile to a sharp cooperative one. On the other hand, several polyamines (ethylenediamine, propanediamine, putrescine, and spermidine) in relatively low concentration, and monovalent cations (Na$^+$, K$^+$, and Li$^+$) in high concentration (approximately 1 M) were able to achieve some degree of thermal stabilization, but did not favor cooperative melting of the tRNA. Thus, as had been found earlier with unfractionated tRNA preparations, charge neutralization could serve as a minimum condition for bringing about renaturation of tRNA$^{Leu}_3$, but was insufficient to accomplish all the results of the binding of Mg$^{++}$ and similar divalent cations. Once the native conformer was attained in the presence of divalent cations, however, this structure could be maintained in the presence of a polyamine even after removal of the divalent cation by dialysis.

Numerous studies of the effect of pH, temperature, and ionic strength on the interconversion of native and denatured tRNA$^{Leu}_3$ led Fresco and his colleagues to formulate a working hypothesis which described the system in terms of native, denatured, and destabilized conformers separated by activation energy barriers. In the presence of Mg$^{++}$ at neutral pH and moderate temperature and ionic
strength, the native form was lowest in energy and therefore most stable. On the other hand, the tRNA molecule could be trapped in the denatured state for relatively long periods of time under these conditions if denatured prior to the addition of Mg++. The time required for conversion to the native conformer was found to vary in relation to the energy available in the system for overcoming the activation energy barrier between the two states. For example, only two minutes were needed for the conversion at 60°, while up to two days were required at room temperature. In the absence of Mg++ at low or moderate ionic strength, the denatured form became the most stable, and the native conformer was observed to collapse to the denatured one in less than one minute. A third destabilized form was postulated to occur (i) in distilled water, (ii) at low pH (around pH 3.5), or (iii) at 60°. This relatively unstable state could be rapidly converted into the native conformation on addition of Mg++. Thus the interconversions of denatured and native conformations could be achieved even in the absence of added heat energy.

Support for the activation energy hypothesis was gained when additional tRNA species were found to display denaturation-renaturation characteristics under conditions which decreased the energy available for interconversion. Solutions of unfractionated tRNA in dilute buffer without
Mg\(^{++}\) exhibited partial or complete loss of alanine, glutamine, lysine, serine, and tryptophan acceptor activities when assayed at 0°. Activity could be regained, however, by brief incubation in the presence of Mg\(^{++}\) at 30°. Thus, as postulated earlier, the phenomenon associated with denaturable tRNA\(^{\text{Leu}}\)\(_3\) appeared to be a manifestation of a general effect which under different conditions could be shown to apply in the case of many if not all tRNAs.

A number of physico-chemical studies were carried out to determine the nature of the conformational differences between the biologically active and inactive forms of tRNA\(^{\text{Leu}}\)\(_3\) (269). While identical in molecular weight, the two forms differed significantly in hydrodynamic properties. The 30 per cent higher intrinsic viscosity and 10 per cent lower sedimentation coefficient of the denatured conformer in comparison to the native structure were consistent with an 8-10 per cent increase in the radius or approximately 25 per cent increase in the volume of an equivalent hydrodynamic sphere. Thus the denatured form appeared to be somewhat more open and swollen and possibly more asymmetric than the native structure. On the other hand, the observed changes were quite small in comparison to those found for completely unfolded tRNA at 85°. Therefore the denatured conformation was only slightly less tightly coiled than the native one.

A comparison of the behavior of the two conformers during gel filtration was consistent with the larger size
of the denatured structure and provided evidence for its unique nature. The peaks of native and denatured tRNA\textsubscript{Leu}\textsuperscript{3} were observed to be symmetrical and of essentially the same width. Thus if the native form represented a unique conformational state (or an equilibrium mixture of similar conformations due to the flexibility of the molecule), then the denatured form must be unique or represent such a rapid equilibrium as well.

In a study of the optical properties of the two conformers, Adams et al. found that very little hypochromicity was lost in going from the native to the denatured form. Since greater than 85% of the thermally reversible hypochromicity of a tRNA molecule was known to result from the formation of helical arrangements of Watson-Crick base pairs (270), the difference in the number of base pairs in the two structures was concluded to be small. A multicomponent wavelength analysis of difference spectra suggested a net loss of 2-3 A\textperiodcentered U base pairs and 1 G\textperiodcentered C pair in the conversion from the native to the denatured form. The observed differences were so small in magnitude, however, that it could not be decided with certainty whether they actually resulted from a net change in the number of base pairs or some more subtle structural difference. Optical rotation studies revealed a shift in the crossover wavelength of the Cotton effect in ORD measurements from 260.8 to 263.3 nm and a similar
shift in the CD maximum from 260.3 to 262.3 nm on conversion from the native to the denatured structure. Comparison with model studies revealed that this red shift was indicative of the disruption of base pairs. The fact that only a small decrease occurred in the rotatory strength of the Cotton effect and in the CD maximum implied again that the net decrease in hydrogen bonding was small, on the order of three to four base pairs. Further analysis based on model compounds suggested the loss of approximately equal numbers of A·U and G·C base pairs, although the same uncertainties that applied to the analysis of the absorption spectra could be considered relevant in these studies as well.

Further evidence that the denatured form was less compact than the native conformer was obtained on digestion of the two forms with pancreatic ribonuclease. The four- to five-fold greater initial rate of digestion observed for the denatured form indicated that it contained many more potentially sensitive sites available to the endonuclease.

The implications of these structural differences in terms of the function of the tRNA molecule were investigated by examining the interaction of the two conformations with biosynthetic enzymes (271). Three enzymatic processes were studied, including the reactions catalyzed by yeast leucyl-tRNA synthetase, yeast nucleotidyl transferase, and
an *E. coli* protein-synthesizing system. An essentially absolute requirement for the native form was demonstrated for enzymatic aminoacylation and deacylation at 20°. To assess the relative abilities of the two conformers to bind the synthetase, however, periodate oxidized samples of each were used to inhibit the aminoacylation of native tRNA³<sub>Leu</sub>. Although less effective than its native counterpart, the denatured form of the oxidized tRNA³<sub>Leu</sub> was found to be a potent inhibitor of the charging reaction. Thus the denatured conformation apparently retains a sufficient amount of the enzyme recognition site to permit association with the synthetase. Studies with nucleotidyl transferase revealed a clear preference on the part of the enzyme for the native form of the tRNA molecule. Denatured tRNA³<sub>Leu</sub> was a poor, but nonetheless definitely recognized, substrate. In the protein-synthesizing system, a marked difference in both the initial rate and the final level of [¹⁴C]-leucine transfer into polypeptide was observed in response to poly (U,G) for the two forms of tRNA³<sub>Leu</sub>. These results suggested an absolute requirement for the native conformation by the system but did not rule out the possibility that the denatured form presented a new anticodon. Such a hypothesis had been proposed to explain the observation that denatured tRNA<sup>Trp</sup> of *E. coli* became bound to ribosomes only in response to polynucleotides which did not contain the normal tryptophan codons
Attempts to demonstrate such an alteration in the fidelity of codon recognition for denatured tRNA$_{\text{Leu}}^\text{Leu}$, however, proved unsuccessful. Studies with a variety of synthetic polynucleotide messengers as well as with intrinsic messenger RNA ruled out the possibility that denatured tRNA$_{\text{Leu}}^\text{Leu}$ responded to any commonly occurring codon. It could not be rigorously proven, however, that the denatured species did not recognize a rarely occurring codon.

While the specificity of the synthetase reaction could possibly be considered to reflect characteristics belonging only to the particular system being studied, the recognition involved in the nucleotidyl transferase reaction and the protein-synthesizing system reflected conformational features common to all tRNAs. Thus the definite preference exhibited by these systems for the native form of tRNA$_{\text{Leu}}^\text{Leu}$ indicated that some structural feature(s) common to all native tRNAs had been modified in the denatured conformer. Lindahl and his associates concluded that the major difference between the native and denatured conformers of tRNA$_{\text{Leu}}^\text{Leu}$ probably occurred at the level of tertiary arrangement since little difference was apparent in the amount and type of secondary structure present in the two forms.

The study of tRNA$_{\text{Leu}}^\text{Leu}$ was given a boost in 1971 with the determination of its nucleotide sequence by two laboratories (238, 239). The independent techniques used
in these analyses resulted in reported sequences which differed in only three out of eighty-five nucleotide residues. Re-analysis at these positions resulted in a corrected nucleotide sequence which was reported by our laboratory in 1973 (136). This sequence is shown in Figure 3 as it appears in the cloverleaf structure. At the same time a method for purifying tRNA\textsubscript{Leu} in large quantities was presented (237), thus making this tRNA species a prime candidate for more extensive physical and chemical study.

Among the first applications of the tRNA\textsubscript{Leu} sequence data was the CD study carried out by Tinoco and coworkers (206). As mentioned in Section 1.4, an attempt was made in this work to determine whether the CD spectra of tRNA\textsubscript{Leu} and other tRNA species could be represented in terms of their primary and secondary structures, or whether the tertiary structure contributed significantly as well. As an extension of this work the nature of the structural differences between the two conformers of tRNA\textsubscript{Leu} was examined. The difference spectrum of the native minus the denatured tRNA\textsubscript{Leu} species was compared with the difference CD of double-stranded minus single-stranded oligonucleotides (60% GC). The results were consistent with the loss of five base pairs in the conversion from the native to the denatured conformer. A similar study using denaturable \textit{E. coli} tRNA\textsubscript{Trp} revealed an anomalous
Figure 3. Nucleotide sequence of yeast denaturable leucine tRNA (tRNA$_{3}^{Leu}$) (136) arranged in the cloverleaf secondary structure. Ofengand's standard numbering system is used.
increase in the CD of the denatured form (as compared to the decrease observed for \( \text{tRNA}_{3}^{\text{Leu}} \)) and yielded a difference spectrum which did not fit well with any combination of double-stranded polymers. Thus the structural change accompanying denaturation of \( \text{tRNA}_{\text{Trp}}^{\text{Trp}} \) was shown to be different from that observed in \( \text{tRNA}_{3}^{\text{Leu}} \).

An additional factor which broadened the scope of \( \text{tRNA}_{3}^{\text{Leu}} \) studies was the purification of its conjugate leucyl-\( \text{tRNA}_{\text{Leu}}^{\text{Leu}} \) synthetase in 1971 (272, 273). Chirikjian et al. reported a molecular weight for the enzyme of 100,000-107,000 as determined by sucrose gradient, hydrodynamic, and equilibrium sedimentation methods. Treatment with SDS and mercaptoethanol indicated the presence of two subunits. \( K_m \) values were determined for L-leucine \( (2.0 \times 10^{-5} \text{ M}) \), ATP \( (1.1 \times 10^{-4} \text{ M}) \), total yeast leucine tRNA \( (4.0 \times 10^{-7} \text{ M}) \), native \( \text{tRNA}^{\text{Leu}}_{3} \) \( (1.0 \times 10^{-7} \text{ M}) \), and denatured \( \text{tRNA}_{3}^{\text{Leu}} \) \( (2.0 \times 10^{-7} \text{ M}) \). Complexes of both the native and denatured conformers with the enzyme were isolated from sucrose gradients. Both complexes appeared to be specific and to form preferentially at pH values slightly below neutral. The leucyl-\( \text{tRNA}_{\text{Leu}}^{\text{Leu}} \) synthetase has also been crystallized in a form which appears to be favorable for X-ray crystallographic analysis (274). The examination of substrate binding to the crystalline enzyme is reportedly in progress, in addition to the sequence analysis and preparation of heavy-atom isomorphous replacements which
are required for structure determination. The availability of the synthetase in pure form also makes possible numerous solution studies which will aid in elucidating the role of tRNA structural features in the synthetase recognition reaction.

Knowledge of the structural differences between the tRNA\textsubscript{Leu}\textsuperscript{3} conformers has also been gained from the equilibrium binding patterns of complementary oligonucleotides to the native and denatured forms (275, 276). The pattern of binding to the native conformer was found to be similar to that observed with other tRNAs. As expected, the anticodon loop and the 3' terminus were available for oligonucleotide binding while the three longest helical regions in the cloverleaf structure (the acceptor stem, the anticodon stem, and the T\textsubscript{Ψ}C stem) were not. The presumed helical structure of the variable stem was shown to be sufficiently weak as to allow competitive binding by probe oligomers. In addition, it is particularly noteworthy that both the D loop and the T\textsubscript{Ψ}C loop interacted only weakly with complementary oligonucleotides. Similar results had been obtained for other tRNAs (176) and seemed to imply that the loop residues were either greatly constrained or otherwise involved in tertiary structural interactions. These findings were in agreement with the close association of the D and T\textsubscript{Ψ}C loops observed in X-ray diffraction studies (135). The most notable
difference in the behavior of native tRNA\textsubscript{3}\textsuperscript{Leu} as compared with other native tRNAs, however, was the competitive binding of oligomers to the tRNA\textsubscript{3}\textsuperscript{Leu} D stem residues. The lack of stability which allowed some disruption of the short helical segment when tetramers were introduced was not observed even in the D stem of tRNA\textsubscript{TyF} which also possesses only three G·C pairs. Such instability in the D stem may quite possibly be linked to the existence of the metastable denatured state of tRNA\textsubscript{3}\textsuperscript{Leu}, and perhaps implies that this molecule has more favorable alternative structures for oligomer binding than do other tRNAs.

The oligonucleotide binding characteristics of the denatured conformer differed from those of the native form in two significant ways. First, the anticodon loop became unavailable for oligomer binding, and second, the D loop and stem became significantly more available. These results implied that the anticodon loop residues entered into tertiary interactions in the denatured form while the D stem base pairing was lost and the tertiary interactions involving the D loop were disrupted. Based on this data, Uhlenbeck \textit{et al.} suggested that the interaction between the D and T\textsubscript{ψ}C loops in the native form might be replaced by the interaction of the anticodon and T\textsubscript{ψ}C loops in the denatured conformer. Since the denatured form has been observed to bind the cognate synthetase (272), these results would appear to imply that unless a major
conformational change occurs on synthetase binding, the
D stem and the anticodon loop are not critical in the
tRNA Leu recognition process. These findings are in
contrast to numerous studies with other tRNA species which
have shown these regions to be important in tRNA-synthetase
recognition (see Section 1.4). It is perhaps important
to note that synthetase-mediated conformational changes
from inactive to active forms have recently been reported
to occur in the case of yeast tRNA Phe (277).

Another technique employed in the study of tRNA Leu
has been that of tritium exchange (182). Using this
method, the native form was shown to possess sixteen more
slowly exchanging hydrogens than the denatured conformer.
This number corresponds to a net increase of combined A·U
and G·C base pairs ranging from three G·C to five A·U
pairs in the transition from the denatured to the native
conformation. Both forms exhibited more slowly exchanging
hydrogens than could be explained in terms of known
secondary structural features (the intact cloverleaf
structure for the native conformer, and disruption of the
D stem for the denatured form). The observed numbers were
thus interpreted to reflect additional hydrogens restricted
by the superposition of "right" or "wrong" tertiary inter­
actions on an existing secondary structure. The differ­
ence between the number of slowly exchanging hydrogens for
the native and denatured conformations was therefore seen
to represent a net sum of broken wrong tertiary interactions, repaired cloverleaf hydrogen bonds, and correctly formed tertiary hydrogen bonds. In comparison, the number of slowly exchanging hydrogens observed for the tRNA$^{Leu}_3$ structure in 1M Na$^+$ was closer to that expected for the cloverleaf. Thus, as had been suggested by earlier studies, the major aberration of the denatured conformer could be corrected by high monovalent cation concentration, but charge neutralization was insufficient to stabilize the entire native tertiary conformation.

The kinetics of the exchange process were found to be even more sensitive to the imposition of tertiary structure than were the total numbers of slowly exchanging hydrogens. Although the native and denatured conformers of tRNA$^{Leu}_3$ had been demonstrated to share many secondary structural features (275, 276), the overall kinetics displayed by the denatured tRNA$^{Leu}_3$ conformer exhibited a six-fold increase in the rate of exchange. In fact, none of the hydrogens in the native conformation were observed to exchange with the fastest rate exhibited by the denatured conformation. The slower exchange in the native form was attributed to such factors as reduced solvent accessibility and restriction of molecular "breathing" by constraining interactions between cloverleaf segments. Such interactions were postulated to include hydrogen bonding between loop residues as well as between loop and helical
residues through sites otherwise occupied by solvent.

The mechanism of renaturation was also investigated by using tritium-exchange to monitor base-pairing perturbations during the transition. Renaturation was found to result in a burst of exchange, suggesting that base pairs were disrupted during the process. Thus part of the base-pairing arrangement of the denatured conformer was demonstrated to be lost on going to the native conformer. These results were in line with the large observed activation energy for the renaturation process and the findings of oligonucleotide binding studies. All approaches appeared to imply that significant structural reorganization was involved in the transition rather than mere superfolding.

On the whole, the constancy of the rate of exchange for the denatured conformation as measured in a variety of solvents supported the conclusion that the inactive form of tRNA\textsubscript{\text{Leu}}\textsuperscript{3} was a well-defined structure rather than a range of conformations. From this data it was predicted that if other tRNAs also possessed such denatured structures, the common lack of native tertiary structure would lead to similar exchange kinetics. The observed rate of exchange for unfractionated yeast tRNA at low ionic strengths (conditions favorable for the existence of denatured tRNA\textsubscript{\text{Leu}}\textsuperscript{3}) was indeed found to be intermediate between the slow native and rapid denatured tRNA\textsubscript{\text{Leu}}\textsuperscript{3}. 
kinetics. This finding was interpreted as being the result of a mixture of native and denatured tRNA species, each tRNA exhibiting different ionic requirements for native form stabilization due to its unique nucleotide sequence. Thus further evidence was obtained in support of the widespread occurrence of conformational mobility among tRNA molecules.

Among the most recent investigations of tRNA\textsuperscript{Leu}\textsubscript{3} structure have been the high resolution nmr studies conducted by Kearns and coworkers (278-280). The 300 MHz spectra of the native and denatured conformers have been observed over a range of temperatures and have been compared with spectra calculated from possible base-pairing schemes using a semiempirical ring current shift theory. Integration of the low-field nmr spectrum of the native conformer indicated the presence of 21+2 base pairs, a number in agreement with the cloverleaf model. In addition, calculated spectra based on the cloverleaf base-pairing scheme agreed well with observed results. The best fit was obtained when the T\textsuperscript{4}C and acceptor stems were assumed to be stacked to form a continuous helix (in agreement with X-ray data (135)), and when residues G13 and G45i were stacked on the adjacent base pairs in the D stem and variable stem, respectively. Further evidence in support of the cloverleaf model for the native conformer was found in thermal melting studies. At 50° the observed
changes in the nmr spectra could be accounted for by the loss of the D stem along with the A·ψ base pair adjacent to the anticodon loop and the A·U terminal base pair in the variable stem. The D stem region had been previously shown to be unstable (275), and terminal A·ψ base pairs were also known to be particularly susceptible to early melting (281). By 62° only the acceptor stem and the anticodon stem remained intact, consistent with earlier studies demonstrating these regions to be the most stable (282). The temperature dependence of the nmr spectrum thus implied that the melting transition of the native conformer was much less cooperative than UV absorbance measurements had indicated (268). This was attributed in part to the fact that the observed loss of nmr resonances corresponded to an earlier stage in helix disruption than was reflected by absorption characteristics. In addition, both single- and double-stranded regions contributed to the optical melting phenomenon (283), while only base-pairing interactions were observed in the low-field nmr spectra. On the basis of these data, some individual resonances in the low-field nmr spectra could be assigned to specific cloverleaf base pairs. Such assignments provide groundwork for future in depth studies of tRNA_{Leu} structural features and metal binding sites.

The well-defined spectrum of denatured tRNA_{Leu} indicated again the unique structure of the inactive
conformer and revealed the presence of $18+2$ base pairs. The difference spectrum of the two conformers provided evidence that the net loss of approximately three base pairs on denaturation resulted from the loss of at least five cloverleaf base pairs and the gain of at least two new base pairs. Analysis of the nmr melting transition of the denatured form implied that the acceptor stem was the only helical region intact at $62^\circ$. Based on this observation, all possible base-pairing schemes for the denatured structure were constructed which fulfilled the following requirements: (i) the retention of the acceptor stem, (ii) a total of approximately 18 base pairs, (iii) the ability to account for the room temperature spectrum of the denatured conformer and for the native-denatured difference spectrum, and (iv) the single-stranded nature of the D loop and stem (from oligonucleotide binding data). Only two possible base-pairing arrangements were found to be consistent with these stipulations. One of these was considered less desirable since it left bases in the anticodon loop unpaired (contradictory to oligonucleotide binding data), did not account successfully for the melting behavior of the denatured conformer, and involved the formation of a weak helical region. The other scheme, shown in Figure 4, was thus favored as the correct model for the secondary structure of denatured $\text{tRNA}^{\text{Leu}}_3$. This model retained the acceptor stem and the $\text{T}\psi\text{C}$ stem, once again assumed to be in a stacked arrangement, as well as the
Figure 4. Model proposed by Kearns et al. (280) for the secondary structure of the denatured conformer of yeast tRNA\textsubscript{Leu}.
variable stem. The D stem and the anticodon stem, however, were replaced by a single helix which involved base pairing of the anticodon region sequence A-ψ-U-m^5C and the TψC loop sequence G-A-A-U. Analysis of the sequential melting behavior of the denatured conformer in terms of this model implied that the minor stem and the new stem were lost by 52°, followed by the loss of the TψC stem as the temperature increased. As mentioned earlier, the acceptor stem alone remained in the 62° spectrum. The reversibility of this melting transition in Mg^{++}-free, low salt solutions indicated that the denatured conformer was indeed more stable than the native conformer under these conditions even though the native conformer contained more base pairs.

The features of the model proposed by Kearns et al. account for many of the observations made concerning the susceptibility of the denatured conformer to nuclease attack, oligonucleotide binding, tritium exchange, etc. Some regions of the cloverleaf are retained which could possibly be responsible for synthetase recognition, although the D stem and anticodon regions most often considered to play roles in this process have been eliminated. Most of all, the model allows specific predictions to be made concerning the structure of the denatured conformer which may be subjected to further experimental tests. Through such studies, knowledge of a general nature will hopefully be gained regarding tRNA structure-function relationships.
1.7 Scope of the Present Studies

In order to verify and extend the results of previous studies, the present investigation of denaturable tRNA$_{\text{Leu}}$ secondary and tertiary structure was undertaken. The problem was approached from each of the three basic standpoints discussed in Section 1.4: modification with a chemical reagent, measurement of physical properties, and comparison with a naturally-occurring tRNA structure. Chemical modification of both the native and denatured conformers was carried out using kethoxal ($\beta$-ethoxy-$\alpha$-ketobutyraldehyde), a reagent which selectively modifies exposed, non-base-paired guanosine residues. Through a comparison of the kethoxal incorporation characteristics of the two conformers, differences in base-pairing patterns as well as possible tertiary interactions were studied. Circular dichroism was employed as a physical probe in order to examine the effects of Mg$^{++}$ binding on tRNA$_{\text{Leu}}$ structure and to further investigate the denaturation process. In addition, this technique was used to study the physical basis for effects produced by kethoxal modification. Finally, during the latter stages of this work a naturally-occurring probe of the denaturation phenomenon was obtained with the isolation and sequence analysis of yeast tRNA$_{\text{CUA}}$. As mentioned previously, this tRNA species differs only slightly from tRNA$_{\text{Leu}}$ in sequence, yet lacks the ability to exist in a
stable denatured form. Before discussing the results obtained from each of these approaches, a brief look will be taken at the potential inherent in each of them for providing insight into the nature of tRNA structure.

