SYNTHESIS AND HYBRIDIZATION STUDIES OF OLIGORIBONUCLEOTIDES

SYNTHESIS AND HYBRIDIZATION STUDIES OF OLIGORIBONUCLEOTIDES CORRESPONDING TO THE COMMON, DOUBLE-STRANDED REGION OF THE DIHYDROURIDINE ARM OF SEVERAL TRANSFER RNA MOLECULES

BY

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ABSTRACT

An improved method for the synthesis of oligoribonucleotides of defined sequence was developed. The general phosphotriester synthesis of Neilson and co-workers was modified by the introduction of a new condensing agent, mesitylenesulfonyl-1,2,4-triazole, and by the replacement of the bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate with its acid salt. These modifications provided significant increases in the yields for the condensation of protected nucleosides - especially in the case of purine residues. Finally, modification of the three-step procedure for the deprotection of protected oligoribonucleotides resulted in the isolation of oligomers of exceptional purity and biological activity.

Oligomers corresponding to natural sequences in transfer RNA molecules were obtained by this improved method of synthesis. These oligomers were then used to study:

1. The formation of short double-stranded RNA helices and
2. The interactions of aminoacyl-tRNA ligases with tRNA fragments.

I wish to dedicate this

thesis to my parents

ACKNOWLEDGEMENTS

I wish to express my gratitude to Dr. Neilson for his guidance and encouragement throughout the course of this project. I am also grateful to the members of my supervisory committee, Dr. S. Bayley and Dr. H.P. Ghosh for their excellent criticisms and advice. Special thanks go to Dr. R. Bell and Mr. D. Hughes of the Chemistry Department for their collaboration with the pmr study.

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ABBREVIATIONS

A^a	adenosine
aa	amino acid
aa-tRNA	aminoacyl-tRNA
ac	acetyl
Ala	alanine
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
A_{260} unit	amount of material contained in 1 ml of a solution having an absorbance of 1.0 at 260 nm.
BAPase	bacterial alkaline phosphatase
bz	benzoyl
C^a	cytidine
cd	circular dichroism
CDP	cytidine-5'-diphosphate
dA	deoxyadenosine
dC	deoxycytidine
DCC	dicyclohexylcarbodiimide
DEAE	diethylaminoethyl
dG	deoxyguanosine
DHU	dihydrouridine
DMF	N, N-dimethylformamide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*

G^a	guanosine
Glu	glutamate
HMPTA	hexamethylphosphoric triamide
ibu	isobutyric
Ile	isoleucine
Lys	lysine
Met	methionine
MMTr	monomethoxytrityl
MST	mesitylenesulfonyl -1,2,4-triazole
mRNA	messenger RNA
NMR	nuclear magnetic resonance
pc	paper chromatography
PD PNPase	primer-dependent polynucleotide phosphorylase
pe	paper electrophoresis
Phe	phenylalanine
Pi	inorganic phosphate
pmr	proton magnetic resonance
PPO	2,5-diphenyloxazole
POPOP	1,4-Bis[2-(5-phenyloxazolyl)]-benzene
PPi	pyrophosphate
ppm	parts per million
Pro	proline
R_F	ratio of distance travelled by sample to that of solvent
RNA	ribonucleic acid
RNase	ribonuclease

Ser	serine
SPDE	spleen phosphodiesterase
SVD	snake venom phosphodiesterase
t	tetrahydropyranyl
T	thymidine
TEAE	triethylaminoethyl
tlc	thin layer chromatography
T_m	melting temperature
TPS	2,4,6-triisopropylbenzenesulfonyl chloride
TPST	TPS-1,2,4-triazole
TPSTT	TPS-tetrazole
Trac	triphenylmethoxyacetyl (trityloxyacetyl)
tRNA	transfer RNA
$tRNA^{Ala}$	tRNA specific for alanine
Tyr	tyrosine
uv	ultraviolet
U^a	uracil
Val	valine
ψ	pseudouridine

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a. mononucleotides and oligoribonucleotides are abbreviated in the standard format (IUPAC-IUB Commission on Biochemical Nomenclature, 1970). pG represents 5'-guanylic acid, Ap represents 3'-adenylic acid and ApG represents adenylyl (3'-5') guanosine.