Glyoxal, β-ethoxy-α-ketobutyraldehyde or kethoxal (registered trademark of the Upjohn Company, Kalamazoo, Michigan), and related 1,2-dicarbonyl compounds, were demonstrated in the late 1950's to exhibit antiviral activity (284) and carcinostatic properties (285). It was subsequently shown that glyoxal and kethoxal, like formaldehyde, caused the inactivation of tobacco mosaic virus through reaction with the viral nucleic acid (286). Unlike formaldehyde, however, the dicarbonyl compounds were found to be specific for guanosine residues forming the adducts shown in Figure 5. Characterization of the reaction products revealed a pronounced change in the UV absorption spectrum of guanylic acid on modification. As shown in Figure 6, the maximum increased slightly in height and was somewhat shifted toward shorter wavelengths. In addition, the shoulder around 270nm decreased in height and became more pronounced. The involvement of the imino group in position 1 of the guanine ring was indicated by the failure of the dicarbonyl derivatives to react with 1-methyl-guanosine. On the other hand, isocytosine (2-amino-4-hydroxy-pyrimidine) and 2-methylamino-6-hydroxy-purine did react, indicating that neither the keto group
Figure 5. Structures of glyoxal, kethoxal, and their respective guanosine adducts as determined by Shapiro et al. (290). The basis of the reagent's specificity for non-base-paired guanosine residues may be seen from the portions of the base structure which are involved in adduct formation.
Glyoxal:

\[
\begin{align*}
\text{O} & - \text{C} \\
\text{H} & - \text{C} \\
\text{H} &
\end{align*}
\]

Kethoxal:

\[
\begin{align*}
\text{H}_5\text{C}_2\text{O} & - \text{C} - \text{C} - \text{C} \\
\text{H} & - \text{O} - \text{O} \\
\text{CH}_3 &
\end{align*}
\]

Dicarbonyl adducts of guanosine:

\[
\begin{align*}
\text{OH} & - \text{N} - \text{H} \\
\text{HO} & - \text{N} - \text{H} \\
\text{R} & - \text{OH} \\
\text{R'} &
\end{align*}
\]

for glyoxal, \( R = \text{H} \)
for kethoxal, \( R = \text{CH}_3-\text{CH(OC}_2\text{H}_5) \)
\( R' = \beta-D\text{-ribofuranosyl} \)
Figure 6. UV absorbance spectra of guanylic acid before and after reaction with glyoxal and kethoxal (286). —— guanylic acid; — — glyoxal adduct of guanylic acid; ——— kethoxal adduct of guanylic acid.
nor the unsubstituted amino group were required for the reaction. The product, which decomposed readily in alkaline solution, was found to exhibit one pK between 1 and 2 and a second pK between 9 and 10.

The potential of kethoxal and related 1,2-dicarbonyl compounds as reagents for the chemical modification of nucleic acids was further explored by Shapiro and Hachmann in 1966 (287). It had been previously suggested that tRNA structural studies would be aided by chemical methods of isotopically labeling nucleotides not involved in secondary structure interactions (288). The portion of the guanine ring involved in adduct formation implied that 1,2-dicarbonyl compounds might provide such reagents, being specific for guanosine residues not involved in hydrogen-bonding. In addition, reagents which increased the specificity of nucleases were in demand, and the removal of the N1 hydrogen of the base in the formation of the glyoxal adduct had been shown to protect the dinucleoside phosphate, guanylyl-cytidine, from hydrolysis by ribonuclease T1 (289).

Further examination of the glyoxal and kethoxal reaction products found them to be stable for days at pH values below 6 but to become increasingly labile as the pH was increased. Studies based on nmr data and the cleavage reaction of the adducts with periodate confirmed the structure shown in Figure 5 (290). The formation
of this structure was rationalized by assuming the initial reaction of the more reactive carbonyl group (the aldehyde) with the more reactive site on the guanine ring (the imino group) (291). This reaction was then followed by a slower cyclization reaction between the ketone group and the less active functional group in guanine (the amino group). It was suggested that the difficulties arising from the lability of the adducts in neutral and alkaline solution could be circumvented by periodate oxidation to the resulting stable acyl-guanosine derivatives.

The first application of kethoxal as a tool in the study of tRNA structure was reported by Litt and Hancock in 1967 (292). In their preliminary study using un­fractionated yeast tRNA, evidence was presented that kethoxal could be used to identify guanosine residues in single-stranded regions of the tRNA molecule. A kinetic analysis of the kethoxalation reaction indicated that approximately 15-20% of the guanosine residues present reacted rapidly with the reagent, while the remaining guanosines were modified at a slower rate. The rapid initial phase was relatively insensitive to the presence of Mg++, but the divalent cation decreased the rate of reaction in the slower phase five-fold relative to the rate observed in EDTA. From this data it was concluded that the initial rate represented the reaction of available non-base-paired guanosine residues. The second
rate, on the other hand, was indicative of the modification of residues participating in secondary structure, accessible to the reagent only due to the "breathing" of the molecule or temporary opening of the double-stranded regions. The modification of essentially all guanosine residues resulted in a decrease in sedimentation constant as well as a decrease in hypochromicity, indicating the loss of a great deal of secondary structure as a result of the blocking of the guanosine hydrogen-bonding sites. Treatment with kethoxal, however, did not lead to degradation of the tRNA, and removal of the reagent under mild conditions could restore a large part of the original acceptor activity and hypochromicity. In addition, tRNA modified with $^{3}$H-labeled kethoxal could be subjected to digestion by pancreatic ribonuclease and the resulting oligonucleotides separated by paper electrophoresis without undue loss of the label. Thus a method was suggested for locating modified residues and thereby identifying single-stranded regions in a specific tRNA structure.

This method was applied to the study of yeast tRNA$^{\text{Phe}}$ by Litt in 1969 (166). In this investigation it was shown that the kethoxal adduct could be stabilized by the presence of borate at a pH as high as 8.0, presumably as a result of complex formation between borate and the vicinal hydroxyl groups of the adduct. This finding greatly enhanced the usefulness of the reagent for
labeling studies. In addition, as had been suggested earlier (289), it was demonstrated that kethoxal modification rendered guanosine residues resistant to attack by T1 ribonuclease. These observations were employed in the analysis of yeast tRNA\textsuperscript{Phe} after limited modification with \[^3\text{H}\]-labeled kethoxal. Kinetic studies demonstrated that in the presence of Mg\(^{++}\) approximately two moles of kethoxal were incorporated per mole of tRNA in the initial rapid phase of the reaction. Digestion with T1 ribonuclease and analysis of the labeled fragments revealed that the sites of modification were located at position 19 in the D loop and at position 33 in the anticodon. The remaining five guanosine residues located in loops of the cloverleaf model were thus indicated to be involved in tertiary interactions with other bases in the molecule. These results provided strong support for the "folded cloverleaf" model of tRNA tertiary structure which had been proposed by Cramer et al. (293). According to this model, the D loop and the T\textsuperscript{Ψ}C loop and stem were folded together to form a compact structure, leaving only guanosine residues 19 and 33 in tRNA\textsuperscript{Phe} free of involvement in tertiary base-pairing interactions. The conclusions are also consistent with the recent X-ray data of Kim et al. (109), with the exception that an additional guanosine at position 44 in the variable region appeared to be free of specific tertiary interactions in the crystal structure. This
residue, however, could quite possibly be buried in the molecule due to other interactions, thus making it unavailable to the modifying reagent.

Since modification of the guanosines at positions 19 and 33 resulted in the loss of approximately 50% of the phenylalanine acceptor activity, an attempt was made to identify one of these sites as the target for inactivation (167). Analysis of kethoxal incorporation after subsequent aminoacylation, however, revealed that both positions were modified in both charged and uncharged tRNA molecules. It was thus proposed that kethoxal could react with the target G to form several or all of the four possible stereoisomers, only some (or one) of which would result in loss of acceptor activity. The observation was also made that modification with glyoxal to an equal extent resulted in the retention of at least 90% of the original acceptor activity. Therefore, the bulkiness of the kethoxal group appeared to play a role in the inactivation process which could not be fulfilled by the smaller, more symmetrical nature of the glyoxal molecule. Inactivation by kethoxal was thus concluded to result from steric hindrance by an appropriately oriented kethoxal group which prevented the synthetase from attaining sufficiently close contact with the tRNA.

Further studies designed to elucidate the site responsible for kethoxal inactivation were carried out
based on the ability of yeast phenylalanine-tRNA synthetase to aminoacylate \textit{E. coli} tRNA$^{\text{Phe}}$ and tRNA$^{\text{Val}}_1$ as well as yeast tRNA$^{\text{Phe}}$ (168). In the case of the homologous charging reaction, the linearity of the degree of inactivation with respect to the degree of kethoxal incorporation, even at low extents of modification, was interpreted as evidence in favor of a single-hit mechanism for inactivation. In light of this finding, the stoichiometry of the inactivation process provided further evidence implicating the importance of the stereoisomerism of the kethoxal adduct. Only 35% inactivation was observed when one mole of kethoxal had been incorporated per mole of tRNA, thus implying that only 35% of the kethoxal molecules had formed adducts oriented properly to destroy acceptor activity. The existence of a unique site in yeast tRNA$^{\text{Phe}}$ which served as the single-hit inactivation target, however, appeared doubtful. \textit{E. coli} tRNA$^{\text{Phe}}$ had been shown to possess a G at position 33 but not at position 19, while the opposite was found to be true for \textit{E. coli} tRNA$^{\text{Val}}_1$. Both tRNAs, however, were inactivated by kethoxal in the heterologous charging reaction due to single modifications at position 33 and position 19 respectively. The sensitivity to modification at position 19 supported the D-stem recognition site proposed by Dudock and colleagues (110, 111). Since the G at position 19 was located near this region, a bulky
modification could easily be visualized to inhibit enzyme binding. On the other hand, the region surrounding position 33 was presumably not directly related to specific synthetase recognition due to sequence differences occurring in this region in *E. coli* tRNA$_{Val}$. As observed in the case of *E. coli* tRNA$_{Phe}$, however, the introduction of a bulky group at that position either interfered sterically with a nonspecific, yet essential, interaction between the tRNA and the synthetase or in some way perturbed the tertiary structure of the molecule so as to result in inactivation. The observation that kethoxal modification at position 33 did not necessarily lead to the inactivation of yeast tRNA$_{Phe}$ but always appeared to inhibit *E. coli* tRNA$_{Phe}$ acceptor activity was attributed to a possible difference in the anticodon structures of the two molecules.

The technique involving modification of exposed, non-base-paired guanosine residues using kethoxal or glyoxal has been extended to the study of other nucleic acid entities as well. Recent reports, for example, have described the use of such reagents in the study of the structures of native and denatured *E. coli* 5S RNA (294) and in the examination of the topography of 16S RNA in intact 30S ribosomal subunits (295). The applicability of kethoxal modification to studies of tRNA bound to its cognate synthetase has also been suggested (168). This
technique would be particularly useful in the further investigation of the yeast tRNA\textsuperscript{Phe}-synthetase complex. Protection of the anticodon region from cleavage by Tl ribonuclease would not be detectable due to the presence of 2'-0'-methyl guanosine, a residue which has been demonstrated to be resistant to Tl digestion even in the free tRNA. Kethoxal, however, has been shown to modify this residue and could therefore be used to study interactions in this region between the bound tRNA and its cognate synthetase.

The potential for the use of kethoxal as a probe of denaturable tRNA\textsubscript{Leu}\textsubscript{3} structure is apparent. Since base-pairing changes have been postulated to occur in the transition from the native to the denatured conformer, differences in kethoxalation patterns should reveal the identity of newly formed single-stranded regions. In addition, alterations in tertiary interactions may be observed through the identification of residues which become more available for modification in the less compact denatured form. During the final stages of this work, a study of E. coli denaturable tRNA\textsubscript{Trp} by reaction with kethoxal was reported (169). Kethoxal was not incorporated into the native form of this tRNA, but was found to modify guanosine residues in the 3' side of the anticodon stem region, the D stem, and the D loop of the denatured conformer. It was concluded from these studies that the
denaturation of tRNA^Trp was accompanied by changes in hydrogen bonding in the anticodon and D stems. Similar changes have been noted in the denatured forms of yeast tRNA^Ser and tRNA^Phe by partial digestion with T1 and T2 ribonucleases (256, 260). The correlation between these results and the observations made in the kethoxalation study of tRNA^Leu will be examined further in Chapter 4.

The effects of kethoxal modification and of the presence of Mg^{++} on the interconversion of native and denatured tRNA^Leu were assessed using the physical probe, circular dichroism. This technique had been shown to differentiate between the two conformers (see Section 1.5) and thus could be used to demonstrate the nature of the form present under prescribed conditions. Furthermore CD spectra have been shown to depend greatly on the secondary and tertiary structure of tRNA molecules. The major positive band around 260-265nm and the negative bands near 236nm and 210nm have been ascribed to the exciton splitting of the π-π* transitions resulting from base stacking (296). Thermal denaturation has been found to result in a red shift of the 260nm band and a blue shift of the band at 210nm, presumably resulting from the separation of base pairs and base unstacking (297). In addition, an increase in the magnitude of the 260nm maximum has been observed to accompany the blue shift of this band when the number of base pairs present is increased (298). It has
been further suggested by Bush and Scheraga that a change in the ratio of the magnitudes of the 260nm maximum to the 210 nm minimum results from a change in the tilt of the bases with respect to the helical axis (299). The negative CD band of low intensity occurring near 295-310 nm has been postulated to result from an n-π* transition (300). The presence of such a transition has been observed in adenosine and uridine derivatives (301, 302), although the band is often visible only in organic solvents. A blue shift of about 15nm which occurs in aqueous solution apparently causes the transition to be masked by the stronger band near 260nm. No evidence for this negative band near 300nm has been found in synthetic dinucleotides or polynucleotides (303), although such a minimum has been observed in several naturally occurring native RNAs (206, 298, 304). This weak transition was observed in the spectra of six of the nine tRNA species studied by Blum et al. (206), its presence presumably being obscured by the major band in the remaining three tRNAs. These and other experimental results have indicated that this band is particularly sensitive to tertiary structure (205). Early studies using unfractionated yeast tRNA demonstrated that the intensity at 295nm disappeared near 50° when the 260nm maximum indicative of base pairing and base stacking had only dropped by 25%. In addition, the spectrum around 295nm actually became positive above 60°, further
indication of a rapid reduction in the negative band as compared to the positive one. The calculations of Blum et al., which did not take into account any tertiary structure, consistently underestimated the value of the 295nm minimum by at least 50% (206). On the other hand, they came close to predicting the observed 260nm bands which apparently result primarily from secondary structural interactions. Furthermore, it has been observed that a large amount of secondary structure is present at low ionic strength in the absence of divalent cations (206, 298) and that the addition of Mg$^{++}$ causes little change in the 260nm region (204). On the other hand, the addition of Mg$^{++}$ was noted to result in the appearance of, or a large increase in, the magnitude of the band near 295nm. This increase has thus been attributed to the formation of the compact tertiary structure induced by Mg$^{++}$ binding. Presumably the band becomes more evident as a result of a red shift in position as residues with an n-π* transition are placed into a hydrophobic, non-hydrogen-bonded environment by the folding of the tRNA molecule. Recently, a fifth Gaussian band has been observed at 185nm, and ORD measurements have implied the existence of a CD band at 165nm (205). Denaturation at elevated temperatures resulted in a 5-10nm red shift and a decrease in magnitude of the 185nm band, as well as the complete disappearance of the 165nm band. Both of these bands were therefore
assumed to be associated with base stacking, presumably in the double-stranded base-paired regions of the molecule. Thus circular dichroism exhibits promise as an effective means of probing the secondary and tertiary structural changes occurring during the interconversion of the two tRNA^{Leu}_3 conformers.

In addition to clarifying the effects of kethoxal modification on the renaturation process, CD measurements were also used to investigate the effects of low ionic strength and the presence or absence of Mg^{++} on the conformation of tRNA^{Leu}_3. Numerous studies using a wide variety of techniques have indicated that Mg^{++} binding is site-specific with maximum structural stabilization occurring well below the concentration required for complete charge neutralization (184, 202, 305, 306). In general four to six divalent ions have been found to bind specific "strong" binding sites with dissociation constants of approximately $10^{-5}-10^{-6} M$, while a larger number of ions (15-25) bind more weakly ($K_d=10^{-3}-10^{-4} M$). The four to six strongly binding ions appear to bind in a cooperative manner (202, 305). Data recently reported by Lynch and Schimmel using a fluorescent probe (200) have shown that this cooperative binding follows a sequential mechanism in which the binding of the first ligand induces or facilitates a structural change, which in turn enables the binding of a second ligand, followed
by another structural change, etc. Their results are analogous to the model proposed by Koshland for the mechanism of cooperative binding of ligands to proteins (307) and do not fit the concerted model of Monod, Wyman, and Changeux (308). Fluorescence (203), CD (204), thermal relaxation kinetics (188), chain cleavage (202) and photochemical cross-linking (157) studies among others have indicated that one of these specific binding sites is located in the region of the uridine occurring at position 8. It is interesting to note that the recent X-ray work of Kim et al. (109) has shown that the purine in position 9 of tRNA molecules often participates in tertiary interactions with the base pair involving residues 12 and 22 in the D stem. Electronegative atoms in these residues form an arrangement which could easily bind a metal ion.

It is possible that the bringing together of chain segments represents an unfavorable process unless cations are able to bind strongly to stabilize the junction. While high Na⁺ concentrations have been found to mimic many of the effects brought about by Mg²⁺, the structural stabilization is apparently less strong. In the presence of Na⁺ the tertiary structure melts first, followed by loss of secondary structure in a multiphasic melting curve (188). Mg²⁺ on the other hand stabilizes the tertiary structure above the Tₘ of some, if not all, of the cloverleaf helices, thus resulting in a sharp melting transition. In
low salt solutions in the absence of Mg$^{++}$, structures have been postulated to exist which possess some "wrong" hydrogen-bonding interactions (188, 200). Cole et al. have envisioned such structures to be extended forms of the molecule in which the tRNA is folded like a hairpin with the anticodon and acceptor stems intact and the regions between them base pairing as much as possible. Presumably such structures would be favored in low salt concentrations since they tend to minimize the phosphate charge repulsion. The breaking of such small aberrant helical segments and the subsequent rearrangement of the molecule to the proper conformation could account for the observed rates and activation energies involved in the renaturation of tRNA molecules on addition of Mg$^{++}$ or high salt (188, 200). The implications of these results with respect to the structural states of tRNA$^{\text{Leu}}_3$ will be examined through the use of circular dichroism as a conformational probe.

The fact that tRNA$^{\text{Leu}}_3$ possesses stable native and denatured conformations comprises in itself a natural probe for the study of tRNA structure. Identical in primary sequence, the active and inactive forms differ only in secondary and tertiary structural interactions. Thus they provide an excellent opportunity for assessing the nature of the structural elements required for biological activity (see Section 1.5). An additional probe, however,
has recently been found in the non-denaturable yeast tRNA species $tRNA^{\text{Leu}}_{\text{CUA}}$ (138). This tRNA molecule has lost the ability to form a stable denatured conformer through the alteration of a small percentage of its nucleotide residues. The nature of these changes, their effect on both the CD spectrum and the renaturation characteristics of the molecule, and their implications concerning possible cellular control mechanisms will be discussed in Chapter 4.

The present studies were thus designed to utilize the above approaches in order to clarify further the nature of the structural differences between the native and denatured forms of yeast $tRNA^{\text{Leu}}_3$. The information obtained regarding the structure-function relationships of this molecule will be presented in the following chapters.
2. MATERIALS AND METHODS

**Purification of yeast tRNA\textsubscript{Leu} \textsuperscript{3}**. Crude tRNA was prepared from stationary phase baker's yeast (Fleischmann) by phenol extraction (309, 310). The aqueous phase was concentrated by ethanol precipitation and further subjected to DEAE-cellulose chromatography to remove low molecular weight contaminants. Purified tRNA\textsubscript{Leu} \textsuperscript{3} was obtained in large quantity from this crude preparation as previously described (237, 311). The crude tRNA was fractionated by chromatography on two successive columns of benzoylated DEAE-cellulose (312), first in the presence of 10 mM MgCl\textsubscript{2} and second in the absence of MgCl\textsubscript{2}. The combined tRNA\textsubscript{Leu} \textsuperscript{3} fractions were then subjected to conditions which led to the selective conversion of the tRNA\textsubscript{Leu} \textsuperscript{3} species to a stable denatured conformation (54). Final purification was achieved by Sephadex G-100 column chromatography which separated this less compact denatured form of tRNA\textsubscript{Leu} \textsuperscript{3} from the more compact structures of contaminating native tRNAs.

**Isolation of crude yeast aminoacyl-tRNA synthetase**. The enzyme preparation was carried out according to a modification of the technique reported by Hoskinson and Khorana (313). Stationary phase baker's yeast cells were disrupted by blending with glass beads at 0-10°. After an initial centrifugation at 16,000 x g for 30 minutes in a Sorvall RC2-B centrifuge to remove cell debris, the clarified supernatant was further subjected to ultracentrifugation.
at 29,000 rpm for 2 hours in a Spinco Model L centrifuge. The enzyme supernatant was subsequently concentrated by ammonium sulfate precipitation, and the 30-75% ammonium sulfate fraction subjected to DEAE-cellulose (Whatman DE-32) chromatography. The combined synthetase fractions were stable for several months at -20° in the presence of 50% glycerol.

**Assay for leucine acceptor activity.** The tRNA samples were assayed for leucine acceptor activity by the filter paper disc method of Nishimura and Novelli (314). Assay solutions contained 0.1 M potassium cacodylate, pH 7.5, 10 mM MgCl₂, 10 mM ATP, 1 A₂₆₀ unit of carrier tRNA (crude yeast tRNA lacking leucine acceptor activity), 0.3 μCi [¹⁴C]-leucine (New England Nuclear, 312 μCi/m mole), 10 μl of the crude yeast synthetase preparation in 50% glycerol, and 0.01-0.03 A₂₆₀ unit of tRNA₃ Leu in a total volume of 0.2 ml. Incubation was carried out for 10 minutes at 37° for renatured samples and at 25° when comparisons of renatured and denatured tRNA₃ Leu activities were desired. The reaction was terminated by transferring 0.1 ml of the assay solution to a 1.5 cm square of Whatman 3MM paper which was subsequently placed in cold 10% trichloroacetic acid (TCA). After three TCA washes to remove unbound [¹⁴C]-leucine from the precipitated [¹⁴C]-aminoacyl-tRNA, the papers were washed once with ethanol-ether (1:1), and finally with anhydrous ether. After air drying, they
were counted for radioactivity in a toluene-based scintillation fluor containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2(4-methyl-5-phenyloxazolyl)benzene (POPOP), using a Beckman LS-255 scintillation counter.

**Preparation of tRNA for kethoxal modification.** Purified tRNA\textsubscript{Leu}\textsuperscript{3} was dialyzed at 4° against three changes of glass distilled, deionized water, each at least 25 times the volume of the sample and of at least four hours duration. Samples to be modified in the native form were renatured by heating at 60° for 5 minutes in the presence of 10 mM potassium cacodylate, pH 7.0, 10 mM MgCl\textsubscript{2}, and 2 mM EDTA, followed by slow-cooling at room temperature. Denatured samples were similarly heated in 10 mM potassium cacodylate, pH 7.0 and 2 mM EDTA (in the absence of MgCl\textsubscript{2}) and quick-cooled in an ice-salt bath at approximately -4°.

**Modification of tRNA with kethoxal.** The modification reaction was performed under conditions similar to those described by Litt (167). The reaction solution contained 0.1 M potassium cacodylate, pH 7.0, 1 mM EDTA, 12-15 mM kethoxal, and 15-30 A\textsubscript{260}/ml tRNA\textsubscript{Leu}\textsuperscript{3}. For native samples the solution also included 10 mM MgCl\textsubscript{2}, while samples in the denatured form were treated both in the presence and absence of 10 mM MgCl\textsubscript{2}. \(^{3}\text{H}\)-kethoxal (8 mC\textsubscript{i}/mmole) was purchased from Schwarz/Mann and non-radioactive kethoxal was a gift from Dr. G. E. Underwood of the Upjohn Company.
To monitor the course of the kethoxalation reaction, aliquots were removed at time intervals and the tRNA precipitated by addition of 0.1 volume of 2M potassium acetate, 2 volumes of cold absolute ethanol, and 25-50 A\textsubscript{260}/ml carrier tRNA (minus leucine acceptor activity). For maximum tRNA recovery, the ethanol precipitate was allowed to equilibrate at least 30 minutes in ice prior to centrifugation and removal of the supernatant. Samples were then washed twice by dissolving in 0.05 M borate, pH 7.5, and precipitating with two volumes of cold absolute ethanol. After a final wash with anhydrous ether the precipitates were dried under vacuum and stored at -20°. Kethoxal adducts appeared to remain intact in samples stored as long as six weeks after such treatment.

The extent of [\textsuperscript{3}H]-kethoxal incorporation was analyzed by dissolving the tRNA precipitates in water and counting aliquots of the solutions in Aquasol (New England Nuclear). Remaining portions of the time samples were subjected to renaturing conditions and assayed for leucine acceptor activity as described above. Less than 2% carry-over of the [\textsuperscript{3}H]-kethoxal counts into the [\textsuperscript{14}C] channel was observed, which was considered to be negligible in the activity determinations.

**Identification of sites of kethoxal incorporation.** In order to determine the location of the rapidly modified, exposed guanosine residues in the native and denatured conformations, the [\textsuperscript{3}H]-kethoxalation reaction
for each conformer was carried out in the presence of MgCl₂ and terminated after 2.5 hours. The [³H]-labeled tRNA Leu, precipitated and washed as described above, was then digested with 20 units of T1 RNase (Sankyo) per A₂₆₀ unit of tRNA for six hours at 37° in 0.05 M borate, pH 7.5. The resulting digests were chromatographed in DEAE-cellulose (Whatman DE-23) columns in the presence of 20 mM Tris-20 mM borate, pH 7.5 and 7 M urea. Aliquots of the column fractions were counted in Aquasol to determine [³H]-kethoxal content, and fractions under each radioactive peak were combined. These labeled fragments were desalted by passage through a Biogel P-2 (Bio-Rad Laboratories) column in the presence of 2 mM Tris-2 mM borate, pH 7.9 (315) and further purified by rechromatography in a DEAE-cellulose column at pH 3.1 in 20 mM borate and 7 M urea (316). The radioactive fragments were again desalted by Biogel P-2 gel filtration in dilute NH₄OH, pH 8.0 prior to removal of the kethoxal groups by overnight incubation in 0.1 M Tris-chloride, pH 7.6 at 37° (292). A third desalting Biogel P-2 column was required for removal of the Tris-Cl prior to base composition analysis. All columns utilized in the isolation of the modified fragments were monitored by a Laboratory Data Control U.V. Monitor at 254 nm.

The periodate -[³H]-borohydride method of base composition analysis developed by Randerath, Yu, and Randerath (317) was used to identify the oligonucleotides.
An outline of this procedure is shown in Figure 7. Each oligonucleotide was first converted to nucleosides by the combined actions of pancreatic ribonuclease, snake venom phosphodiesterase, and alkaline phosphatase. The nucleosides were then treated with periodate to form the dialdehydes which were in turn reduced with $[^3H]$-potassium borohydride to produce the $[^3H]$-labeled trialcohols. The trialcohol mixture was subsequently subjected to two-dimensional thin layer chromatography, and the compounds identified by fluorography. The radioactive regions of the thin layer sheets were eluted and counted in order to obtain quantitative base composition data. A comparison of the base compositions of the labeled oligonucleotides with the known sequence of tRNA$^{\text{Leu}}_3$ permitted identification of the sites of rapid kethoxalation in the native and denatured conformers.

**Preparation of tRNA for circular dichroism (CD) studies.**

Modification of tRNA$^{\text{Leu}}_3$ using non-radioactive kethoxal was carried out according to the procedure described above. Samples modified in the denatured conformation were treated in the absence of MgCl$_2$. The extent of modification was monitored by assaying time samples for the percent leucine acceptor activity remaining after modification and subsequent exposure to renaturing conditions. In order to modify exposed guanosines only, the reaction was terminated for both native and denatured samples after
Figure 7. Outline of the periodate-[³H]-borohydride method of base composition analysis developed by Randerath, Yu, and Randerath (317).
Oligonucleotide

\[ \text{Pancreatic RNase} \]
\[ \text{Snake venom phosphodiesterase} \]
\[ \text{Alkaline phosphatase} \]

Nucleosides

\[ \text{NaIO}_4 \]

Nucleoside

Dialdehydes

\[ \left[ ^3\text{H} \right] - \text{KBH}_4 \]

\[ \left[ ^3\text{H} \right] - \text{labeled} \]

Trialcohols

\[ \text{Thin layer chromatography} \]
\[ \text{and Fluorography} \]

\[ \text{HOCH}_2\text{O} \]

\[ \text{Base} \]

\[ \text{HOCH}_2\text{O} \]

\[ \text{Base} \]
the modified denatured conformer had lost 70-75% of its renaturable acceptor activity. This extent of modification and concomitant loss of activity corresponded to that used in the previous investigation of the sites of rapid kethoxal incorporation. Native and denatured tRNA species modified in this fashion will be designated N_s and D_s respectively in further discussions. (A summary of all such abbreviations which will be used throughout the text to designate various forms of tRNA_{Leu}^3 is given in the List of Abbreviations.) For more extensive modification, the kethoxalation reaction was allowed to proceed until all renaturable activity was lost by the denatured conformer (N_L and D_L). Since little activity was lost by the modified native conformer during either time period, only the sample reacted for the longer period of time (N_L) was used in the CD studies.

Two dialysis procedures were employed in the removal of Mg^{++} from tRNA_{Leu}^3 samples prior to CD analysis in the denatured conformation. The first procedure consisted of dialysis against at least three changes of double-distilled water as described above. The second, more extensive dialysis procedure was similar to that used in the CD studies reported by Tinoco and coworkers (206). Samples were dialyzed at 4° against two changes at six-hour intervals of each of the following buffers: (i) 0.5 M NaCl, 10 mM EDTA, 10 mM potassium cacodylate, pH 7.0; (ii) 0.2 M
NaCl, 1 mM EDTA, 10 mM potassium cacodylate, pH 7.0; (iii) 1 mM EDTA, 10 mM potassium cacodylate, pH 7.0; and (iv) 0.01 mM EDTA. Each buffer contained an additional 10 mM borate for the dialysis of kethoxalated tRNA samples. The volume of each change was at least 100 times greater than the volume of the sample.

**Synthesis of poly (U₂,G).** The copolymer was synthesized according to the method of R. M. Bock (318). Four mg/ml UDP (P-L Biochemicals) and 2 mg/ml GDP (P-L Biochemicals) were incubated at 30° in the presence of 0.1 M Tris-Cl, pH 9.0, 2.5 mM MgCl₂, 0.5 mM EDTA, 1 M urea, and 21 mg of polynucleotide phosphorylase (P-L Biochemicals) in a total volume of 4.0 ml. The reaction was carried out in a capillary viscometer, and the extent of polymerization was monitored by measuring the increase in viscosity of the solution with time. After reaching maximum viscosity, the reaction was terminated by shaking with an equal volume of water-saturated phenol. The aqueous layer was washed twice more with phenol and finally with ether before the polymer was precipitated by addition of 0.2 M potassium acetate and two volumes of absolute ethanol. Final separation of the poly (U₂,G) product from reaction components was accomplished using Sephadex G-100 column chromatography.

**Modification of poly (U₂,G) and 2'-3' GMP with kethoxal.** Samples of the synthetic copolymer, as well as
of the mononucleotide 2'-3' GMP (P-L Biochemicals), were kethoxalated according to the extensive modification conditions described above. The reaction with poly \((U_2,G)\) was terminated by ethanol precipitation as for the tRNA samples, while the 2'-3' GMP reaction was ended by separation of the reaction components on a Biogel P-2 column. The UV spectra of nonmodified and kethoxalated samples of both the nucleotide and the copolymer were measured at room temperature using a Cary 14 recording spectrophotometer.

**Measurement of CD spectra.** The \(A_{260}\) of solutions used in the CD studies was between 0.5 and 2.0. For \(tRNA_{3}^{Leu}\) the extinction coefficient per mole of residue at 260 nm was taken to be 7400, as reported by Blum et al. (206). The molar extinction coefficients used for nonmodified and kethoxalated 2'-3' GMP were 11,700 (206) and 11,000 (286) respectively, and those for nonmodified and kethoxalated poly \((U_2,G)\) were 9900 (319) and 9600. These values were derived from reported extinction coefficients at different wavelengths and extrapolated to 260 nm using the observed UV spectra. The \(\varepsilon_{260}\) of kethoxalated poly \((U_2,G)\) was calculated from the values for its components using the approximate 5% hypochromicity observed for the nonmodified copolymer. For comparison with CD melting data, the change in UV absorption with temperature of native and denatured \(tRNA_{3}^{Leu}\) samples was monitored at 260 nm using a
Gilford Model 200 spectrophotometer. A thermocouple inserted into the blank solution was used to determine cell temperature.

CD spectra were measured using a Jasco Model J-20 spectropolarimeter. Cell temperatures between 16° and 85° were controlled using a calibrated Brinkman Lauda K-2/RD water bath. During the thermal transition studies, the cell was allowed to equilibrate at least fifteen minutes at each temperature prior to beginning the scan. Spectra of "native" tRNA samples were measured at 25° in solutions of 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA, and 10 mM MgCl₂. "Denatured" tRNA spectra were similarly obtained in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, and 1 mM EDTA plus or minus 10 mM MgCl₂ as indicated. Baseline spectra of the solvent in the same cell were obtained before and after each set of CD spectra, permitting correction for baseline drift during the melting curve determinations.

The mean residue ellipticity, [θ], for each sample was calculated from observed data using the following equation:

\[ [\theta] = \frac{F(E-B)S\left(\epsilon_{260}\right)}{L\left(A_{260}\right)} \]

where \( F \) = a calibration factor for the instrument based on a \( \Delta\epsilon \) value of 2.2 liter/cm-mole for camphorsulfonic
acid (320); E = the height in cm of the experimental curve at any given wavelength; B = the location of the baseline at the same wavelength; S = the scale of the instrument in millidegrees/cm used in recording the spectrum; \( \varepsilon_{260} \) = the extinction coefficient of the sample per mole of residue at 260 nm and 25°; L = the light path length in mm; and \( A_{260} \) = the absorbance of the sample at 260 nm measured at 25° in a 1 cm light path. For tRNA samples the \( A_{260} \) values were expressed in terms of the native conformation. The conversion of denatured tRNA readings to comparable native \( A_{260} \) values was based on the observed 3.8% hyperchromicity of the \( \text{D}_{\text{Mg}^{++}} \) conformer (see List of Abbreviations) and 6.8% hyperchromicity of the \( \text{D}_{\text{EDTA}} \) conformer with respect to the native configuration. Values for \( \Delta \varepsilon \), the difference in the molar extinction coefficients of the sample for left- and right-handed circularly polarized light, were calculated from the mean residue ellipticity values according to the following equation:

\[
\Delta \varepsilon = \varepsilon_L - \varepsilon_R = \frac{[\theta]}{3300} .
\]

All CD spectra presented are expressed in terms of these calculated \( \Delta \varepsilon \) values.
3. RESULTS

3.1 Kethoxalation Studies

A comparison of the number of exposed, non-base-paired guanosine residues in the native and denatured forms of tRNA$^\text{Leu}$ was obtained by modification of the two conformers with kethoxal for varying lengths of time as described in Chapter 2. The observed kinetics of kethoxal incorporation into each conformer are illustrated in Figure 8A. The denatured structure, both in the presence and absence of Mg$^{++}$, was observed to incorporate kethoxal at an initial rate approximately four times faster than the native conformer. Attempts to linearize the curves, however, in order to obtain a discrete number of pseudo-first-order rate constants for the modification reaction (kethoxal present in large excess), were unsuccessful. The multiplicity of rate constants indicated by this analysis would be expected considering the integral involvement of the guanosine residues in the complex structure of the polynucleotide molecule. Reactivities of individual residues could be influenced not only by interactions with neighboring nucleotides, but also by secondary and tertiary structural features of the molecule which could lead to interactions with distant residues in the chain. Variations in the nature and strength of such higher order
Figure 8. Kinetics of kethoxalation and inactivation of yeast tRNA$^{\text{Leu}}_3$. Leucine tRNA (27 $A_{260}$ units) in native (○—○) or denatured conformation in the presence (□——□) or absence (Δ——Δ) of Mg$^{++}$ was treated with 19 μmoles of [$^3$H]-kethoxal under the conditions described in Chapter 2. Samples were taken at time intervals. The RNA was precipitated and washed with ethanol, redissolved in water, and counted for [$^3$H]-kethoxal incorporation (Figure 8A) or measured for leucine acceptor activity after the RNA solution was incubated at 60° in the presence of 10 mM potassium cacodylate, pH 7.0, 10 mM MgCl$_2$ (Figure 8B).
% Leucine Acceptor Activity

μ mole Kethoxal /μ mole t RNA

T (hours)

0 10 20 30 40 50 60 70 80 90

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
structure could further influence the rate at which individual guanosine residues became exposed to the modifying reagent during the course of the reaction. Nonetheless, the existence of at least two distinct classes of reaction rates is indicated by the biphasic nature of the incorporation curves. The initial, steeper slopes represent the reaction of ketoxal with exposed, non-base-paired guanosine residues, while the second region of the curves may be attributed to the reaction taking place at less readily available residues. It is interesting to note that similar initial reaction rates were observed for ketoxal incorporation into the denatured conformer in both the presence and absence of Mg\(^{++}\). The rate of incorporation during the second phase of the reaction, however, was slightly greater when Mg\(^{++}\) was absent. This phenomenon indicates the existence of higher order structural features in the denatured conformer which are stabilized by the addition of Mg\(^{++}\). Further evidence regarding this stabilization was obtained from CD analysis which will be reported in Section 3.2.

On the whole, however, the ketoxalation reaction indicated a much greater stability for the native structure than for the denatured conformer. After twenty-four hours, when essentially all twenty-one possible guanosine residues were modified in the denatured conformer in the absence of Mg\(^{++}\) (eighteen in the presence of Mg\(^{++}\)), only
an average of five residues had been modified in the native form under the same conditions. Thus residues initially concealed within the secondary and tertiary structure of the molecule were much more readily exposed in the denatured conformation.

The percent leucine acceptor activity remaining in the kethoxalated samples after exposure to renaturing conditions is shown in Figure 8B. The ability of the native conformer to accept \(^{14}\text{C}\)-leucine appeared to be affected only slightly by kethoxalation. On the other hand, tRNA modified in the denatured conformation exhibited a drastic reduction in activity which coincided with increased kethoxal incorporation. After twenty-four hours, essentially all renaturable acceptor activity had been lost by the denatured form as compared to only 50% loss of activity by the native structure. This 50% loss, coinciding with the incorporation of approximately five \(\mu\)moles of kethoxal per \(\mu\)mole tRNA was comparable to that observed in the denatured structure after approximately one hour of reaction time. It was of interest to determine whether the initial inactivation of the denatured conformer was the result of the modification of residues essential in the charging reaction (i.e. directly involved in the interaction with the cognate synthetase) or of residues required for base pairing in the process of refolding to the native, active conformation. CD analysis
of the modified tRNA species provided one approach to this problem, and the results are described in Section 3.2.

In order to study the differences in location of exposed, rapidly-reacting guanosine residues in the native and denatured conformers, it was necessary to maximize the modification of exposed residues, while minimizing the reaction with less accessible guanosines. This goal was accomplished by terminating the kethoxalation reaction at the "break-point" in the incorporation curve (or the point of intersection of the initial and secondary slopes), which was found to occur after 2.5 hours of reaction time under the conditions described in Chapter 2. In addition, the 2.5 hour time point marked the divergence of the incorporation curves for the denatured conformer in the presence and absence of Mg++. This occurrence provided further indication of the onset of a secondary set of reaction rates due to changes in the availability of the guanosine residues being modified. At the time of reaction termination, approximately 9 μmoles of kethoxal had been incorporated per μmole tRNA in the denatured state with a concomitant loss of approximately 70% of the leucine acceptor activity present after renaturation. Approximately 2 μmoles of kethoxal per μmole tRNA had been incorporated by the native conformer, accompanied by a loss of less than 10% of its acceptor activity.
The identification of these sites of rapid $^{3}\text{H}$-kethoxal incorporation was achieved by TL RNase digestion of the labeled $\text{tRNA}_{3}^{\text{Leu}}$ conformers. The DEAE-cellulose chromatographic patterns of the digests are shown in Figure 9. Two radioactive peaks occurred in the native column while seven were observed in the denatured $\text{tRNA}_{3}^{\text{Leu}}$ digest, numbers 1 and 6 corresponding to the location of the label in the native tRNA. Quantitative determination of the extent of modification occurring in each labeled fragment could not be accurately obtained due to the loss of kethoxal at varying rates from the fragments during the lengthy process of their purification. Such calculations would have been further complicated by the fact that kethoxalated fragments often chromatograph differently than their non-modified counterparts due to borate complexation by the kethoxal-guanosine adduct and the accompanying addition of a negative charge (166). Thus quantitative yield data would be required not only for all fragments containing a given modified guanosine residue, but also for the corresponding non modified fragment as well. A significantly larger initial amount of tRNA would be required to permit such determinations. It is apparent from a qualitative viewpoint, however, that the overall extent of kethoxal incorporation into the native conformer (as indicated by the height of the radioactive peaks) is much lower than that observed in the denatured structure. In
Figure 9. Chromatography of T1 RNase digests of $^{3}H$-kethoxalated tRNA$^{\text{Leu}}_{3}$. Leucine tRNA (15 A$_{260}$ units) in native (Figure 9 A) or denatured (Figure 9 B) conformation was treated with 6.5 µmoles of $^{3}H$-kethoxal for 2.5 hours under the conditions described in Chapter 2. The samples were digested with 300 units of T1 RNase (Sankyo) at 37° in 0.05 M borate, pH 7.5 for 6 hours. Each of the digests was chromatographed in a column of DEAE-cellulose (0.9 x 65 cm) equilibrated with 7 M urea, 20 mM Tris-borate, pH 7.5. The columns were eluted with 800 ml of the same buffer containing a linear gradient of NaCl from zero to 0.5 M. Absorbance at 254 nm (---) was monitored (range = 0.32 A$_{254}$), and radioactivity (---) was measured as described in Chapter 2.
addition, the incorporation into radioactive peaks 2, 3, and 5 of the denatured tRNA digest appears to be somewhat lower than that of peaks 4, 6, and 7. Clearly the majority of the radioactivity is found in the large fragment(s) occurring in peak 7.

After purification and dekethoxalation, the oligonucleotides were subjected to base composition analysis as described in Chapter 2 and outlined in Figure 7 (317). The results of this analysis are shown in Table I. From these data and the known sequence of tRNA\textsubscript{Leu}\textsuperscript{3}, the modified fragments were identified.

The only nucleotide possibly chromatographing at the position of peak 1 could have been kethoxalated-Gp (*Gp), retarded in the column due to borate complexation. Several factors, however, tended to strongly discount this possibility. First, formation of free *Gp would require the presence of two adjacent guanosine residues and modification of at least the guanosine in the 3' position. This situation is found to occur in several locations in the denatured conformer, and in all cases both adjacent guanosine residues appear to be modified. Cleavage after either one of the kethoxalated residues, however, would be hindered due to the inhibition of Tl RNase by kethoxal modification. Consequently, the concurrent cleavage at both positions required for the release of free *Gp would be highly unlikely, and the amount of radioactivity in
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TABLE I
Identification of Kethoxalated Fragments from Denatured tRNA$^{Leu}_3$ Digestion
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peak 1 would be expected to be much less than that observed in the denatured tRNA digest. The most convincing argument, however, lies in the fact that peak 1 is observed in the native as well as the denatured tRNA digest. In the native conformer, two adjacent guanosine residues do indeed appear to be modified (see analysis of peak 6). The guanosine in the 5' position, however, is methylated at the 2' position of the ribose and is therefore resistant to Tl RNase. Thus no $^*Gp$ could be released. Due to these considerations peak 1 was identified as $[^3H]$-kethoxal, removed from the tRNA during the six hour incubation with Tl RNase at $37^\circ$ and pH 7.5.

Radioactive peak 2 chromatographed in the position of a dinucleotide, and base composition analysis confirmed its identity as A-$^*Gp$, the only dinucleotide ordinarily released by Tl RNase digestion of intact, denatured tRNA$^{Leu}_3$. This dinucleotide, however, occurs in two locations in the tRNA molecule, once in the dihydrouridine (D) loop and again in the TΨC stem. The location of other modified residues in the D loop appeared to indicate that this region of the molecule is relatively exposed in the denatured conformer in comparison to the TΨC region where no other modified guanosine residues were observed. Thus the modification was assigned to the A-Gp position in the D loop. As in the case of the other observed fragments which terminated in kethoxalated guanosine residues, the
A-Gp dinucleotide was obtained in relatively low yield. This result may be attributed to the inhibition of Tl RNase action by removal of the N1 proton from the guanine base in the formation of the kethoxal adduct (290). As in the case of 1-methyl guanosine, however, some cleavage apparently does occur. The overwhelming excess of Tl RNase used (20 units/A260 unit tRNA) makes this observation even more likely. Longer oligonucleotides of lower yield have also been observed in the region of peak 7. Although these longer fragments were not present in sufficient quantity to allow unequivocal base composition analysis, preliminary studies using the 3'-32P labeling technique of Szeto and Söll (321) have indicated that one of these fragments contains the sequence A-G-C-Gm-G-D-C-D-A-A-G-Gp. These results provide further basis for the assignment of the A*-Gp modification to the D loop region.

Peak 3 appeared in the tetranucleotide region of the chromatographic pattern and was clearly identified as the tetranucleotide C-C-U-Gp known to occur in tRNA3Leu. Once again, kethoxalation of the terminal guanosine residue resulted in a relatively low yield of the small, modified fragment. It is interesting to note that cleavage after m2G, which is not usually observed in Tl RNase digestion of the intact denatured tRNA3Leu molecule (322), did occur in the modified denatured conformer (see also the analysis of peak 7). This result could possibly be attributed to
the large amount of nuclease used in these experiments as well as to a decrease in the number of more readily available guanosine "substrates" for the enzyme due to kethoxalation.

The position of peak 4 in the chromatographic pattern indicated that the fragment was at least a pentanucleotide in length. (Addition of an extra negative charge due to borate complexation by the kethoxal-guanosine adduct could not account for the degree of displacement observed from the usual position of the U-U-U-Gp tetranucleotide.) Furthermore, the higher yield observed for the fragment indicated that the terminal guanosine was more than likely not kethoxalated. For these reasons the oligonucleotide was identified as the pentanucleotide U-U-U-G-m^2Gp even though low yields of the m^2G trialcohol were observed.

The labeled oligonucleotide in peak 5 chromatographed along with the leading edge of the UV absorbance peak corresponding to the heptanucleotide A-ψ-U-m^5C-A-A-m^1G from the anticodon region of tRNA^Leu_3. Since base composition analysis revealed the presence of ψ and since only one ψ was likely to occur in a Tl RNase digestion product of this size from tRNA^Leu_3, molar ratios were normalized on the basis of the amount of ψ present. Although the anticodon fragment itself possesses a ψ, the only guanosine it contains is methylated at the N1 position and thus is resistant to kethoxalation. The nucleotide composition of
the fragment was, in fact, found to be consistent with the adjacent hexanucleotide sequence C-ψ-C-A-*G-*Gp which would have been delayed in elution from the column due to borate complexation by its modified residues. The only other region containing ψ in tRNA_{Leu}^{3} occurs in the TψC loop. The failure to observe any indication of the presence of ribothymidine, as well as the better agreement of the base composition data with the C-ψ-C-A-G-Gp sequence, however, discounted the possibility of fragment 5 being derived from the TψC region. The height of radioactive peak 5 was observed to be lower than that of peak 4 which contained only one modified residue. On the other hand, peak 5 exhibited approximately twice the radioactivity found in each of the two peaks 2 and 3 in which the kethoxalated guanosine occurred in the terminal position. On this basis it was concluded that both guanosine residues in the peak 5 oligonucleotide were modified, with kethoxalation of the terminal residue leading to the observed low yield of the fragment.

Base composition analysis of the large oligonucleotide in peak 6 led to its identification as C-G^{m}-G-D-C-D-A-A-Gp. Since this fragment eluted in the same position as the single modified oligonucleotide (peak 2) in the native tRNA_{Leu}^{3} column, both were assumed to possess the same composition and sequence. Molar ratios were based on a value of 2.0 for D due to the fact that any Tl RNase
fragment from tRNA$^{\text{Leu}}_3$ containing D should contain two and only two D residues. It should be noted that the 2'-O-methyl-G (G$^m$) species is resistant to periodate oxidation and therefore was not identified by the method used in the base composition analysis. This modified nucleotide is also normally resistant to Tl RNase and thus, unlike the adjacent guanosine, was not necessarily kethoxalated to permit formation of the observed fragment. According to the results of kinetic studies, however, two sites of modification were expected to occur in the native conformer under the kethoxalation conditions used. From the evidence presented here, these sites are identified as the two adjacent G$^m$-G residues occurring in the D loop of tRNA$^{\text{Leu}}_3$. If the corresponding region of the denatured structure is also exposed to modification, as indicated by peak 6 in the denatured tRNA$^{\text{Leu}}_3$ digest, it would seem likely that both of these adjacent guanosine residues would be modified in the denatured form as well. In addition, the area under radioactive peak 6 may best be explained in terms of the modification of all three guanosine species present. This peak was somewhat larger in height and width than peak 4, which represented a single internal kethoxalation. It was not as large, however, as would be expected to result from two internal modifications alone. A third kethoxalation at the terminal position would place the observations in keeping with results from
other fragments which terminated in a modified guanosine and contained one (peaks 2 and 3) and two (peak 5) kethoxal groups respectively.

As mentioned earlier, the area under radioactive peak 7 contains a number of large oligonucleotides which result from the inhibition of T1 RNase by kethoxalation. Due to limitations in the amount of material available for isolation and analysis, however, only the major species was analyzed for base composition. As indicated in Table I the identity of this fragment was determined to be C-*\textsuperscript{m}G-D-C-D-A-A-\textsuperscript{*}G-\textsuperscript{*}G-C-m\textsuperscript{2}Gp. Thus the "unusual" cleavage after m\textsuperscript{2}Gp noted with regard to fragment 3 was again observed to occur in the formation of this oligonucleotide. All four susceptible guanosine species were concluded to be modified on the basis of their resistance to T1 RNase digestion and the results from peak 6. The relatively low yield observed for D may be attributed to the decomposition of the base by KBH\textsubscript{4} during analysis, and for this reason the molar ratios were normalized on the basis of adenosine content.

The location of the exposed guanosine residues in the cloverleaf structure of tRNA\textsubscript{Leu}\textsubscript{3} as determined by reactivity with kethoxal is illustrated in Figure 10. The two sites of kethoxal incorporation indicated by the kinetic studies to be characteristic of the native molecule are found to be located at positions 17 and 18 in the D loop. In the
Figure 10. Sites of kethoxalation in yeast tRNA\textsubscript{Leu}. Arrows indicate positions of kethoxalation in the native (N→) and denatured (D→) conformations. The thickness of the arrow indicates the extent of kethoxalation.
denatured conformation these two guanosine residues are again modified, in addition to the following seven residues: # 9 preceding the D stem, # 15 in the D loop, # 22 and # 23 in the D stem, and # 29, # 41, and # 42 in the anticodon stem. The apparent extent of modification at each residue is indicated by the thickness of the arrow in Figure 10. As previously noted, however, the lower level of kethoxalation observed at some positions is quite possibly a reflection of the incomplete release of the respective fragments during Tl RNase digestion. Further discussion of the implications of these results will be presented in Chapter 4.
3.2 Circular Dichroism Studies

In order to clarify and extend the results of the kethoxalation studies, circular dichroism measurements were made of both nonmodified and kethoxalated tRNA$_3^{Leu}$ species. These investigations were basically designed to further investigate (i) the stabilizing effect of Mg$^{++}$ on the denatured conformer as indicated by the kinetics of kethoxalation; (ii) the ability of the denatured conformer to renature after kethoxal modification and concomitant loss of acceptor activity; and (iii) the effect of kethoxalation on the stability of the tRNA$_3^{Leu}$ molecule with increasing temperature. In addition, the CD spectra of tRNA$_{CUA}^{Leu}$ over a range of temperatures were measured and compared with the results from tRNA$_3^{Leu}$ studies.

In order to determine the effect, if any, of kethoxalation itself on the nature of the CD spectra, modified and nonmodified 2'-3' GMP were first investigated. The kethoxalation of GMP was carried out as described in Chapter 2, and the identity of the reaction product was verified by UV absorbance spectra as shown in Figure 11. Addition of acid to a pH of approximately 1.5 resulted in the decrease and slight red shift of the 250 nm maximum of the kethoxalated spectrum as reported for the nonmodified species (323). In contrast, adjustment to high pH values (approximately pH 12) resulted in the loss of the kethoxal group and the eventual conversion of the
Figure 11. Stability of the kethoxal adduct of 2'-3' GMP as monitored by UV absorbance spectra. 2'-3' GMP was treated with kethoxal under the conditions described in Chapter 2. The reaction was terminated by separating the reaction components in a Biogel P-2 column equilibrated with 10 mM borate, pH 7.3. UV absorbance spectra for the kethoxalated 2'-3' GMP were obtained at neutral pH (pH 7.3) (---), at acid pH (approximately pH 1.5) (---), and at alkaline pH (approximately pH 12). Spectra were recorded both immediately upon addition of base (- - - -), as well as one hour later (-----).
modified GMP spectrum to the alkaline spectrum of non-modified GMP. The CD spectra of the nonmodified and modified GMP species are shown in Figures 12 and 13 respectively. As expected, the CD signal for the mononucleotide was small, primarily reflecting the optical activity induced in the base chromophore by the optically active ribose moiety (205). It is of interest to note that the spectrum of 2'–3' GMP (Figure 12) is very similar to that of 5' GMP (303), indicating that the position of the phosphate group on the ribose has very little effect on the CD signal. Although slight differences between the spectra of kethoxalated and nonmodified samples at 25° and 60° were observed, the amount of noise present in the signal, as indicated by the crossbars, was sufficiently large to essentially negate most of these apparent discrepancies. The major conclusion which may be reached from this data, therefore, lies in the observation that no large changes may be expected to occur in the kethoxalated tRNA spectra due simply to alterations in the mononucleotide spectra. As will be observed later, any differences of the size noted in the kethoxalated GMP spectra would in fact be masked by the larger signals resulting from nucleotide interactions in a polynucleotide chain.

Since interactions between monomers specifically oriented with respect to one another provide the major basis for the observed CD spectra of polynucleotides, the
Figure 12. CD spectra of 2'-3' GMP in 10 mM potassium cacodylate, pH 7.0, 10 mM MgCl$_2$, and 10 mM borate at 25° (----) and 60° (— — —). Relative levels of signal noise in various regions of the spectra are indicated by the crossbars.
Figure 13. CD spectra of kethoxalated 2'-3' GMP in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, and 10 mM MgCl₂ at 25° (-----) and 60° (-----). Relative levels of signal noise in various regions of the spectra are indicated by the crossbars.
possible effects of kethoxalation on such interactions were further investigated by studying the random copolymer poly \((U_2,G)\). Differences in the UV absorbance spectra of the nonmodified and kethoxalated copolymer are shown in Figure 14. As in the case of the GMP monomer, the effect of kethoxalation on the absorbance characteristics of the polynucleotide were small. A greater sensitivity to the modification of the polynucleotide, however, was exhibited by CD measurements. It is interesting to note in this regard the relationship of the CD spectra of poly \((U_2,G)\) to those of poly U over a similar range of temperatures. Such a comparison reveals that significant alterations in both the height and position of the spectral bands occur as a result of the insertion of G residues into the poly U chain (324). The base-stacking arrangements brought about by the addition of guanosine are further altered by the introduction of the kethoxal modification, as may be seen in Figures 15 (nonmodified poly \((U_2,G)\)) and 16 (kethoxalated poly \((U_2,G)\)). The maximum occurring at 262 nm, which has been shown to reflect base-stacking interactions (296), was decreased by 25% at 25° in the kethoxalated sample. At higher temperatures where base stacking is minimized, the differences between the modified and nonmodified spectra essentially disappeared. The minimum at 239 nm did not appear to be as sensitive to kethoxalation, decreasing in absolute value by only 10% at 25°. Both modified and nonmodified
Figure 14. UV absorbance spectra of nonmodified (——) and kethoxalated (— — —) poly (U₂,G) in 10 mM cacocylate, pH 7.0, 10 mM borate, and 10 mM MgCl₂.
Graph showing absorbance ($E \times 10^{-3}$) versus wavelength ($\lambda$ in nm). The graph has two curves, one solid and one dashed, with absorbance peaking around 270 nm and decreasing towards the ends of the spectrum.
Figure 15. Temperature dependence of the CD spectra of poly (U₂G). Spectra were measured in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 10 mM MgCl₂ at the temperatures indicated.
Figure 16. Temperature dependence of the CD spectra of kethoxalated poly (U₂,G). Spectra were measured in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 10 mM MgCl₂ at the temperatures indicated.
\[ \Delta \varepsilon \text{ (l/cm-mole)} \]
minima were observed to decrease in absolute value with the loss of base-stacking interactions which accompanied an increase in temperature.

Analysis of the melting characteristics of the copolymers is shown in Figure 17. Although the height of the peak at 262 nm is influenced by kethoxalation, the normalized temperature effect over the range 16°-85° is essentially the same for the two copolymers. In each case half of the total change ($T_{1/2}$) occurred at 65°. A similar relationship between the melting behaviors of the two copolymers is observed on analysis at both 239 nm and 290 nm. Cooperativity in the melting process is observed, but is small in comparison to that exhibited by more ordered polynucleotides such as tRNA molecules.

The effect of kethoxal modification is thus seen to be much greater in a polynucleotide chain than in a mononucleotide. A significant reservation, however, must be kept in mind in the application of the poly (U$_2$G) data to CD measurements of modified tRNA. Due to the effects of secondary and tertiary structure, many of the guanosine residues in a tRNA molecule are protected from kethoxalation. As a result, only a small percentage of the total residues will be modified, and the effect of kethoxalation on the CD spectra should be much less than that observed for the synthetic copolymer.

The CD spectra of nonmodified tRNA$_{_{Leu}}^3$ conformers at 25° are shown in Figure 18. The native spectrum (solid
Figure 17. Comparison of the CD thermal transitions of nonmodified and kethoxalated poly (U<sub>2</sub>,G) at 262 nm. Spectra were recorded in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 10 mM MgCl<sub>2</sub>.

For nonmodified poly (U<sub>2</sub>,G) (○—○):

\[ \Delta \varepsilon(16^\circ) = 8.12 \text{ liter/cm-mole} \]

\[ \Delta \varepsilon(85^\circ) = 0.98 \text{ liter/cm-mole} \]

For kethoxalated poly (U<sub>2</sub>,G) (□—□):

\[ \Delta \varepsilon(16^\circ) = 6.13 \text{ liter/cm-mole} \]

\[ \Delta \varepsilon(85^\circ) = 1.00 \text{ liter/cm-mole} \]

An estimated uncertainty of ±0.07 liter/cm-mole is present in the calculated \( \Delta \varepsilon \) values.
Figure 18. CD spectra of nonmodified tRNA$_{3}$Leu at 25° in the native (----), D$_{Mg}^{++}$ (— — —), and D$_{EDTA}$ (----) conformations. All spectra were obtained in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA in the presence or absence of 10 mM MgCl$_{2}$ after treatment as indicated in List of Abbreviations.
line) exhibits a slight increase around 295 nm, followed by a dip at 290 nm. The maximum occurs at 261 nm and the curve is fairly level from 233 until it drops after 220, forming only a slight hump in the neighborhood of 222. Distinct differences from this pattern, however, are observed in the denatured spectra, both in the presence and absence of Mg\textsuperscript{++}. These spectra have lost the "dip" around 290, and their maxima exhibit a red shift as well as a decrease in magnitude. In the absence of Mg\textsuperscript{++} and the presence of 1mM EDTA (D\textsubscript{EDTA}, see List of Abbreviations), the maximum of the denatured conformer occurs at 262 nm. Addition of 10 mM Mg\textsuperscript{++} (D\textsubscript{Mg\textsuperscript{++}}) results in an increase in magnitude and a further red shift of the maximum to 263.5 nm. The D\textsubscript{EDTA} spectrum crosses above the D\textsubscript{Mg\textsuperscript{++}} curve at 255 nm, and both spectra exhibit a more distinguishable minimum at 233 nm than the native conformer, possibly due to less overlap from the negative band presumed to occur in the region of 210 nm (296). The red shift of the 260 nm maximum and the apparent blue shift of the 210 nm minimum observed for the denatured conformer are comparable to results observed on thermal denaturation of tRNA molecules (297).

When tRNA\textsuperscript{Leu\textsubscript{3}} was dialyzed versus distilled water and the heat denaturation process carried out in the absence of EDTA, conversion to these denatured conformers did not occur. As shown in Figure 19, a CD spectrum quite similar
Figure 19. Interconversion of tRNA\textsubscript{Leu} conformers. All CD spectra were obtained at 25° in 10 mM potassium cacodylate, pH 7.0, 10 mM borate with or without 1 mM EDTA and MgCl\textsubscript{2} (1 mM and 10 mM). After recording the D\textsubscript{H\textsubscript{2}O} spectrum (---), 1 mM EDTA was added to the tRNA\textsubscript{Leu} sample, resulting in a 6.8% hyperchromic shift in UV absorbance at 260 nm. The D\textsubscript{EDTA} (-----) spectrum was then recorded, followed by addition of 1 mM MgCl\textsubscript{2} (——) (3.4% hypochromic shift with respect to the D\textsubscript{EDTA} conformer), and 10 mM MgCl\textsubscript{2} (— —) (0.4% hyperchromic shift with respect to the 1 mM MgCl\textsubscript{2} sample). Renaturation was carried out by heating the sample to 60° in the CD cell for approximately 5 minutes, followed by slow-cooling over a period of one hour. The native sample (••••) exhibited 3.8% hypochromicity with respect to the D\textsubscript{Mg\textsuperscript{2+}} species.
to that of the native structure was obtained at 25°. Addition of 1 mM EDTA resulted in the conversion of this form (DH$_2$O) to the D$_{EDTA}$ structure, apparently removing residual Mg$^{++}$ and completing the denaturation process. Further addition of 1 mM Mg$^{++}$ produced a spectrum between that of the D$_{EDTA}$ and the D$_{Mg}^{++}$ forms, while in the presence of 5 mM Mg$^{++}$ (not shown) the spectrum was essentially identical to that obtained at 10 mM Mg$^{++}$. Renaturation by heating to 60° and slow cooling in the CD cell converted the tRNA to the native structure. Although the native spectrum at 25° was quite similar to the DH$_2$O spectrum, the positive band at 260 nm was slightly broader and greater in magnitude than the DH$_2$O curve. These differences, although small, have proven to be reproducible, occurring not only between spectra obtained on the same day from a single sample, but also between spectra recorded on different days, using different tRNA samples, and both in the presence and absence of borate.

A series of native and denatured spectra for tRNA$_{Leu}^3$ which had been modified with kethoxal in the native conformation (N$_L$) is shown in Figure 20. A great deal of similarity may be seen to exist between these curves and those of the nonmodified tRNA species (Figure 18). Slight decreases in each of the CD maxima, however, were observed to occur in the N$_L$ spectra, particularly in the native conformation. In addition, the dip at 290 nm for the
Figure 20. CD spectra of $N_L$ trNA$_3^{Leu}$ at 25° under native (---), $D_{Mg}^{++}$ (---), and $D_{EDTA}$ (-----) conditions. After modification, samples to be denatured were subjected to extensive dialysis as described in Chapter 2 prior to heat denaturation treatment in the presence of EDTA. Solvent conditions were those described in Figure 18.
modified native conformer was not as pronounced as in the nonmodified molecule, perhaps as a result of a slight red shift in the major positive band. At low wavelengths, the spectra of the two native tRNAs were essentially identical, while the denatured $N_L$ spectra were slightly lower than their nonmodified counterparts.

A comparable set of spectra for a tRNA$_3^{Leu}$ preparation in which the guanosine residues exposed in the denatured conformation had been kethoxalated ($D_S$) is presented in Figure 21. In this case the $D_{EDTA}$ spectrum (the form in which the tRNA was modified) was found to be essentially identical to that for $N_L$. In the presence of $Mg^{++}$, however, the $D_S$ species exhibited a further decrease in the CD maximum as compared to its $N_L$ counterpart. An increase in magnitude (absolute value) in the region of the 233 nm negative band was also noted for the $D_S$ species. Somewhat surprisingly, the $D_S$ molecule, which had lost approximately 70% of its renaturable leucine acceptor activity, did appear able to convert to a native-like structure. Although essentially identical to the $N_L$ form at low wavelengths, the native $D_S$ spectrum appeared to be slightly broader than the $N_L$ spectrum above 260 nm. This broadening was accompanied by an apparent red shift of the maximum to 261.5 nm. In addition, the dip around 290 nm was much less pronounced in the native $D_S$ spectrum, perhaps as a further result of the broadening of the major band.
Figure 21. CD spectra of $D_S$ tRNA$_{Leu}^3$ at 25° under native (----), $D_{\text{Mg}}$ ++ (-----), and $D_{\text{EDTA}}$ (----) conditions. Solvent conditions were those described in Figure 18.
Figure 22 illustrates the results obtained from CD studies of extensively kethoxalated tRNA$^{\text{Leu}}_3$ (D$_L$) which completely lacked renaturable leucine acceptor activity. The D$_{\text{EDTA}}$ maximum was further lowered in height in comparison to the N$_L$ and D$_S$ spectra, and the signal did not decrease as rapidly as previous curves after 222 nm, indicating a possible decrease in magnitude of the 210 nm minimum as well. On addition of Mg$^{++}$, the D$_{\text{Mg}^{++}}$ maximum was found to exhibit a slight red shift to 264 nm.

Subjecting the sample to renaturing conditions resulted in a spectrum similar to that of the D$_{\text{Mg}^{++}}$ form below 258 nm. At longer wavelengths, however, the spectrum fell between those of the D$_{\text{EDTA}}$ and D$_{\text{Mg}^{++}}$ forms, possessing a maximum at 263 nm.

The differential stabilities of the modified and non-modified tRNA$^{\text{Leu}}_3$ species were studied by examining their thermal denaturation characteristics using circular dichroism. As a background for this investigation, the UV absorbance melting profiles were obtained for the native and D$_{\text{Mg}^{++}}$ conformers of nonmodified tRNA. These results appear in Figure 23. Both tRNA species exhibited cooperative melting curves over the temperature range of 15°-85° with a $T_{1/2}$ value of 76°. The most notable feature of these results, however, was the decrease in absorbance exhibited by the D$_{\text{Mg}^{++}}$ species between 35° and 60°. This increase in hypochromicity could perhaps be
Figure 22. CD spectra of $D_L$ tRNA$_3^{Leu}$ at 25° under native (----), $D_{Mg}^{++}$ (— — —), and $D_{EDTA}$ (----) conditions. Solvent conditions were those described in Figure 18.
Figure 23. Thermal transitions of native (-----), and 
D_{Mg}^{++} (-----) conformers of tRNA_3^{Leu} as measured by UV 
absorbance in 10 mM potassium cacodylate, pH 7.0, 10 mM 
MgCl_2, 1 mM EDTA.
% TOTAL CHANGE IN A$_{260}$
attributed to the conversion of the tRNA to its more hypochromic, native conformer at slightly elevated temperatures in the presence of Mg++. This possibility was subjected to further analysis by circular dichroism in order to verify the nature of the structure present at each temperature. The native tRNA melting curve, on the other hand, was essentially temperature independent until the overall structure began to disrupt at approximately 50°.

The series of CD curves obtained with increasing temperature for the nonmodified tRNA in the native, D_Mg++, and D_EDTA forms are shown in Figures 24-26 respectively. Although not all scans which were obtained are included in these figures, it may still be seen that, in contrast to UV absorbance data, the CD maxima are temperature dependent throughout the temperature range investigated (16°-85°). As the tRNA species melted, a decrease in the CD maxima and a corresponding broadening of the positive band occurred. Such broadening would be expected considering the fact that circular dichroism is based on a difference in UV extinction coefficients, and absorption bands are observed to increase in width with increasing temperature. Accompanying this broadening was a red shift in the position of the maxima with increasing temperature. The CD maxima of non-base-paired polynucleotides such as the synthetic copolymer poly (U2,G) (Figure 15) do not shift in this fashion, and therefore this change is considered
Figure 24. Temperature dependence of the CD spectra of native tRNA\textsubscript{Leu}\textsuperscript{3}. Spectra were measured in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA, 10 mM MgCl\textsubscript{2} at the temperatures indicated.
Figure 25. Temperature dependence of the CD spectra of D$_{Mg}^+$ tRNA$_{3}^{Leu}$. Spectra were measured in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA, 10 mM MgCl$_2$ at the temperatures indicated.
Figure 26. Temperature dependence of the CD spectra of $\text{D}_{\text{EDTA}} \text{tRNA}^{\text{Leu}}_3$. Spectra were measured in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA at the temperatures indicated.
to be primarily indicative of the loss of base-pairing interactions (269, 299). In the native conformer the combined peak broadening and red shift eventually obscured the dip occurring in the region of 290 nm. Only small differences at low wavelengths were noted to occur with melting.

As shown in Figure 25, the $\text{D}_{\text{Mg}^{++}}$ tRNA species was, indeed, observed to shift from the denatured conformer to a native-like form at moderate temperatures as had been suggested by the UV absorbance melting curve. This conversion began at approximately 32° and was essentially completed by 40°. Above that temperature the spectra behaved in a manner characteristic of the native species. It should be noted, however, that the signals observed during this conversion are time dependent. Thus the total renaturation process would eventually occur at any of the temperatures shown if given sufficient time (as long as two days at 25°). Measurement of the extent of this time dependence over a range of temperatures could in fact be used in determining the thermodynamic parameters $\Delta G^\circ$ and $\Delta H^\circ$ for the renaturation process (see Chapter 4). During the length of time required for the CD measurements, however (approximately twenty minutes for equilibration and thirty minutes for the scan at each temperature), the conversion exhibited a fairly uniform temperature dependence among the $\text{D}_{\text{Mg}^{++}}$ samples of the nonmodified, $\text{N}_{\text{L}}$. 
and $D_S$ trNA$^\text{Leu}_3$ species.

In the absence of Mg$^{++}$ ($D_{\text{EDTA}}$), as shown in Figure 26, no such shift to the native spectrum was found to occur with increasing temperature. In addition, no dip appeared around 290 nm, and a distinct decrease in the $T_{1/2}$ value was noted. The eventual loss of the increase in the region of 222 nm indicated a possible broadening of the negative band at 210 nm.

Similar sets of CD spectra were obtained with increasing temperature for each of the kethoxalated trNA$^\text{Leu}_3$ species $N_L$, $D_S$, and $D_L$. These curves are not shown due to their general similarity to the comparable nonmodified trNA curves. The differences which occurred among them, however, may best be analyzed by looking at the change in the CD signal with temperature for each species at specific wavelengths. The wavelengths found to be of particular interest in the trNA$^\text{Leu}_3$ CD spectra included (i) the wavelength at which the maximum occurred at 25° (261-263 nm), (ii) the "dip" region at 290 nm, and (iii) the minimum at 233 nm. The results of these analyses are presented in Figures 27-48, and a summary of the observed melting curve parameters is given in Table II.

The melting curves obtained at the $\lambda_{\text{max}}$ for each of the trNA species are presented according to two formats. First, the actual $\Delta\varepsilon$ values at $\lambda_{\text{max}}$ are plotted as a function of temperature, thus providing an indication of
### TABLE II

**Summary of CD Melting Curve Parameters**

<table>
<thead>
<tr>
<th>Sample Conditions</th>
<th>Position of Maximum at 25° (nm)</th>
<th>( \lambda_{\text{max}} )</th>
<th>( T_{1/2} ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{Xmax} ) 290 nm</td>
<td>25° (°C)</td>
<td>233 nm</td>
</tr>
<tr>
<td>Nonmodified tRNA(^{\text{Leu}}) (_3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>261</td>
<td>70</td>
<td>64.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{Mg}}^{++} )</td>
<td>263.5</td>
<td>70.5</td>
<td>65.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{EDTA}} )</td>
<td>262</td>
<td>43</td>
<td>37.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{H}_2\text{O}^{+}}^{+\text{Mg}}^{++} )</td>
<td>261</td>
<td>70</td>
<td>67.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{H}_2\text{O}^{-}}^{+\text{Mg}}^{++} )</td>
<td>261</td>
<td>55</td>
<td>54.5</td>
</tr>
<tr>
<td>( N_L ) tRNA(^{\text{Leu}}) (_3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>261</td>
<td>70</td>
<td>69.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{Mg}}^{++} )</td>
<td>263.5</td>
<td>71</td>
<td>65.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{EDTA}} )</td>
<td>262</td>
<td>42</td>
<td>36.5</td>
</tr>
<tr>
<td>( D_S ) tRNA(^{\text{Leu}}) (_3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>261.5</td>
<td>70</td>
<td>69.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{Mg}}^{++} )</td>
<td>263.5</td>
<td>70</td>
<td>68.5</td>
</tr>
<tr>
<td>( \text{D}_{\text{EDTA}} )</td>
<td>262</td>
<td>42</td>
<td>37.5</td>
</tr>
<tr>
<td>( D_L ) tRNA(^{\text{Leu}}) (_3)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>263</td>
<td>67.5</td>
<td>48</td>
</tr>
<tr>
<td>( \text{D}_{\text{Mg}}^{++} )</td>
<td>264</td>
<td>67.5</td>
<td>47</td>
</tr>
<tr>
<td>( \text{D}_{\text{EDTA}} )</td>
<td>262</td>
<td>39.5</td>
<td>35.5</td>
</tr>
</tbody>
</table>

Nonmodified tRNA\(^{\text{Leu}}\) \(_{\text{CUA}}\)

| Native | 261 | 69 | 64 |

* see List of Abbreviations and figure legends for detailed descriptions of sample conditions
the range of Δε values observed for each of the samples. In conjunction with these data are shown the change in the λ_max position with temperature. Therefore it is possible to compare changes in base-stacking interactions (change in Δε) and base-pairing characteristics (shift in λ_max) among the tRNA samples. Secondly, the data are presented in a normalized form as the percent total change in Δε over the range of temperatures investigated. This representation illustrates the rate and nature of the overall change occurring for each sample, making it possible to determine relationships between the extent of change which has occurred for each of the conformers at any given temperature. Melting curve data obtained at the λ_max (25°) have been further summarized in Table III to facilitate these comparisons.

The melting curves of the native, D_Mg++, and D_EDTA forms of nonmodified tRNA_{Leu} at their respective λ_max values are shown in Figures 27 and 28. In the presence of Mg^{++} half of the total change in Δε occurs at 70°. This value represents the melting of the native structure for both the native and the D_Mg++ transitions, since the D_Mg++ form undergoes renaturation below 40°. Evidence of this renaturation process is further demonstrated by the shift in both the Δε and λ_max values over the temperature range 30°-40°, resulting in curves which essentially parallel those of the native conformer. According to Figure 27,
TABLE III

Summary of $T_{1/2}$ Values at $\lambda_{max}$ (25°) for Nonmodified and Kethoxalated $\text{tRNA}^\text{Leu}_3$ Species

<table>
<thead>
<tr>
<th>Solvent Conditions</th>
<th>Nonmodified</th>
<th>$T_{1/2}$ (°C)</th>
<th>$D_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>70</td>
<td>70</td>
<td>67.5</td>
</tr>
<tr>
<td>$D_{\text{Mg}^{++}}$</td>
<td>70.5</td>
<td>71</td>
<td>67.5</td>
</tr>
<tr>
<td>$D_{\text{EDTA}}$</td>
<td>43</td>
<td>42</td>
<td>39.5</td>
</tr>
<tr>
<td>$D_{\text{H}_2\text{O}^+\text{Mg}^{++}}$</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$D_{\text{H}_2\text{O}^-\text{Mg}^{++}}$</td>
<td>55</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 27. CD thermal transitions of nonmodified tRNA$_3^{\text{Leu}}$ conformers. Solvent conditions were those described in Figure 18. Solid symbols represent the wavelength of the CD maximum ($\lambda_{\text{max}}$) at each temperature for the native (●—●), D$_{\text{Mg}^{++}}$ (■—■), and D$_{\text{EDTA}}$ (▲—▲) conformers. Open symbols indicate $\Delta\varepsilon$ values observed at the $\lambda_{\text{max}}$ in each spectrum. An estimated uncertainty of $\pm0.07$ liter/cm-mole is present in the calculated $\Delta\varepsilon$ values. The uncertainty becomes slightly greater at higher temperatures due to the relatively poor signal to noise ratios resulting from the hyperchromicity and reduced optical activity of the tRNA samples.
Figure 28. Relative change in $\Delta \varepsilon$ as a function of temperature for nonmodified tRNA$^{\text{Leu}}_3$ conformers, measured at the $\lambda_{\text{max}}$ observed in the 25° CD spectrum. Solvent conditions were those described in Figure 18.

For the native conformer (o--o):

$\lambda_{\text{max}}(25°) = 261$ nm
$\Delta \varepsilon(16°) = 9.52$ liter/cm-mole
$\Delta \varepsilon(85°) = 1.06$ liter/cm-mole

For the $\text{D}_{\text{Mg}}^{++}$ conformer (□—□—□):

$\lambda_{\text{max}}(25°) = 263.5$ nm
$\Delta \varepsilon(16°) = 8.68$ liter/cm-mole
$\Delta \varepsilon(85°) = 1.40$ liter/cm-mole

For the $\text{D}_{\text{EDTA}}$ conformer (Δ----Δ):

$\lambda_{\text{max}}(25°) = 262$ nm
$\Delta \varepsilon(16°) = 7.62$ liter/cm-mole
$\Delta \varepsilon(85°) = 1.34$ liter/cm-mole

An estimated uncertainty of $\pm 0.07$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
this change is accompanied by an increase in both base stacking (increase in \( \Delta \varepsilon \)) and base pairing (blue shift of \( \lambda_{\text{max}} \)), in agreement with the results of previous studies (206, 269, 280). Both \( \Delta \varepsilon \) and \( \lambda_{\text{max}} \) values revealed cooperative denaturation behavior in the neighborhood of the \( T_{1/2} \) value. Although the nature of the CD measurements does not permit accurate detection of small changes in \( \lambda_{\text{max}} \) (even differences of 0.5 nm are of borderline significance), it is nonetheless interesting to note that the change in the \( \lambda_{\text{max}} \) for the native form appears to be relatively independent of temperature prior to 50°. This change also exhibits a slightly higher \( T_{1/2} \) value (73.5°) than the \( \Delta \varepsilon \) curve. Thus the shift in \( \lambda_{\text{max}} \) position as a result of base pair disruption more nearly parallels the change observed in UV absorbance with increasing temperature. The \( D_{\text{EDTA}} \) form, lacking Mg\(^{++}\), is markedly less stable, exhibiting a \( T_{1/2} \) value of approximately 43°.

In Figures 29 and 30, the corresponding curves for the \( D_{\text{H}_2\text{O}} \) species with and without the addition of Mg\(^{++}\) are shown. In the presence of Mg\(^{++}\) the melting curve was identical to that of the native conformer. Without added Mg\(^{++}\), however, the initial normalized temperature effect remained the same while the overall stability of the molecule decreased (\( T_{1/2} = 55° \)). Nonetheless, the \( D_{\text{H}_2\text{O}} \) conformation in the absence of Mg\(^{++}\) possessed greater stability than the \( D_{\text{EDTA}} \) form. Apparently, therefore,
Figure 29. CD thermal transitions of $D_{H_2O} tRNA_{Leu}^{Leu}$ in the presence and absence of Mg$^{++}$. Solvent conditions were those described in Figure 18. Solid symbols represent the $\lambda_{max}$ at each temperature for the spectra obtained in the presence (■ — ■) and absence (▲—▲) of Mg$^{++}$. Open symbols indicate $\Delta \varepsilon$ values observed at the $\lambda_{max}$ in each spectrum. An estimated uncertainty of $\pm 0.10$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
$\Delta \varepsilon_{\lambda_{\text{max}}}$ (1/cm-mole)

Graph showing $\lambda_{\text{max}}$ (nm) on the x-axis and $T$ (°C) on the y-axis.
Figure 30. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_{H_2O}^{Leu}$ in the presence and absence of $Mg^{++}$, measured at the $\lambda_{max}$($25^\circ$). Solvent conditions were those described in Figure 18.

In the presence of $Mg^{++}$ (□ --- □):

$$\lambda_{max}(25^\circ) = 261 \text{ nm}$$
$$\Delta \varepsilon(16^\circ) = 9.56 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = 0.94 \text{ liter/cm-mole}$$

In the absence of $Mg^{++}$ (Δ---Δ):

$$\lambda_{max}(25^\circ) = 261 \text{ nm}$$
$$\Delta \varepsilon(16^\circ) = 9.06 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = 1.29 \text{ liter/cm-mole}$$

An estimated uncertainty of $\pm 0.10$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
even very small levels of Mg$^{++}$ are able to confer some degree of structural stability on the tRNA molecule.

The melting curves obtained for the $N_L$ species are shown in Figures 31 and 32. On the whole, they were quite similar to those observed for the nonmodified species. Thus kethoxalation was shown to have little effect on the structural features of the molecule, in agreement with the retention of over 90% of the biological activity. The major difference between nonmodified and $N_L$ behavior may be seen in the slight decrease in the $\Delta \varepsilon$ values for the $N_L$ species at lower temperatures. Therefore some degree of base stacking in the molecule is apparently disrupted by modification of the D loop residues. This base stacking is not primarily responsible for the thermal stability of the molecule, however, since the $T_{1/2}$ value is the same for both nonmodified and $N_L$ samples.

In the $D_S$ species (Figures 33 and 34), the melting curves of both $\Delta \varepsilon$ and $\lambda_{max}$ values once again reflected the occurrence of the renaturation process in the $D_{Mg}^{++}$ form. In addition to the initial loss in base stacking (indicated by the lower $\Delta \varepsilon$ as compared to nonmodified tRNA), however, the $D_S$ molecule did not exhibit as great an increase in base-stacking interactions on renaturation as did the nonmodified and $N_L$ species. Thus the number and/or location of the kethoxalated residues in the $D_S$ species (Figure 10) resulted in the prevention of proper base
Figure 31. CD thermal transitions of \( N_L \) tRNA\(^{\text{Leu}} \) conformers. Solvent conditions were those described in Figure 18. Solid symbols represent the \( \lambda_{\text{max}} \) at each temperature for the native (●—●), \( \text{D}_{\text{Mg}}^{\text{++}} \) (■—■), and \( \text{D}_{\text{EDTA}} \) (▲—▲) conformers. Open symbols indicate \( \Delta \varepsilon \) values observed at the \( \lambda_{\text{max}} \) in each spectrum. An estimated uncertainty of \( \pm 0.08 \) liter/cm-mole is present in the calculated \( \Delta \varepsilon \) values.
\[ \Delta \varepsilon_{\lambda_{\text{max}}} \quad (1/\text{cm-mole}) \]
Figure 32. Relative change in Δε as a function of temperature for N₄₃ tRNA³-Leu conformers, measured at the λ_max (25°). Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

λ_max(25°) = 261 nm
Δε(16°) = 8.55 liter/cm-mole
Δε(85°) = 0.86 liter/cm-mole

Under D_Mg++ conditions (□—□—□):

λ_max(25°) = 263.5 nm
Δε(16°) = 8.46 liter/cm-mole
Δε(85°) = 1.35 liter/cm-mole

Under D_EDTA conditions (Δ—Δ—Δ):

λ_max(25°) = 262 nm
Δε(16°) = 7.14 liter/cm-mole
Δε(85°) = 1.21 liter/cm-mole

An estimated uncertainty of ±0.08 liter/cm-mole is present in the calculated Δε values.
Figure 33. CD thermal transitions of \( D_{S} tRNA_{3}^{\text{Leu}} \) conformers. Solvent conditions were those described in Figure 18. Solid symbols represent the \( \lambda_{\text{max}} \) at each temperature for the native (●—●), \( D_{\text{Mg}^{++}} \) (■——■), and \( D_{\text{EDTA}} \) (▲——▲) conformers. Open symbols indicate \( \Delta\varepsilon \) values observed at the \( \lambda_{\text{max}} \) in each spectrum. An estimated uncertainty of ±0.075 liter/cm-mole is present in the calculated \( \Delta\varepsilon \) values.
Figure 34. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_\text{S} \text{tRNA}^\text{Leu}_3$ conformers, measured at the $\lambda_{\text{max}}(25^\circ)$. Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

$$\lambda_{\text{max}}(25^\circ) = 261.5 \text{ nm}$$
$$\Delta \varepsilon(16^\circ) = 8.72 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = 0.99 \text{ liter/cm-mole}$$

Under $D_{\text{Mg}^{++}}$ conditions (o—o—o):

$$\lambda_{\text{max}}(25^\circ) = 263.5 \text{ nm}.$$  
$$\Delta \varepsilon(16^\circ) = 8.27 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = 0.95 \text{ liter/cm-mole}$$

Under $D_{\text{EDTA}}$ conditions (Δ—Δ—Δ):

$$\lambda_{\text{max}}(25^\circ) = 262 \text{ nm}$$
$$\Delta \varepsilon(16^\circ) = 7.14 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = 1.32 \text{ liter/cm-mole}$$

An estimated uncertainty of $\pm 0.075$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
stacking in the modified regions of the molecule. As in the case of the NL species, however, the overall thermal stability of the molecule was not significantly affected by these changes.

Greater differences were observed for the DL form as shown in Figures 35 and 36. In the presence of Mg\textsuperscript{++} the loss in base-stacking interactions was accompanied by a decrease in the cooperativity of the melting transitions. Thus the sites of Mg\textsuperscript{++} interaction appeared to be affected by the extensive ketoxalation. A slight decrease in the overall stability of the DL species as compared to the previous tRNA samples is also implied by the lower T\textsubscript{1/2} values of the \Delta\epsilon melting transitions. As indicated by the change in \lambda\textsubscript{max} with temperature, however, the lower level of base pairing which does occur in the DL species possesses a thermal stability comparable to that of the less extensively modified tRNAs.

The melting curves obtained for the tRNA samples at 290 nm are shown in Figures 37-42. The changes which occurred at this wavelength were much more complex than the simple melting phenomenon observed in the region of the maximum. These complexities are due in part to the relatively small size of the signal at 290 nm and the ease by which it is masked by the broadening and shifting of the positive band with increasing temperature. As in the case of the \lambda\textsubscript{max} measurements, the actual \Delta\epsilon values at
Figure 35. CD thermal transitions of D₅tRNA³Leu conformers. Solvent conditions were those described in Figure 18. Solid symbols represent the $\lambda_{\text{max}}$ at each temperature for the native (●), $\text{D}_{\text{Mg}}^{++}$ (■), and $\text{D}_{\text{EDTA}}$ (▲) conformers. Open symbols indicate $\Delta\varepsilon$ values observed at the $\lambda_{\text{max}}$ in each spectrum. An estimated uncertainty of ±0.07 liter/cm-mole is present in the calculated $\Delta\varepsilon$ values.
\[ \Delta \varepsilon_{\lambda_{\text{max}}} \text{ (1/cm-mole)} \]

\[ \lambda_{\text{max}} \text{ (nm)} \]

\[ T(\circ) \]

\[ (\circ)^{\circ} \]

\[ (\triangle)^{\circ} \]

\[ (\square)^{\circ} \]

\[ (\bullet)^{\circ} \]

\[ (\blacktriangle)^{\circ} \]

\[ (\blacklozenge)^{\circ} \]

\[ (\blacktriangleleft)^{\circ} \]
Figure 36. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_L$ tRNA$^{Leu}_3$ conformers, measured at the $\lambda_{\text{max}}(25^\circ)$. Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

\begin{align*}
\lambda_{\text{max}}(25^\circ) &= 263 \text{ nm} \\
\Delta \varepsilon(16^\circ) &= 7.89 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) &= 1.06 \text{ liter/cm-mole}
\end{align*}

Under $D_{\text{Mg}^{++}}$ conditions (o—o—o):

\begin{align*}
\lambda_{\text{max}}(25^\circ) &= 264 \text{ nm} \\
\Delta \varepsilon(16^\circ) &= 8.68 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) &= 1.19 \text{ liter/cm-mole}
\end{align*}

Under $D_{\text{EDTA}}$ conditions ($\Delta$—$\Delta$—$\Delta$):

\begin{align*}
\lambda_{\text{max}}(25^\circ) &= 262 \text{ nm} \\
\Delta \varepsilon(16^\circ) &= 6.98 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) &= 1.17 \text{ liter/cm-mole}
\end{align*}

An estimated uncertainty of $\pm 0.07$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
290 nm are shown in Figure 37 to illustrate the range of values involved in each transition. Due to the relatively small magnitude of the changes, however, the trends exhibited by each tRNA species in the overall transition are more clearly demonstrated by a comparison of the percent total change in \( \Delta \varepsilon \) as a function of temperature. For this reason the data for the modified tRNA species are presented in this latter form only. The initial and final \( \Delta \varepsilon \) values for each curve, however, are presented in the figure legends. In addition, values are given for the estimated uncertainty in these figures, based on the two major sources of uncertainty in the CD measurements: (i) the baseline drift during the scan and (ii) the level of noise in the signal. Although the present study was designed primarily for investigation of the \( \lambda_{\text{max}} \) transitions, it should be noted that significantly higher tRNA concentrations may be used in the study of melting behavior at 290 nm where the UV absorbance of the tRNA is low. Thus further information regarding the details of the transitions at this wavelength may be obtained for comparison with the trends observed here.

For the nonmodified tRNA in the native conformation (Figures 37 and 38), the \( \Delta \varepsilon \) value remained stable until approximately 50°, at which point the signal was observed to decrease. A possible explanation for the initial stability in the signal may be traced to the
Figure 37. CD thermal transitions of nonmodified tRNA$^\text{Leu}$$_3$ conformers, measured at 290 nm. Solvent conditions were those described in Figure 18. Values of $\Delta \varepsilon$ as a function of temperature are shown for the native (○—○), $D_{\text{Mg}^{2+}}$ (□—□), $D_{\text{EDTA}}$ (Δ—Δ), and $D_{\text{H}_2\text{O} + \text{Mg}^{2+}}$ (○—○) conformers. An estimated uncertainty of $+0.035$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
Figure 38. Relative change in $\Delta \varepsilon$ as a function of temperature for nonmodified tRNA$_3^{\text{Leu}}$ conformers, measured at 290 nm. Solvent conditions were those described in Figure 18.

For the native conformer (○—○):

$\Delta \varepsilon(16^\circ) = 0.448 \ \text{liter/cm-mole}$

$\Delta \varepsilon(85^\circ) = 0.654 \ \text{liter/cm-mole}$

For the $D_{\text{Mg}^{++}}$ conformer (□—□):

$\Delta \varepsilon(16^\circ) = 1.392 \ \text{liter/cm-mole}$

$\Delta \varepsilon(85^\circ) = 0.529 \ \text{liter/cm-mole}$

For the $D_{\text{EDTA}}$ conformer (Δ—Δ):

$\Delta \varepsilon(16^\circ) = 0.859 \ \text{liter/cm-mole}$

$\Delta \varepsilon(85^\circ) = 0.703 \ \text{liter/cm-mole}$

An estimated uncertainty of ±0.035 liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
proposed sensitivity of the negative band in the 290 nm region to tertiary structure (205). Thus prior to 50° the decrease in the positive signal of the major band (Figures 27 and 28) was essentially balanced by a corresponding decrease in the absolute value of the negative signal (an actual increase in Δε value). When the tertiary structure reflected by the negative band was lost near 50° (205), the effect of the positive band became predominant, and the signal decreased, indicative of the continued loss of base-stacking interactions. Although the present results are not sufficient to prove these relationships, they do provide implications which are worthy of further investigation. Above approximately 65° the disruption of the base-pairing structure of the molecule led to a red shift in the positive band and a resulting rapid increase in the 290 nm signal. Fifty percent of this increase had occurred by 70°, in agreement with the 261 nm melting curve data. In the case of the D_{Mg}^{++} structure, an initial decrease in the Δε_{290} paralleled the formation of the structural features characteristic of the native conformer and resulted in the appearance of the "dip" at 290 nm. A relative minimum value for the signal was obtained when this conversion was complete. Again the signal remained constant, decreasing just prior to the increase indicative of secondary structure disruption. Once more approximately half of the total increase had occurred when the 261 nm
$T_{1/2}$ of 70.5° was reached. When the denatured conformer was prepared in the presence of EDTA and the absence of 
Mg$^{++}$ ($D_{EDTA}$), the structure was again seen to relax somewhat to reach a minimum signal around 37.5°. Without Mg$^{++}$ to bring about the stabilization of the native conformer, the ordered structure was rapidly lost, resulting in an increase in the signal which coincided with the red shift in the major positive band and the previously observed $T_{1/2}$ of 43°. Once the red shift was essentially complete, the signal at 290 nm decreased along with the decreasing signal of the positive band.

Figure 39 illustrates the melting curves for the $D_{H_2O}$ tRNA species with and without Mg$^{++}$. In the presence of Mg$^{++}$, the signal at 290 nm remained stable until approximately 40°, at which point it began to increase until leveling off over the 55°-65° region. This observed increase could possibly be attributed to a rapid decrease in the absolute value of the negative signal (with respect to the decreasing positive signal), reflecting a loss of tertiary interactions. A similar $\Delta \varepsilon$ value was reached for the native, $D_{Mg^{++}}$, and $D_{H_2O} + Mg^{++}$ forms near 60° (Figure 37), perhaps indicative of the formation of a common "relaxed" intermediate prior to the overall disruption of secondary structure. The 290 nm $T_{1/2}$ value of 73.5° observed for the $D_{H_2O}$ species in the presence of Mg$^{++}$ was comparable to the 70° value observed at 261 nm. In the
Figure 39. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_{H_2O} tRNA_{Leu}^{3}$ in the presence and absence of $Mg^{++}$, measured at 290 nm. Solvent conditions were those described in Figure 18.

In the presence of $Mg^{++}$ (○—○—○):

$\Delta \varepsilon(16^\circ) = 0.057 \text{ liter/cm-mole}$
$\Delta \varepsilon(85^\circ) = 0.411 \text{ liter/cm-mole}$

In the absence of $Mg^{++}$ (Δ----Δ):

$\Delta \varepsilon(16^\circ) = 0.377 \text{ liter/cm-mole}$
$\Delta \varepsilon(85^\circ) = 0.895 \text{ liter/cm-mole}$

An estimated uncertainty of $\pm 0.060 \text{ liter/cm-mole}$ is present in the $\Delta \varepsilon$ values.
absence of 

Mg++, the "near-native" structure of the 

D_{H_2O}
molecule melted in a pattern similar to that of the D_{EDTA}
species, passing through a minimum near 40° and a maximum
near 65°. The 290 nm T_{1/2} value of 53.5° for the 

D_{H_2O}
minus Mg++ structure agreed well with the 55° value ob-
served at 261 nm. It was somewhat higher and thus indica-
tive of greater structural stability, however, than the
43° and 43.5° values observed for the D_{EDTA} structure at
261 nm and 290 nm respectively.

In Figure 40 the melting curves of the kethoxalated
N_L structure are shown to follow a pattern similar to those
of the nonmodified species. Furthermore, the T_{1/2} values
of 69.5°, 72°, and 42.5° for the native, D_{Mg++}, and D_{EDTA}
forms respectively were in close agreement with the values
obtained for the nonmodified tRNA (see Table II). Like-
wise, the values compared quite favorably with the 70°,
71°, and 42° temperatures recorded for the N_L species at
261 nm.

The melting curves for the D_S tRNA species are
illustrated in Figure 41. Once again the patterns ex-
hibited by the curves of the renatured species and the
D_{EDTA} species (the form in which the tRNA was modified)
were similar to those of the nonmodified molecule. The
T_{1/2} values of 70.5° for the native and 40.5° for the
D_{EDTA} forms were also relatively close to the nonmodified
values of 70° and 43.5° at 290 nm and to the D_S values of
Figure 40. Relative change in $\Delta \varepsilon$ as a function of temperature for $N_L$ tRNA$_{Leu}$ conformers, measured at 290 nm. Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

$\Delta \varepsilon(16^\circ) = 0.373$ liter/cm-mole
$\Delta \varepsilon(85^\circ) = 0.559$ liter/cm-mole

Under D$_{Mg}^{++}$ conditions (□ — □ — □):

$\Delta \varepsilon(16^\circ) = 1.429$ liter/cm-mole
$\Delta \varepsilon(85^\circ) = 0.590$ liter/cm-mole

Under D$_{EDTA}$ conditions (Δ——Δ):

$\Delta \varepsilon(16^\circ) = 0.819$ liter/cm-mole
$\Delta \varepsilon(85^\circ) = 0.684$ liter/cm-mole

An estimated uncertainty of $\pm 0.040$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
Figure 41. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_8$ tRNA$_{Leu}^{\text{Le}}$ conformers, measured at 290 nm. Solvent conditions were those described in Figure 18.

Under native conditions (○—○):

\[ \Delta \varepsilon(16^\circ) = 0.480 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon(85^\circ) = 0.534 \text{ liter/cm-mole} \]

Under $D_{Mg}^{++}$ conditions (□——□):

\[ \Delta \varepsilon(16^\circ) = 1.630 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon(85^\circ) = 0.560 \text{ liter/cm-mole} \]

Under $D_{EDTA}$ conditions (Δ——Δ):

\[ \Delta \varepsilon(16^\circ) = 0.801 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon(85^\circ) = 0.676 \text{ liter/cm-mole} \]

An estimated uncertainty of $\pm 0.040$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
70° and 42° at 261.5 nm. The melting curve for the D_{Mg}^{++} form, however, appeared to differ slightly from those of the nonmodified and N_L species. A gradually sloping, less cooperative decrease in \Delta \varepsilon_{290} was observed for the D_S species prior to reaching its minimum value at approximately 60°. Thus the sharp decrease to 37.5° followed by leveling off indicative of formation of the stable native structure was not observed. It is possible that this behavior reflects the failure of the D_S structure to form correctly a portion of the native conformation which is required for biological activity. It may be recalled that the overall spectrum of the "renatured", yet 70° inactivated, D_S molecule was similar to the native nonmodified CD spectrum, with the primary exception that the dip at 290 nm was not as pronounced in the D_S form (Figure 21). A sufficient amount of the native structure was present, however, to provide a $T_{1/2}$ value of 71°, essentially identical to the 70.5° value observed for the nonmodified species.

In the case of the D_L tRNA_{Leu}^{3} molecule (Figure 42), only the D_{EDTA} curve was comparable to the nonmodified 290 nm curve in both shape and resulting $T_{1/2}$ value (41° for the D_L form as compared to 43.5 for the nonmodified species). As observed at the $\lambda_{max}$, however, a large decrease in cooperativity was noted in the native and D_{Mg}^{++} curves as compared with the spectra of less extensively modified tRNAs. Both D_L
Figure 42. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_L$ tRNA$^{Leu}_3$ conformers, measured at 290 nm. Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

$\Delta \varepsilon(16^\circ) = 1.123$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = 0.545$ liter/cm-mole

Under $D_{Mg}^{++}$ conditions (□—□—□):

$\Delta \varepsilon(16^\circ) = 1.637$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = 0.574$ liter/cm-mole

Under $D_{EDTA}$ conditions (Δ——Δ):

$\Delta \varepsilon(16^\circ) = 0.987$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = 0.603$ liter/cm-mole

An estimated uncertainty of ±0.035 liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
curves reached a minimum just prior to their $\lambda_{\text{max}} T_{1/2}$ of 67.5°. Although little or no increase was observed after this minimum for both species, the temperature midway between this point and the final decrease in signal (71.5° and 73.5° for the native and $D_{Mg^{++}}$ forms, respectively) corresponded to the $T_{1/2}$ values of the $\lambda_{\text{max}}$ melting curves (Figure 35).

The effect of renaturation on the structural stability of tRNA$_{Leu}^\text{3}$ may also be seen in the melting curves obtained at 233 nm. The actual $\Delta \varepsilon$ values for the nonmodified species over the temperature range 16°-85° are shown in Figure 43. Again, due to the small magnitude of the change in the signal, however, the trends exhibited by each tRNA conformer are more easily visualized from comparisons of the percent change in $\Delta \varepsilon$ as a function of temperature. Thus this format only will be used for describing the melting behavior of all modified tRNA species.

As seen in Figures 43 and 44, the native, nonmodified tRNA structure was shown to melt cooperatively at the 233 nm wavelength. The $T_{1/2}$ value for this melting transition, however, as well as for the other denaturation curves obtained at this wavelength, was somewhat lower than those observed at higher wavelengths. Thus a value of 64.5° was noted for the native conformation at 233 nm as compared to the value of 70° obtained at 261 nm and 290 nm. In the presence of Mg$^{++}$, the denatured form exhibited a decrease...
Figure 43. CD thermal transitions of nonmodified tRNA\textsubscript{Leu}\textsuperscript{3} conformers, measured at 233 nm. Solvent conditions were those described in Figure 18. Values of $\Delta\varepsilon$ as a function of temperature are shown for the native (o--o), D\textsubscript{Mg}\textsuperscript{++} (o—-o), and D\textsubscript{EDTA} (Δ----Δ) conformers. An estimated uncertainty $\pm 0.070$ liter/cm-mole is present in the $\Delta\varepsilon$ values.
\[ \Delta \varepsilon_{233} \text{ (l/cm-mole)} \]

\[ T(\degree C) \]

Graph showing changes in \( \Delta \varepsilon_{233} \) with temperature (T) in degrees Celsius.
Figure 44. Relative change in $\Delta \varepsilon$ as a function of temperature for nonmodified tRNA$^\text{Leu}$ conformers, measured at 233 nm. Solvent conditions were those described in Figure 18.

For the native conformer (o---o):

\[
\Delta \varepsilon(16^\circ) = -0.635 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) = -0.847 \text{ liter/cm-mole}
\]

For the $D_{\text{Mg}^{++}}$ conformer (□ --- □):

\[
\Delta \varepsilon(16^\circ) = -0.549 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) = -0.945 \text{ liter/cm-mole}
\]

For the $D_{\text{EDTA}}$ conformer (Δ----Δ):

\[
\Delta \varepsilon(16^\circ) = -0.148 \text{ liter/cm-mole} \\
\Delta \varepsilon(85^\circ) = -1.254 \text{ liter/cm-mole}
\]

An estimated uncertainty of $\pm 0.070$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
in Δɛ similar to the $D_{\text{EDTA}}$ form until approximately 37.5°, at which temperature the conversion to the more stable native conformer occurred. As in the transitions monitored at longer wavelengths, the secondary structural interactions began to disrupt after approximately 55°, and a $T_{1/2}$ value of 65.5° was observed, comparable to that of the native structure. The greatest total change in Δɛ at 233 nm occurred in the melting of the $D_{\text{EDTA}}$ conformer, which exhibited a $T_{1/2}$ value of 37.5°.

A similar "renaturation" phenomenon was observed in the case of the $D_{\text{H2O}}$ samples as shown in Figure 45. Both of these denatured tRNA species melted similarly until 50°, the approximate temperature at which their melting curves would intersect that of the renaturing $D_{\text{Mg}^{++}}$ nonmodified species. At that point the presence of $\text{Mg}^{++}$ led to a shift in the structure to the native form, while in the absence of $\text{Mg}^{++}$, the melting transition continued without interruption. The $T_{1/2}$ value of 67.5° in the presence of $\text{Mg}^{++}$ was similar to that of the native structure. On the other hand, the $D_{\text{H2O}}$ species without $\text{Mg}^{++}$ exhibited a $T_{1/2}$ of 54.5°. This value, significantly larger than the 37.5° observed in the presence of EDTA, again indicated a greater stability for this conformation than for the $D_{\text{EDTA}}$ species. As mentioned earlier, this increased stability may possibly result from the incomplete removal of $\text{Mg}^{++}$ during the dialysis procedure.
Figure 45. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_\text{H}_2\text{O} \text{ tRNA}^\text{Leu}_3$ in the presence and absence of $\text{Mg}^{++}$, measured at 233 nm. Solvent conditions were those described in Figure 18.

In the presence of $\text{Mg}^{++}$ (○-○-○):

$$\Delta \varepsilon(16^\circ) = -0.740 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = -0.997 \text{ liter/cm-mole}$$

In the absence of $\text{Mg}^{++}$ (△-△-△):

$$\Delta \varepsilon(16^\circ) = -0.604 \text{ liter/cm-mole}$$
$$\Delta \varepsilon(85^\circ) = -1.051 \text{ liter/cm-mole}$$

An estimated uncertainty of ±0.100 liter/cm-mole is present in the $\Delta \varepsilon$ values.
The 233 nm melting curves obtained for the $N_L$ tRNA$^{Leu}_3$ species appear in Figure 46. These melting transitions were essentially identical to those for nonmodified tRNA, exhibiting $T_{1/2}$ values of 64°, 65.5°, and 36.5° for the native, $D_{Mg}^{++}$, and $D_{EDTA}$ conformers, respectively.

Figure 47 reveals a similar pattern in the melting curves for the $D_S$ modified tRNAs. The $T_{1/2}$ values for the native and $D_{EDTA}$ forms of 64° and 37.5° were in agreement with the data obtained from the spectra of the nonmodified tRNA. Although the $D_{Mg}^{++}$ curve essentially paralleled the native curve after 65°, a slight, presumably insignificant, shift in the curve resulted in a $T_{1/2}$ value of 68.5° for the $D_{Mg}^{++}$ species as compared to the native 65.5°.

As expected, the melting transitions for the $D_L$ forms differ notably from their less extensively modified counterparts, as shown in Figure 48. The $T_{1/2}$ value for the $D_{EDTA}$ species was slightly lowered to 35.5° while those of the native and $D_{Mg}^{++}$ species were significantly decreased to 48° and 47° respectively. These differences, which presumably resulted from the inability of the molecule to form the stable native structure, were greater at 233 nm than at the previously discussed wavelengths.

CD measurements were also employed to investigate the differences between tRNA$^{Leu}_3$ and its non-denaturable counterpart, tRNA$^{Leu}_{CUA}$. The sequence of this similar yeast tRNA molecule as determined by Randerath et al. (138) is
Figure 46. Relative change in Δε as a function of temperature for N_L tRNA_{3}^{Leu} conformers, measured at 233 nm. Solvent conditions were those described in Figure 18. Under native conditions (○—○):

\[ \Delta \varepsilon (16^\circ) = -0.678 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon (85^\circ) = -0.839 \text{ liter/cm-mole} \]

Under D_{Mg}^{++} conditions (□—□—□):

\[ \Delta \varepsilon (16^\circ) = -0.531 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon (85^\circ) = -0.758 \text{ liter/cm-mole} \]

Under D_{EDTA} conditions (Δ----Δ):

\[ \Delta \varepsilon (16^\circ) = -0.195 \text{ liter/cm-mole} \]
\[ \Delta \varepsilon (85^\circ) = -1.127 \text{ liter/cm-mole} \]

An estimated uncertainty of ±0.075 liter/cm-mole is present in the calculated Δε values.
Figure 47. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_S$ tRNA$^{Leu}_3$ conformers, measured at 233 nm. Solvent conditions were those described in Figure 18.

Under native conditions (o—o):

$\Delta \varepsilon(16^\circ) = -0.813$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = -0.915$ liter/cm-mole

Under $D_{Mg}^{++}$ conditions (□—□—□):

$\Delta \varepsilon(16^\circ) = -0.272$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = -0.841$ liter/cm-mole

Under $D_{EDTA}$ conditions (Δ—Δ—Δ):

$\Delta \varepsilon(16^\circ) = -0.219$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = -0.977$ liter/cm-mole

An estimated uncertainty of $\pm 0.075$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
Figure 48. Relative change in $\Delta \varepsilon$ as a function of temperature for $D_\text{L} \ tRNA_3^{\text{Leu}}$ conformers, measured at 233 nm. Solvent conditions were those described in Figure 18.

Under native conditions (o-o):

$\Delta \varepsilon(16^\circ) = -0.694$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = -0.920$ liter/cm-mole

Under $D_{\text{Mg}^{++}}$ conditions (□—□—□):

$\Delta \varepsilon(16^\circ) = -0.446$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = -0.919$ liter/cm-mole

Under $D_{\text{EDTA}}$ conditions (∆—∆—∆):

$\Delta \varepsilon(16^\circ) = -0.182$ liter/cm-mole  
$\Delta \varepsilon(85^\circ) = -0.905$ liter/cm-mole

An estimated uncertainty of $\pm 0.075$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
shown in Figure 49, and its CD spectrum at 25° appears in Figure 50. The occurrence of the CD maximum at 260.5 and the overall features of the curve were similar to those of native tRNA$_3^{Leu}$ (Figure 18). Details of the two spectra, however, were observed to differ notably. The major positive band for tRNA$_{CUA}^{Leu}$ was not only lower in magnitude but also somewhat shifted to shorter wavelengths. In addition, the dip in the region of 290 nm was more prominent than in tRNA$_3^{Leu}$, even crossing slightly below the baseline. The negative band at 233 nm, on the other hand, was significantly decreased in magnitude. It is interesting that differences of this extent occur in the CD spectra of two such closely related tRNAs which differ so slightly in nucleotide sequence.

The melting transitions of tRNA$_{CUA}^{Leu}$ were also investigated by UV absorbance and circular dichroism measurements. The UV absorbance curves after subjecting the tRNA to both renaturing and denaturing conditions (not shown) were quite similar to those of native tRNA$_3^{Leu}$ (Figure 23), and also exhibited a $T_{1/2}$ value of 76°. The CD melting curve data in terms of actual $\Delta\varepsilon$ and $\lambda_{max}$ values as well as the percent change in $\Delta\varepsilon$ as a function of temperature are shown in Figures 51-53. The transitions at 260.5 nm and 233 nm were similar to those of native tRNA$_3^{Leu}$ and exhibited comparable $T_{1/2}$ values of 69° and 64°, respectively. Although the $T_{1/2}$ at 290 nm of 69° agreed with its tRNA$_3^{Leu}$
Figure 49. Nucleotide sequence of yeast non-denaturable tRNA$^{Leu}_{CUA}$ (138) arranged in the cloverleaf secondary structure. Shaded areas indicate identical nucleotides in tRNA$^{Leu}_{CUA}$ and tRNA$^{Leu}_3$. 
Figure 50. CD spectrum of tRNA\textsubscript{\text{Leu}}\textsubscript{\text{CUA}} at 25° in 10 mM potassium cacodylate, pH 7.0, 10 mM borate, 1 mM EDTA, and 10 mM MgCl\textsubscript{2}.
Figure 51. CD thermal transition of tRNA^Leu$_{CUA}$. Solvent conditions were those described in Figure 50. Solid symbols (●—●) represent the $\lambda_{\text{max}}$ value at each temperature. Open symbols (○——○) indicate $\Delta \varepsilon$ values observed at the $\lambda_{\text{max}}$ in each spectrum. An estimated uncertainty of ±0.07 liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
Figure 52. CD thermal transition of tRNA$_{\text{CUA}}^{\text{Leu}}$
measured at 290 nm (o---o) and 233 nm (□—□—□).
Solvent conditions were those described in Figure 50. An estimated uncertainty of ±0.070 liter/cm-mole is present in the calculated Δε values.
Figure 53. Relative change in $\Delta \varepsilon$ as a function of temperature for tRNA$^{\text{Leu}}_{\text{CUA}}$, measured at $\lambda_{\text{max}}(25^\circ)$, 290 nm, and 233 nm. Solvent conditions were those described in Figure 50.

At $\lambda_{\text{max}}(25^\circ)$ (o—o):

$\Delta \varepsilon(16^\circ) = 7.81$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = 1.87$ liter/cm-mole

At 290 nm (□ — — — □):

$\Delta \varepsilon(16^\circ) = -0.218$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = +0.561$ liter/cm-mole

At 233 nm (Δ----Δ):

$\Delta \varepsilon(17^\circ) = -0.436$ liter/cm-mole

$\Delta \varepsilon(85^\circ) = -1.010$ liter/cm-mole

An estimated uncertainty of $\pm 0.070$ liter/cm-mole is present in the calculated $\Delta \varepsilon$ values.
counterpart, the overall nature of the melting curve below 69° differed notably from that of the denaturable tRNA_Leu^3 species (Figures 37 and 38). The observed discrepancies in the behavior of the two molecules most likely result from the differences in strength of the negative band occurring in this region. The possible relationship of this negative band to structural features important in the denaturation-renaturation process, as well as in the attainment of biological activity by the modified tRNA_Leu^3 molecules, remains to be clarified.
4. DISCUSSION

The results of the present studies provide information regarding the structural behavior of tRNA\textsubscript{Leu}\textsuperscript{3} in solution and give rise both to general implications concerning tRNA structure and to numerous suggestions for further investigation. In the kethoxalation studies, data were obtained which agree well with the findings derived from other experimental approaches to the study of tRNA\textsubscript{Leu}\textsuperscript{3}. A comparison of the location of the two exposed guanosine residues in the native cloverleaf structure with the nine positions of modification in the denatured base-pairing scheme proposed by Kearns and coworkers (280) is shown in Figure 54. In the native form the two conserved guanosine residues in the dihydrouridine loop are modified, thus implying that this portion of the tRNA sequence is positioned on the surface of the molecule. Such results are not surprising considering that these guanosines lie between the two variable regions of the D loop. If a common tertiary organization is indeed characteristic of tRNA molecules in general, it would require the positioning of this portion of the molecule on the surface in order to accommodate the observed differences in length among tRNA sequences. The X-ray crystallographic data of Kim \textit{et al.} have confirmed this prediction in the case of yeast tRNA\textsubscript{Phe} (109). In addition, the G at position 17 (using
Figure 54. Location of guanosine residues exposed to kethoxal modification in the secondary structures of native (Figure 54 A) and denatured (Figure 54 B) tRNA<sub>Leu</sub><sup>3</sup>. In each of the conformers, guanosine residues which are not involved in Watson-Crick base-pairing interactions yet are resistant to kethoxalation are designated by shading.
the proposed standard numbering system) has been found to be particularly susceptible to T1 RNase attack in tRNA\textsuperscript{Phe} (326), and the isotope labeling method of Gamble and Schimmel has shown the Gl7-Gl9 sequence to be among the most exposed regions in this tRNA with regard to tritium incorporation (171). Oligonucleotide binding studies have also indicated the availability of these residues in tRNA\textsuperscript{Phe} for hybridization interactions (128, 175). It is interesting to note that removal of the Y base in the anticodon region of tRNA\textsuperscript{Phe} decreases binding of the oligonucleotide U-C-C-C (complementary to residues 17-20) by five-fold (128). This decrease is accompanied by a similar decrease in the binding ability of the anticodon region of the molecule. Thus it is possible to envision an interaction between the two loops which may normally be prevented by the presence of the hydrophobic base. Since numerous studies have implicated the anticodon region of the tRNA molecule to be involved in synthetase recognition and aminoacylation, such a change in the anticodon structural characteristics could serve to explain the decrease in acceptor activity on loss of the Y base and the requirement for a hydrophobic modification at the Y position to allow proper aminoacylation (143).

Although a great deal of data implies that the conserved guanosine residues are on the surface of the molecule, several arguments indicate that they may not
always be available for chemical modification. The fact that the nature of these residues has been conserved in all tRNA molecules involved in protein synthesis implies that they may play a significant role in maintaining the structure-function relationships of the molecule, and thus might be concealed from modifying reagents by intramolecular structural interactions. X-ray data has in fact implied that in the crystal structure of tRNA$^{\text{Phe}}$ these residues are unavailable for modification due to involvement in tertiary hydrogen-bonding interactions with the $\psi$-C sequence in the T$\psi$C loop, a region also conserved among all non-initiator tRNA molecules participating in ribosomal protein synthesis (109). In addition, kethoxalation studies using yeast tRNA$^{\text{Phe}}$ and $\text{E. coli}$ tRNA$^{\text{Val}}$ have shown that while the G at position 19 is modified, the neighboring G17 and G18 are not (168). Such modification does seem to occur in tRNA$^{\text{Leu}}_3$, however, and thus it appears plausible that the reactivity of these residues depends not only on the method of investigation used, but also on the particular tRNA species under investigation.

Other studies have in fact indicated that although tertiary interactions involving residues G17 and G18 are weakened in tRNA$^{\text{Leu}}_3$, they have not been abolished. For example, oligonucleotide binding studies of the native tRNA$^{\text{Leu}}_3$ species have indicated a low level of binding by the D loop which suggests oligomer competition with
existing tRNA structure (275). It should also be noted that the extent of modification at positions 17 and 18 in the tRNA_{Leu}^3 native structure appeared to be significantly lower than that observed for the denatured conformer (see Chapter 3) which is known to possess a more "open" structure (182, 269, 280). The fact that these residues are kethoxalated in native tRNA_{Leu}^3, however, reflects a relatively "loose" structure in comparison with some tRNAs, which might be correlated with the longer variable arm, the longer β region in the D loop, or some other structural aspect of the molecule resulting from its particular nucleotide sequence (e.g. the anticodon conformation (127, 128), the ability to form a stable, denatured conformer (138), etc.).

It may also be recalled in this regard that the major difference in intramolecular distances determined by fluorescence energy transfer measurements and X-ray crystallographic data involved the distance between the D and TΨC loops. The solution studies using E. coli tRNA_{Met}^f and tRNA_{Glu}^2 (198) indicated a greater distance between these two regions of the molecule than that implied by crystallographic measurements based on yeast tRNA_{Phe} (232). It remains to be determined whether this discrepancy results from (i) different degrees of interaction between these two regions of the molecule in the solution and crystal states, (ii) an increase in the intramolecular distance
due to the size of the bulky fluorescent probes, or (iii) characteristic differences in the individual tRNA molecules themselves.

In addition to the two modified residues in native tRNA$^{Leu}_3$, a number of guanosines exist which remain unreacted even though not involved in traditional Watson-Crick cloverleaf base-pairing interactions. As indicated in Figure 54 such resistant residues appear at positions 9, 13, 15, 45b, 45i, 55, and 65.

Tertiary involvement of the region which includes residues 9, 13, and 15 has been supported by many experimental approaches. For example, treatment of E. coli tRNA$^{Leu}_l$ and tRNA$^{Met}_f$ with a carbodiimide derivative has shown residues U8 and G9 to be resistant to modification (127). In addition, oligonucleotide binding studies have shown the A-G-D16 sequence in the 5' portion of the tRNA$^{Phe}_D$ loop to be unavailable for trimer binding (175). In full agreement with these findings are both the observed resistance of G15 to T1 RNase digestion (325) and the results of isotope labeling experiments using tRNA$^{Phe}$, which show the A-G15 residues to be concealed within the tertiary structure (171). Possible tertiary interactions involving residues 9 and 15 have also been indicated by X-ray data as shown in Figure 2. It may be further noted with regard to residue G13, that tRNAs of the class D3VN (see Section 1.4) always possess a purine at position 13 while position 21 is always filled by an adenosine (109).
Kim et al. have suggested the possibility of purine-purine (non-Watson-Crick) hydrogen bonding between these two residues, along with the concomitant involvement of the conserved A21 residue in tertiary interactions with the base at position 9. In light of the additional observations which have been made concerning the existence of a Mg$^{++}$-binding site in this region (157, 188, 200-202), as well as the apparent requirement for a specific ordered structure in the D-stem region for synthetase recognition (110-113), it is not surprising that residues 9, 13, and 15 are resistant to kethoxalation in the native form of the tRNA$^{_{3\text{Leu}}}$ molecule.

As in the case of the D loop residues, evidence pertaining to the unmodified G at position 45b in the variable region indicates variability not only among native tRNA structures, but among techniques used to monitor residue availability as well. Treatment of E. coli tRNA$^{_{3\text{Tyr}}}$ with methoxyamine and carbodiimide derivatives did result in the modification of residues located in the loop region of the long variable stem. In addition, oligonucleotide binding (175) and tritium labeling (171) were observed to occur in this region of tRNA$^{_{\text{Phe}}}$, although to "intermediate" and "low" extents, respectively. On the other hand, the tRNA$^{_{\text{Phe}}}$ variable region was shown to be resistant to T1 RNase attack (260) and to kethoxalation (166-168). The low level of availability observed in some instances may perhaps be explained in terms of the
proposed tertiary interactions involving this region of
the tRNA^{Phe} molecule (109). A better model for compari-
son with tRNA^{Leu}_{3}, however, may be found in the yeast de-
naturable tRNA^{Ser}_structure. This molecule has been shown
to possess a long variable region also (as have all serine-
specific tRNAs), which is resistant to T1 RNase digestion
in the native molecule (260). Additional indications of
the involvement of such long variable regions in structural
interactions may also be seen in the study of tRNA Mg^{++}-
binding characteristics. In tRNA molecules possessing
such long regions, a class of binding sites is observed
which appears to be indicative of the folding back of the
variable arm onto the main structure (146). In addition,
aberrant structures involving the long variable regions
of E. coli tRNA^{Tyr}_I and tRNA^{Tyr}_II have been indicated to
appear during the melting transitions of these molecules
(147). Formation of the proposed "incorrect" hydrogen-
bonded structures, however, requires the prior melting of
tRNA tertiary structure to free the residues in the
variable arm loop for subsequent interactions. It is also
interesting to note the degree to which the length of the
variable arm is conserved among tRNAs of the same amino
acid specificity. In fact, the only similarity to be
noted among all leucine-specific tRNAs sequenced thus far
is the presence of a long variable region. Similarly, the
D3VN classification has been found to apply to all serine-
specific tRNAs, being conserved in phage, bacterial, yeast, and even mammalian tRNA species. This preservation throughout evolution seems to imply a functional importance for the variable region which is quite likely to be reflected in conformational interactions. The imposition of a specific tertiary structure on the region could thus render the region unavailable for modification reactions.

The guanosine residue at position 45i is most likely prevented from reaction with kethoxal by its relationships with neighboring residues. Although it does not form Watson-Crick hydrogen bonds with U43 to form a fourth base pair in the variable stem, high resolution nmr data has indicated that G45i is stacked on the adjacent A-U45h base pair. In addition, the conserved nature of nearby residues at positions 43 and 46 imply their involvement in tertiary interactions. In most tRNA molecules the conserved pyrimidine at position 46 is complementary to the conserved purine at position 15. Furthermore in all tRNAs belonging to the D3VN class, position 43 contains a pyrimidine which is always anticomplementary to the position 25 purine. A proposed trans base pairing between these latter two residues would result in identical structures for the G-U and A-C combinations (109). In agreement with this prediction is the protection of the pyrimidine at position 43 from chemical modification (127). In E. coli tRNA\textsuperscript{Leu}\textsubscript{1} the G at position 45k,
corresponding to G45 in tRNA^Leu\textsubscript{3}, is also resistant to modification \cite{127}. Although methylation of m\textsubscript{2}G25 in tRNA^Leu\textsubscript{3} possibly reduces its interactions with U43, it is likely that the two residues remain in close proximity. The failure of G45 to be kethoxalated is in agreement with its being concealed by such secondary and tertiary stacking arrangements.

The involvement of G55 of the T\Psi C loop in tertiary interactions has been indicated by all methods of investigation employed. Residues in the T\Psi C region have been found to be resistant to chemical modification (including kethoxalation) \cite{127, 162, 166-169}, as well as to isotope labeling \cite{171}, T1 RNase digestion \cite{260}, and oligonucleotide binding \cite{128, 175}. Although the G55 residue itself (a conserved purine) was not indicated by X-ray studies to participate directly in tertiary hydrogen-bonding interactions in tRNA^Phe \cite{109}, the T-\Psi-C residues on its 5\textsuperscript{'} side and the A on its 3\textsuperscript{'} side were implicated as playing specific roles in such structural features. The fact that some variability is allowed at position 55 (A\leftrightarrow G) among tRNA sequences confirms the observation that specific interactions are not required at this position. A purine structure, however, is apparently necessary for proper tRNA folding and function as the T\Psi C loop participates in forming the tRNA tertiary conformation.
The failure of kethoxal to modify G65 would be expected from the location of the residue within the acceptor stem. Although Watson-Crick base-pairing does not occur with the anticomplementary U6 residue, G65 is nonetheless apparently involved in and concealed by the base-stacking interactions of the helix. This observation, along with those previously mentioned, thus indicates a close correlation between the observed pattern of kethoxalation in native tRNA$_3^{Leu}$ and the results that would be predicted on the basis of known secondary and tertiary interactions in tRNA molecules.

The pattern of kethoxalation in the denatured tRNA$_3^{Leu}$ conformer is shown in Figure 54 as it would appear in the base-pairing scheme proposed by Kearns et al. (280). Once again residues 17 and 18 in the D loop are modified, although in this case modification occurs to a greater extent than observed in the native molecule. These results imply that the weak tertiary interactions involving the D loop in the native conformer are essentially lost in the conversion to the denatured form. The additional modification of residues 9 and 15, previously concealed by this tertiary structure, also indicates the loss of specific tertiary interactions in this region. The reaction of G22 and G23 with the reagent reflects a loss of base-pairing among the D stem residues, and modification of G29, G41, and G42 implies the destruction of the anti-
In accordance with the Kearns base-pairing scheme, however, some non-base-paired guanosine residues remain unmodified in the denatured molecule. These include residues 10, 13, 45b, 45i, and 65. In the case of \( \text{m}^2\text{G}10 \), the kethoxalation of neighboring residue 9 makes it appear unlikely that extensive structural interactions are responsible for the lack of modification. A more probable explanation would appear to lie in the methylation at the N2 position of the residue. As may be seen from the structure of the kethoxal adduct of guanosine (Figure 5), such methylation could sterically hinder formation of the modified product and thus essentially prevent the modification reaction at this position.

The failure of kethoxal to modify G13 also does not appear to result from concealment within a compact structure. Nearby G15 is observed to be modified, and the loss of the D stem helix reduces the possibility of the purine-purine interaction with A21 which is postulated to occur in the native structure. In addition the G9 residue, believed to interact with G13 and A21 in the native molecule, is kethoxalated in the denatured species. The possibility exists that the G13 residue is indeed modified and is contained in one of the longer oligonucleotides found in peak 7 of the Tl digest (Figure 8B). On the other hand, some form of intramolecular interaction...
involving this residue may occur which is not apparent at this time. Further experimentation will be required to clarify this point.

As in the native conformer, the lack of modification of residues 45b and 45i in the variable region of the denatured molecule could perhaps result from involvement in base-stacking interactions with the neighboring residues in the variable stem helix. It is also possible, however, that the variable stem region might be involved in structural interactions which serve to stabilize the denatured conformation. Such interactions are believed to occur in the case of *E. coli* tRNA\textsubscript{Tyr} \textsc{I} and tRNA\textsubscript{Tyr} \textsc{II} which differ in sequence only in the loop region at the tip of their variable stems (147). Differences in the melting characteristics of the two molecules have thus been attributed to the differences in stability of intermediate structures formed as a result of variable region interactions. The nature of such possible interactions involving the variable stem of tRNA\textsubscript{Leu} \textsc{III} is not clear at this point. It is interesting to note, however, that the non-denaturable tRNA\textsubscript{Leu} \textsc{CUA} species differs from tRNA\textsubscript{Leu} \textsc{III} in three of the four residues in the loop region at the tip of the variable stem. In contrast, the remainder of the variable region, as well as much of the remainder of the molecule, is identical in sequence to the denaturable tRNA\textsubscript{Leu} \textsc{III} species.
As in the native structure the guanosine at position 65 is not kethoxalated due to its involvement in stacking interactions in the acceptor stem helix. In fact, its resistance to modification may be considered a further indication of the retention of the helical nature of this region of the molecule in the denatured conformation. This factor may in turn be responsible for the ability of the denatured conformer to bind the cognate synthetase. The disruption or alteration of other structural features (most likely the D stem and/or the anticodon structure), however, results in prevention of the subsequent aminoacylation reaction.

The results of the kethoxalation studies are also in good agreement with other investigations of the structure of denatured tRNA$_{Leu}^3$. The identity of oligonucleotides obtained in partial T1 RNase digestions of the denatured tRNA$_{Leu}^3$ implied that the D loop was the most readily digested region of the molecule (322). The D stem residues were also susceptible to the enzyme, while the anticodon loop was relatively resistant to degradation. In addition, oligonucleotide binding studies of the denatured conformer demonstrated the former D loop and stem regions to exhibit strong oligomer binding characteristics indicative of essentially random RNA segments. On the other hand, the anticodon loop became inaccessible to the complementary oligomers. Thus the
base-pairing of the D stem and the interaction between the D and TψC loops in the native conformer again appeared to be replaced by interactions involving the anticodon and the TψC loops in the denatured form. It may be noted that the anticodon stem, which NMR data indicated to be disrupted in the denatured form, was nonetheless found to be unavailable for oligomer binding. As observed by Uhlenbeck et al., however, the method used for binding data analysis could lead to a low binding constant if the conformation of a single-stranded RNA segment were constrained, and thus its affinity for the complementary tetramer reduced. The observation of positive binding should therefore be considered more significant than the indication of negative binding.

Kethoxalation data were also in agreement with the results of tritium exchange studies. The transition from denatured to native conformers was shown to result in a gain of 3-5 base pairs reflecting disruption of "wrong" structural interactions, repair of the cloverleaf hydrogen bonds, and formation of native tRNA tertiary interactions. Such observations were also in agreement with the large activation energy observed for the renaturation process (268).

Strikingly similar results have been obtained in the study of other denaturable tRNA species. Recently Streek and Zachau have examined the native and denatured forms of
yeast tRNA$^\text{Phe}$ and tRNA$^\text{Ser}$ (259, 260). Both tRNA species exhibited hyperchromic changes on going from the native to the denatured conformer similar to those observed for tRNA$^\text{Leu}$ and indicative of differences in base-pairing and base-stacking interactions. Further characterization of these differences was carried out using limited nuclease digestion by T1, T2, and pancreatic RNases, sheep kidney nuclease, and acid RNase from hog spleen. Internucleotide bonds susceptible to the nucleases were considered to be located in accessible non-hydrogen-bonded and weakly stacked regions of the tRNA structure. In the study of tRNA$^\text{Phe}$, D stem and anticodon stem residues which were protected in the native structure became exposed in the denatured conformation. On the other hand, residues in the anticodon loop became inaccessible after denaturation, as observed for tRNA$^\text{Leu}$. In contrast, however, certain D loop residues appeared to be less available in denatured tRNA$^\text{Phe}$ than in the native form, implying the possible involvement of these residues in the structural interactions of the denatured molecule.

A reduction in the rate of digestion of the anticodon region was also found to accompany the denaturation of tRNA$^\text{Ser}$. In addition, splitting of native tRNA$^\text{Ser}$ in the anticodon region resulted in the loss of its ability to attain the denatured conformation. Thus the anticodon region was suggested to play a role in stabilization of
the denatured conformation. As in other denaturable tRNA molecules, the D stem (the least stable tRNA helical region) appeared to be disrupted. In addition, however, the variable stem of tRNA\textsuperscript{Ser} appeared to become exposed in the denatured state, contrary to the observations concerning tRNA\textsubscript{Leu} behavior.

Studies have also been conducted to elucidate the nature of the denatured form of \textit{E. coli} tRNA\textsuperscript{Trp} (169, 255, 326). As for the other denaturable species, hyperchromicity is observed (327) and renaturation requires considerable activation energy (328). A mutant species of tRNA\textsuperscript{Trp} which possesses U-G-A suppressor activity has also been isolated and shown to be more stable to denaturation than the wild type molecule. Unlike most suppressor species in which the mutation is in the anticodon sequence, tRNA\textsuperscript{Trp}_{su\textsuperscript{UGA}} differs from the wild type by a single base change at position 23 in the D stem (35). This G→A change results in the replacement of a G·U "base pair" in the D stem with a more stable A·U pair. The stability imparted to the molecule by this alteration again implies that the disruption of the D stem is involved in the denaturation process. In addition, once the denatured conformer of the suppressor tRNA species is formed, it is found to be less stable than that of the wild type. Thermodynamic studies of the renaturation process for both tRNA species thus suggested that the G23 in the wild
type molecule usually pairs with a C residue in the denatured conformation, therefore serving to stabilize the denatured structure. Possible base-pairing schemes were proposed which incorporated both the known stability of the acceptor stem and the previous observation that photochemical cross-linking of residues 8 and 13 did not affect denaturation (326). Thus the region containing these residues either was conserved in structure during the denaturation process or became a part of a largely unstructured region. The first of these possibilities was included in a model which involved base pairing of D-stem region residues 23-26 with residues 42-45a in the short variable region. A second model, placing residues 8 and 13 in a relatively unstructured region, included pairing of residues 20-23 in the D stem with TΨC stem residues 59-62. The latter scheme involved a G23-C59 base pair and thus was favored on the basis of the relative stabilities of the mutant and wild-type species.

Recent studies have also been reported involving kethoxal modification of native and denatured tRNATrp (169). Although no incorporation of the reagent into the native conformer was observed, five exposed guanosines appeared to be characteristic of the denatured structure. These included guanosines 10, 41, and 43 in the D stem and anticodon stem regions as well as the two conserved guanosines at positions 17 and 18 in the D loop.
Differences in the relative reactivities of the exposed residues were also observed, with G41 on the 3' side of the anticodon stem being the most reactive with kethoxal, and residues 10 and 17 being the next most reactive. The failure to kethoxalate G21 and G23 of the 5' portion of the D stem is in agreement with the postulated base-pairing of these residues in the denatured conformation (326).

The results of such studies have thus revealed several features which appear to be characteristic of the tRNA denaturation process in general. As might be expected from the low stability of the D stem in comparison with the other cloverleaf helices, all stable denatured conformers studied thus far seem to have lost the D stem base-paired structure. In addition, all or a portion of the anticodon stem appears to be disrupted, with the resulting involvement of the anticodon region in non-cloverleaf base-pairing interactions. On the other hand, the stable acceptor stem has generally been retained in models of denatured tRNAs and may account for the observed ability of these molecules to bind their cognate synthetases. The loss of D stem and/or anticodon region structure, however, appears to parallel a loss in the ability of the denatured tRNAs to be aminoacylated.

Beyond this point, however, differences are observed in the nature and stability of the denatured structures which appear to result from individual differences in primary sequence. Thus base-pairing
schemes in the stable denatured conformers apparently occur on the basis of the possible sequence interactions available, and various combinations of cloverleaf structural regions are involved. These variations account for the fact that the CD difference spectra of denatured versus native species of tRNA$_{Leu}^3$ and tRNA$_{Trp}^T$ differ markedly (180). They are also reflected in the loss of denatured structure stability in tRNA$_{Trp}^{s_{u+UGA}}$ and its absence in tRNA$_{Leu}^{CUA}$ which differ slightly in sequence from their denaturable counterparts (35, 138). These observations serve to further confirm the proposal by Fresco and co-workers that conformational mobility is characteristic of all tRNA molecules (268). Those species possessing alternate structures of sufficient stability may be trapped in inactive, denatured conformations which may, in fact, be more stable than their native counterparts in the absence of Mg$^{++}$ (268, 280). Although the existence of these denatured species in vivo remains to be demonstrated, the possible role of such conformational changes in the control of tRNA activity within the cell may easily be envisioned.

A further look into the nature of the denatured structure of tRNA$_{Leu}^3$ may be obtained from the results of the CD measurements. A comparison of the native and D$_{EDTA}$ spectra of the unmodified species at $25^\circ$ reveals a shift in both the height and position of the CD maximum accompanying denaturation. Comparison with model
compounds such as the copolymer poly (U₂, G) has indicated that such a decrease in the maximum at 260 nm may be attributed primarily to the loss of base-stacking interactions, while a red shift in this maximum is indicative of the loss of base pairs (261, 300, 330). Thus the tRNA molecule appears to have lost both base-stacking and base-pairing interactions in the transition from the native to the D\textsubscript{EDTA} conformer. These results are consistent with those of other experimental approaches and agree well with the model proposed by Kearns \textit{et al.} for the denatured base-pairing structure (Figure 54). Since the nmr data were obtained under Mg\textsuperscript{++}-free conditions similar to those employed for the CD spectra of D\textsubscript{EDTA}, it may be assumed that the hydrogen-bonding patterns observed in the nmr studies also apply to the CD measurements. Thus the changes in position and height of the D\textsubscript{EDTA} spectrum as compared to that of the native conformer at 25° may be considered equivalent to an overall loss of four base pairs (net loss of five G•C pairs and gain of one A•U pair) as well as to the decrease in base-stacking interactions accompanying the loss of the D and anticodon stems and the overall conversion from native to denatured tertiary structure. The 6.9% hyperchromicity observed for the D\textsubscript{EDTA} form in comparison to the native molecule is also in agreement with these changes.

The addition of Mg\textsuperscript{++} to the D\textsubscript{EDTA} form of the molecule results in an increase in height of the positive
CD band indicative of additional base-stacking inter-
actions. Such an increase would be expected if the
molecule assumes a more compact structure in the presence
of the divalent cation. Stabilization of this type is
further indicated by the 3.0% hypochromicity of the D_{Mg}^{++}
form in relation to the D_{EDTA} form, with a resulting
decrease in hyperchromicity to 3.8% with respect to the
native form. This remaining hyperchromicity accounts for
the decrease in UV absorbance of tRNA_{Leu}^{3} over the tempera-
ture range 35°-45° (Figure 23) which accompanies the
proposed conversion from the denatured to the native con-
formation. It is also in agreement with the decreased
rate of modification of the denatured conformer in the
presence of Mg^{++} during the secondary phase of the
kethoxalation reaction. Thus the addition of Mg^{++} seems
to reduce the flexibility of the denatured conformer,
possibly by the imposition of tertiary structural inter-
actions on the Kearns base-pairing scheme.

At the same time, however, the further red shift of
the D_{Mg}^{++} positive band appears to indicate the further
loss of base-pairing interactions. If the observed shift
of approximately 1 nm for the D_{EDTA} spectrum is indicative
of a loss of four base-pairs, then from a qualitative
point of view, the additional shift of a comparable 1.5 nm
may be considered to imply a similar reduction in the
number of base pairs present. This loss could possibly
be attributed to the disruption of the newly-formed base pairs involving anticodon and TΨC loop residues. The stability of this short helix is not known at the present time, although it is possible that its composition of three A•U base-pairs and one G•m5C base pair results in a relatively low degree of stability in comparison with the acceptor and TΨC stems, both of which are longer and possess a higher G•C content. The major source of instability, however, would probably be found in conformational constraints placed on the regions of the molecule involved. Such constraints could lead to a significant reduction in the expected affinity of the complementary bases in the new stem region (128). The further imposition of tertiary interactions on the overall structure, perhaps involving residues G13 and G45b (resistant to kethoxalation in the D_{Mg}++ form) and resulting in increased base-stacking and decreased flexibility, might serve to increase these constraints to the point that the new interactions are no longer possible. The existence of such constraints on these regions of the molecule would further serve to explain the absence of oligonucleotide binding for the anticodon stem residues in the D_{Mg}++ form (275), while kethoxalation studies imply that they are not involved in hydrogen-bonding interactions.

It should be kept in mind, however, that these analyses rely heavily on the qualitative interpretation
of the observed changes in height and position of the CD maxima. Although the CD data thus do not prove the disruption of the new helix in the $D_{\text{Mg}^{++}}$ form, their implications do provide useful predictions which may be tested by other experimental approaches. Perhaps the most revealing of such approaches would be the nmr study of the denatured conformer in the presence of $\text{Mg}^{++}$. It would also be of interest to determine, by both CD and nmr measurements, whether increased concentrations of a monovalent cation (e.g. 1 M Na$^+$) would be able to produce the same conformational effect.

In addition, the CD data imply that the renaturation of $\text{tRNA}_{\text{Leu}}^3$ is a two-step process. The first step to occur on addition of $\text{Mg}^{++}$ to the denatured form is the rapid conversion of the $D_{\text{EDTA}}$ state to the $D_{\text{Mg}^{++}}$ conformation. In this step a number of base-stacking interactions are gained, although they are possibly different than those characteristic of the native molecule. The $D_{\text{Mg}^{++}}$ form, which is present at the 10 mM $\text{Mg}^{++}$ concentration used in measuring enzyme activity, retains less than 10% of the leucine acceptor activity of the native structure although it does appear to bind the cognate synthetase (271). In the second, slow renaturation step the molecule finds its way back to the native conformation. This process requires the breaking of incorrect structural interactions and the reforming of the native secondary and tertiary
structure characteristics. At room temperature this rearrangement requires days, while at slightly elevated temperatures it may be completed in a matter of hours or minutes. Quite similar results were obtained in the study of the mechanism for the renaturation of yeast tRNA$^{\text{Ser}}$ and tRNA$^{\text{Phe}}$. Streek and Zachau reported that the hyperchromicity which accompanied the denaturation of these molecules disappeared much faster during the course of renaturation than the amino acid acceptor activity was regained. Thus their results also implied that the refolding process involved a rapid increase in base stacking, followed by a slower conformational change exhibiting little further increase in base-stacking interactions. Only this slow process restored the biological activity of the two tRNA species.

It is interesting to note in this regard that the differences in the height and position of the positive CD bands of the two conformers of tRNA$^{\text{Leu}}_3$ would make it possible to monitor the conversion of the D$_{\text{Mg}^{++}}$ form to the native structure as a function of time and temperature. Such measurements could then be used to obtain values for the activation energy and the activation enthalpy parameters which are characteristic of this portion of the renaturation process. The determination of these values would provide added insight into the nature of the changes which occur during the refolding process.
The fact that Mg$^{++}$ becomes tightly bound during the formation of the native conformer is evidenced in the CD spectrum of tRNA$^\text{Leu}_3$ which has been dialyzed extensively against distilled water ($\text{D}_{\text{H}_2\text{O}}$). Although a slight decrease in base stacking may be observed, the overall spectrum is quite similar to that of the native conformer. This retention of conformation appears to result from the presence of Mg$^{++}$ since addition of EDTA leads to a rapid collapse of the structure to the $\text{D}_{\text{EDTA}}$ conformation. The affinity of the tRNA for the small amount of Mg$^{++}$ present is apparently quite high as evidenced by the failure of the tRNA to convert to the denatured conformation after heating at 60° for 5 minutes (in the absence of EDTA) and quick-cooling to -4°. Such treatment would disrupt all secondary and tertiary interactions in the tRNA molecule, except perhaps the acceptor stem which nmr data has shown to be intact at 62°. Thus either the Mg$^{++}$ is interacting with the acceptor stem, or it is reincorporated into the tRNA structure as the molecule rapidly refolds. In either case, its presence is sufficient to allow formation of a native-like structure. In the absence of added Mg$^{++}$ this structure possesses greater thermal stability than the $\text{D}_{\text{EDTA}}$ conformation but less than the native structure. In the presence of added Mg$^{++}$ it is observed to undergo conversion to the completely native conformer at moderate temperatures. Further indication that the "native-like"
structure of the $D_{H_2O}$ species lacks some of the structural characteristics of the native conformation is obtained from assays for amino acid acceptor activity. At 25° the $D_{H_2O}$ conformation exhibits only 35% of the native activity. During the ten minute assay at 37° (in the presence of Mg$^{++}$), however, sufficient renaturation takes place to increase the level of activity to 60% of the native conformer. These results provide additional evidence that some energy barrier must be overcome to allow conversion of the $D_{H_2O}$ structure to the native conformation.

The results obtained with the $D_{H_2O}$ conformer are in agreement with the study of Danchin (146) which indicated the existence of two classes of Mg$^{++}$ binding sites in tRNA molecules. The first of these classes exhibits strong cooperative binding characteristics which suggest the occurrence of accompanying conformational changes at free cation concentrations as low as 3 μM. Thus the conformational change resulting from the binding of one ion facilitates the binding of the next, up to a total of six ions, at which point the conformational change is complete. This binding at low cation concentrations is believed to occur at specific Mg$^{++}$ binding sites. Subsequent independent binding (up to approximately 25 Mg$^{++}$ ions per tRNA molecule) appears to be more random and probably serves to reduce the electrostatic repulsion of the tRNA
phosphate groups. This latter function might also be filled by high monovalent cation concentrations which have been shown to induce the formation of tRNA structures similar, but not identical, to those formed in the presence of Mg\(^{++}\) (146, 268). Although the first class of binding sites accounts for some of the conformational changes which occur during renaturation, the secondary binding of Mg\(^{++}\) at independent sites was shown to be required for the interaction of tRNA with its cognate synthetase. A third class of binding sites of intermediate strength was observed in tRNA molecules possessing a long variable region which was able to fold back onto the main structure. In light of these results, it would be of interest to determine the amount of Mg\(^{++}\) present per molecule of the \(\text{D}_{\text{H}_2\text{O}}\text{tRNA}^{\text{Leu}}_3\) species, both before and after heat treatment at 60\(^\circ\). Further investigation of the changes which occur during titration of the \(\text{D}_{\text{H}_2\text{O}}\) species with EDTA, as well as of the conformational changes accompanying increased Mg\(^{++}\) concentration, could provide valuable information regarding the role of the Mg\(^{++}\) ion in determining tRNA structure and stability.

Modification of native \(\text{tRNA}^{\text{Leu}}_3\) with kethoxal results in little conformational change as indicated by the CD spectrum and amino acid acceptor activity measurements. The slight decrease in the CD maximum at 25\(^\circ\) as compared to the nonmodified species suggests that a small loss of
base-stacking interactions may accompany the modification of the D loop residues. Since these residues have been shown to be partly shielded from oligomer binding and kethoxalation in the native molecule, it is not surprising that the addition of a bulky kethoxal group appears to affect the interactions in which they participate. This interference could also account for the slight decrease in acceptor activity of the molecule (less than 10%), since the structure of the D stem region has been shown to be important in aminoacylation reactions (110). The slight alteration in structure, however, does not affect the overall thermal stability of the molecule as indicated by the similarity in the melting curves and $T_{1/2}$ values for the nonmodified and $N_L$ species.

The CD spectra of tRNA$_3^{Leu}$ modified to a limited extent in the denatured form ($D_S$) are also quite similar to those of the nonmodified and $N_L$ species. It appears that the additional kethoxal groups have little or no effect on the $D_{EDTA}$ form of the molecule, while modification interferes slightly with formation of some of the base-stacking interactions in the $D_{Mg^{++}}$ conformer. It is surprising, however, that kethoxalation of D stem and anticodon stem residues does not appear to affect significantly the re-formation of a "native-like" structure. Although the "native" $D_S$ spectrum is slightly broader than that of the $N_L$ species, resulting in a
decreased dip in the 290 nm region, its similarity in terms of the position and height of the positive CD band as well as its overall appearance implies that much of the base-stacking and base-pairing nature of the nonmodified molecule is conserved. It thus becomes necessary to investigate further the nature of the kethoxal adduct (Figure 5). At first glance it becomes apparent that modification interferes with normal base-pairing interactions involving the N1 and N2 positions of the guanine ring. Examination of space-filling models, however, reveals that the newly formed hydroxyl groups of the kethoxal adduct may be positioned in a manner which enables them to participate in base-pairing interactions in place of the guanine NH groups. Needless to say, the presence of the bulky kethoxal moiety would necessitate an increase in the distance between the hydrogen-bonded bases and might require other alterations in the normal position of the bases with respect to one another as well. In addition, all three hydrogen-bonding interactions between C and G might not be conserved in the kethoxalated base pair due to constraints imposed by the polynucleotide chain on the relative orientation of the bases. Arguments in support of the possibility of *G·C base pairing, however, may be gained from X-ray crystallographic studies of purine and pyrimidine interactions (330). Considerable variability has been observed in terms of the types of
hydrogen bonds that are found between such molecular
species. In addition an important variable in such inter-
actions has been found to reside in differences between
hydrogen bond lengths. Thus it is possible to envision
hydrogen-bonding variations which could make possible the
formation of helices involving *G.C base pairs. The
effect of such alterations on the polynucleotide backbone,
and thus on tRNA conformation, however, remains to be
determined.

Although the CD data suggests that such hydrogen
bonding does occur in the modified D$_S$ molecule, further
evidence will be required to confirm these results. 
Computer analysis of the effect of kethoxalation on the
structure of a G-containing polynucleotide helix could
provide an indication of the perturbations which would
occur in the backbone of the chain as a result of residue
modification. Thus information could be obtained regarding
the extent to which *G.C base pairing might be compatible
with known helical structure. In addition, a study of
the CD behavior of the modified and nonmodified base-
pairing copolymer poly (C,G) could provide more direct
evidence regarding the existence and stability of *G.C
base pairs. Furthermore, an nmr investigation of the
structure of kethoxalated (D$_S$) tRNA$_3^{Leu}$ could possibly
reveal first-hand information regarding the existence of
*G.C base-pairing interactions in the "renatured" D$_S$
molecule. It has been found that low field resonances from base pairs in a double helix are shifted upfield from their intrinsic positions as a result of ring current fields generated by neighboring bases (279). Although the *G·C base pairs themselves would not be expected to exhibit a low field resonance (due to the lack of involvement of the guanosine ring NH proton in the proposed pairing interaction), the behavior of A·U base pairs in the anticodon stem might provide evidence regarding the formation of a helical structure. Experiments along this line are currently in progress in collaboration with Dr. D. R. Kearns at the University of California, San Diego.

It should also be noted that the kethoxalated Dₕ species appears to possess the same degree of thermal stability exhibited by the nonmodified and Nₜ molecules. This observation could in part be explained in terms of the re-formation of the modified D-stem and anticodon-stem helical structures. It would seem likely, however, that the stability of the resulting helices would be lower than that of their nonmodified counterparts. It may be recalled in this regard that the D stem is already relatively unstable in the native conformer, being susceptible to competitive oligonucleotide binding. Thus the effect on the overall tRNA structure of additional destabilizing interactions in this region might be negligible. Additional arguments would be required,
however, to explain the behavior of the anticodon stem, which is significantly more stable than the D stem in the nonmodified structure. The most likely explanation, therefore, for the observed thermal stability of the D$_S$ tRNA$^\text{Leu}_3$ species lies in the assumption that the major basis for such stability is found in some structural feature of the molecule other than the D and anticodon stem helices. Such stabilization of overall structure beyond the $T_m$ of less stable helical regions has been observed in the cooperative melting behavior of tRNA molecules in the presence of Mg$^{++}$ and has been attributed to the imposition of tertiary interactions on the basic cloverleaf structure (191). Thus modification of the D and anticodon stems might not be observed to affect greatly the overall melting behavior of the D$_S$ tRNA molecule.

In spite of its thermal stability, however, the "native-like" D$_S$ species has lost approximately 70\% of its leucine acceptor activity. The cause of this inactivation may stem from one or more of several factors. First, the positive CD band of the D$_S$ spectrum is seen to exhibit a slight red shift, continuing the trend begun by the N$_L$ spectrum. Thus a further weakening or disruption of base-pairing interactions apparently occurs to some degree in the D$_S$ conformer. Although these interactions do not appear to influence the stability of the molecule to
thermal denaturation, it is possible that changes occur which specifically prevent its recognition by the synthetase and/or its subsequent aminoacylation. Enzyme inhibition studies using $D_g\ tRNA_{3}^{Leu}$ could be carried out in order to distinguish between these two possibilities.

In addition to conformational differences, however, a possible cause of inactivation might arise from the modification of a single guanosine residue which is required for interaction with the synthetase. The identification of such a residue could be determined by following the rate of kethoxal incorporation at each modified position in correlation with the loss of overall acceptor activity. Attempts to identify such an inactivation target in $tRNA^{Phe}$, however, have thus far been unsuccessful (167, 168).

It should particularly be noted with regard to the CD studies of the modified $D_g\ tRNA_{3}^{Leu}$ species that the possibility of dekethoxalation during the melting curve determinations has not been eliminated. Complexation with borate, however, which was present in all solutions for CD study, has been shown to prevent decomposition of the kethoxal-guanosine adduct for as long as 43 hours at pH 8.0 and 25° (166). In addition, although some $[^{3}\text{H}]$-kethoxal was lost during the six hour Tl RNase digestion in the presence of borate at 37° and pH 7.5, as well as during the subsequent days of fragment purification at
room temperature, pH 7.5, a large portion of the initial adduct remained intact. It seems unlikely, therefore, that the amount of dekethoxalation which might have occurred during the CD measurements at pH 7.0 over the temperature range 30°-40° would alone be sufficient to allow for the observed return to the native-like spectrum.

The larger alterations in the D_2 CD spectra reflect further decreases in base-stacking interactions in the extensively kethoxalated molecule. In addition, a slight destabilization is observed to occur in the melting profile which is accompanied by an overall decrease in the cooperativity of the melting transition. The observed red shift of all curves on melting, however, in addition to the degree of stability retained, indicates that hydrogen-bonding does occur in the molecule. If essentially all G residues are modified, these base-pairing interactions imply that kethoxalated residues may indeed participate in hydrogen bonds. On the other hand, quantitative incorporation data is not available for the non-radioactive kethoxal derivatives, and it is possible that some guanosines remained unmodified or subsequently lost their modifying groups. Nonetheless, these experiments demonstrate that it is possible to kethoxalate sufficient residues in a tRNA molecule to prevent not only enzymatic aminoacylation, but also the ability to re-form the basic features of the native tRNA structure. Enzyme inhibition
studies might prove useful in determining whether or not the $D_L$ molecules retain sufficient amounts of structure to be recognized at all by the cognate synthetase. Whether the failure to form the native conformation is the result of an overall reduction in ability to form base-stacking and base-pairing interactions or to the modification of guanosines at specific locations strategic in the refolding process remains to be determined.

Further clarification of the structural features involved in the formation of the stable denatured conformer of $tRNA_{3}^{Leu}$ may be obtained from a comparison with the non-denaturable $tRNA_{CUA}^{Leu}$. As illustrated in Figure 49, 66 of a total of 85 nucleotides are identical in the two species. It is particularly significant to note in this regard that the sequence of the D stem is the same in both molecules. The fact that two of the three base pairs in this short stem region involve modified nucleotides had previously been postulated to account for the instability of the stem and perhaps for the ability of the $tRNA_{3}^{Leu}$ molecule to attain a stable, non-cloverleaf, denatured conformation (322). The identical D-stem sequence of non-denaturable $tRNA_{CUA}^{Leu}$ discounts this possibility. It does not necessarily imply, however, that the stability of the region is identical in the two molecules, since higher order interactions may also have an effect on the structure in the D stem and loop region.
Among the most significant differences between the two molecules lies in their anticodon sequences. Since the anticodon region has been implicated to be involved in the formation of the denatured base-pairing scheme, it may easily be seen that sequence changes in the region could affect the denaturation process. Indeed the sequence changes observed at the 5' end of the anticodon \((\text{m}^5\text{C}\rightarrow\text{U})\) and at position 56 in the \(\Psi\text{C}\) loop \((\text{A}\rightarrow\text{m}^1\text{A})\) act to effectively prevent the formation of the new helix in the proposed denatured structure. Thus these changes would significantly reduce stabilization of the denatured conformation.

Additional changes which might prove to be important in the denaturation process occur in the loop of the variable stem. The guanosine in this region of the \(\text{tRNA}_{3}^{\text{Leu}}\) molecule is not modified in the kethoxalation reaction involving either the native or the denatured conformer. Therefore, the possibility exists that it and its neighboring residues are involved in stabilizing tertiary interactions in both structures. The fact that three out of the four nucleotides in this region differ between the \(\text{tRNA}_{3}^{\text{Leu}}\) and \(\text{tRNA}_{3}^{\text{Leu}}\) species could thus also have an effect on the degree of stability and consequently the likelihood of formation of the denatured conformation. It would be of interest to carry out kethoxalation studies and more complete CD measurements with the \(\text{tRNA}_{3}^{\text{Leu}}\) species as described for \(\text{tRNA}_{3}^{\text{Leu}}\). A comparison of such results might
provide further information concerning the nature of secondary and tertiary structural differences between the two molecules.

A further note of interest regarding the possible evolutionary relationship of these two very similar tRNAs may be made with regard to the identity of the 3' residue in the anticodon sequence and the adjacent m^1G56. The nature of the modified purine at position 56 has been indicated by a number of studies to be related to the codon recognized by a tRNA molecule. Thus most tRNAs which exhibit m^1G at residue 56 recognize a codon beginning with C, while the majority of tRNAs which recognize codons beginning with U possess ms^2i^6A or i^6A at this position (see Section 1.4). The former rule is observed in the sequence of tRNA\textsubscript{Leu}\textsubscript{CUA}, but tRNA\textsubscript{Leu}\textsuperscript{3}, which recognizes the codon U-U-G provides an exception to the latter observation. It thus appears possible that tRNA\textsuperscript{Leu}\textsubscript{3} might have arisen from the tRNA\textsubscript{Leu}\textsubscript{CUA} species as a result of gene duplication and subsequent base substitution mutations. The fact that mutations appear to have been conserved and perhaps even selected in the tRNA\textsubscript{Leu}\textsuperscript{3} molecule which seem to be advantageous for the stabilization of the denatured conformer (i.e. in the anticodon and variable stem loop regions) could be construed as evidence in favor of a significant biological role for the denaturation process.
Although the existence of denatured tRNA species has not yet been demonstrated to occur in vivo, the occurrence of such structures in vitro provides evidence of a structural flexibility which could conceivably play a significant role in tRNA function. Such flexibility, for example, could serve as a possible means of controlling the level of active tRNA within the cell or as a basis for enabling the tRNA molecule to participate in its many functional interactions with a variety of RNA and protein species. The studies presented here provide further evidence regarding the conformational changes available to the tRNA molecule in solution and the extreme sensitivity of such changes to the immediate environment in terms of divalent ion concentration. Extension and clarification of these results is now in progress in the study of tRNA$_{3}^{Leu}$ using the powerful techniques of high resolution nmr spectroscopy and X-ray crystallography. As stated by Kim et al. in recently presenting some of the most definitive data available concerning the crystal structure of a tRNA molecule, we are just at the beginning of the process of understanding tRNA structure-function relationships. Yet we have come a long way from the early sequence work of Holley, and the expanding number of pieces in the puzzle are beginning to fit together.
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Erin Christine Raschke Hawkins was born in Austin, Texas in 1949. She attended public schools in Austin and graduated as valedictorian of her class from Stephen F. Austin High School in 1967.

She attended the University of Texas at Austin where she majored in chemistry and became a member of Alpha Lambda Delta, Mortar Board, Phi Lambda Upsilon, Iota Sigma Pi, Phi Kappa Phi, and Phi Beta Kappa. During this time she also worked as a research assistant in the laboratory of Dr. William Shive under the direct supervision of Dr. Glenn Cunningham. She graduated summa cum laude in December 1970, receiving a Bachelor of Science degree with special honors in chemistry.

In January, 1971, she married Kerry Mahler Hawkins and moved to San Francisco, California. While there, she began her graduate studies at the University of California at Berkeley under the direction of Dr. Stuart Linn, and received her Master of Arts degree in biochemistry in April, 1972.

On completion of her husband's military obligation, they moved to Baton Rouge, Louisiana, where she continued her graduate studies at Louisiana State University under the direction of Dr. Simon Hsin Chang. She will complete the requirements for the Doctor of Philosophy degree with a major in biochemistry and a minor in microbiology in
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EXAMINATION AND THESIS REPORT

Candidate: Erin Raschke Hawkins

Major Field: Biochemistry

Title of Thesis: Studies on the Secondary and Tertiary Structure of Yeast Denaturable Leucine Transfer Ribonucleic Acid

Approved:

[Signatures]

Major Professor and Chairman
Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

April 23, 1975