FIGURES

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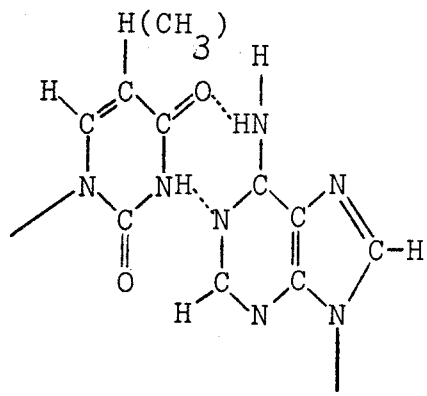
1. INTRODUCTION

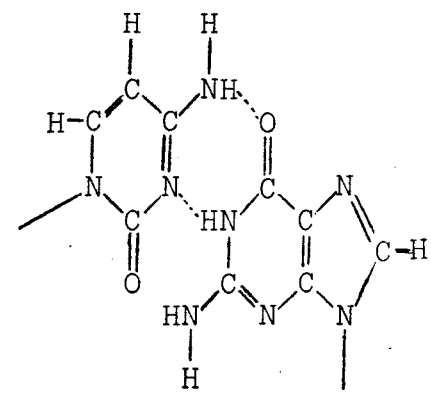
Crick's 'Central Dogma' (Crick, 1958) makes the assumptions that genetic information passes in one direction: from DNA to RNA to protein; and that this information is stored in the linear arrangement of the monomeric units in these macromolecules. These two concepts are of fundamental importance in molecular biology.

The flow of information from DNA to RNA (transcription) can be easily understood in terms of Watson-Crick hydrogen bonding (dA with T and dG with dC, see Figure 1). However the process of translating a linear polymer of nucleotides (RNA) into a linear polymer of amino acids (protein) presents a major problem. It was inconceivable to Crick that RNA (or DNA) could function as a direct template for amino acids. He therefore predicted the existence of an 'adaptor' molecule to which the amino acid could be covalently attached and would then interact with the template by a specific pattern of hydrogen bonds (Crick, 1955). This 'adaptor' hypothesis was elaborated to include the provision that there must be at least one 'adaptor' molecule and its corresponding charging enzyme for each amino acid.

Zamecnik and his colleagues have been credited with the actual discovery of the 'adaptor' molecule. They demonstrated that amino acids were covalently attached to a low-molecular weight RNA species by an ATP-dependent reaction and that these charged, RNA molecules efficiently incorporated amino acids into proteins at the ribosome - the organelle of protein synthesis (Hoagland et al., 1958).

Figure 1. Standard Watson-Crick Base Pairing.





Uracil(Thymine) Adenine Cytosine Guanine

Although originally termed soluble RNA (sRNA) the 'adaptor' molecule is now referred to as transfer RNA (tRNA).

1.1 Transfer RNA

Transfer RNAs are the smallest biological macromolecules. They are involved in a number of complex interactions with both proteins and other classes of RNA. However, it is because of the relatively small size of tRNA that a major effort has been made in attempting to understand the relationship between its structure and its function.

tRNAs range from 73 to 93 nucleotides in length and have an average molecular weight of 25,000. Their sedimentation co-efficient is about 4S. In addition to the usual nucleosides adenosine, guanosine, cytidine and uridine a number of modified bases are also observed.

The first tRNA to be sequenced was yeast $tRNA^{Ala}$ (Holley et al., 1965). Of the conformations proposed for this tRNA one secondary structure has become universally accepted. It has been possible to adopt the same two-dimensional 'cloverleaf' structure (see Figure 2.) for the over 60 to 70 species of tRNA from various sources that have been sequenced to date (Barrell and Clark, 1974). This is inspite of their different primary structures.

The 'cloverleaf' is formed in the following manner. The 5' and 3' ends of the molecule base pair to form the terminal arm (usually seven base pairs). Three or four bases at the 3' end remain unpaired. As the amino acid is esterified to the 3'-terminal base, this region of the tRNA is known as the amino acid acceptor arm. To the left, a loop of eight to twelve unpaired bases is closed off by a neck of three to four base pairs. As this loop often contains the modified nucleoside dihydro-

uridine (DHU) this region is named the DHU arm. At the bottom there is a loop of seven unpaired bases with a neck of five base pairs. The anticodon (three bases which specifically hydrogen bond to the complementary triplet codon of mRNA) is located in this loop and the region is therefore known as the anticodon arm. To the right there is another loop of seven base pairs with a neck of five base pairs. The common occurrence of -TpψpC- in this loop has lead to this region being termed the TψC arm. Between the anticodon arm and the TψC arm there is a region of variable length - the extra arm.

From a comparison of the secondary structures of many tRNAs several common features are noted:

1. The 3' end of all tRNAs terminate in -CpCpAOH.
2. The 5' terminus bears a 5'-phosphate and this terminal base is often pG.
3. The anticodon loop has the sequence -PypUpNpNpNpPu- (where -NpNpN- represents the anticodon.) and
4. The last base pair before the loop of the TψC arm is G≡C and the sequence -GpTpψpCpPupA- is always present in the loop. Also the last base at the 3' end of the loop is a pyrimidine. The only exceptions to this are the eukaryote initiator tRNAs (eg. yeast $tRNA_f^{Met}$ where -ApUpC- replaces -TpψpC-, Simsek and RajBhandary, 1972). The significance of this exception will be discussed later.

One feature of tRNA that distinguishes it from other nucleic acids is the high percentage of modified nucleosides (for review see Nishimura, 1972 and references therein.). These modified components can range from simple methylated nucleosides such as N^2-methyl guanosine in

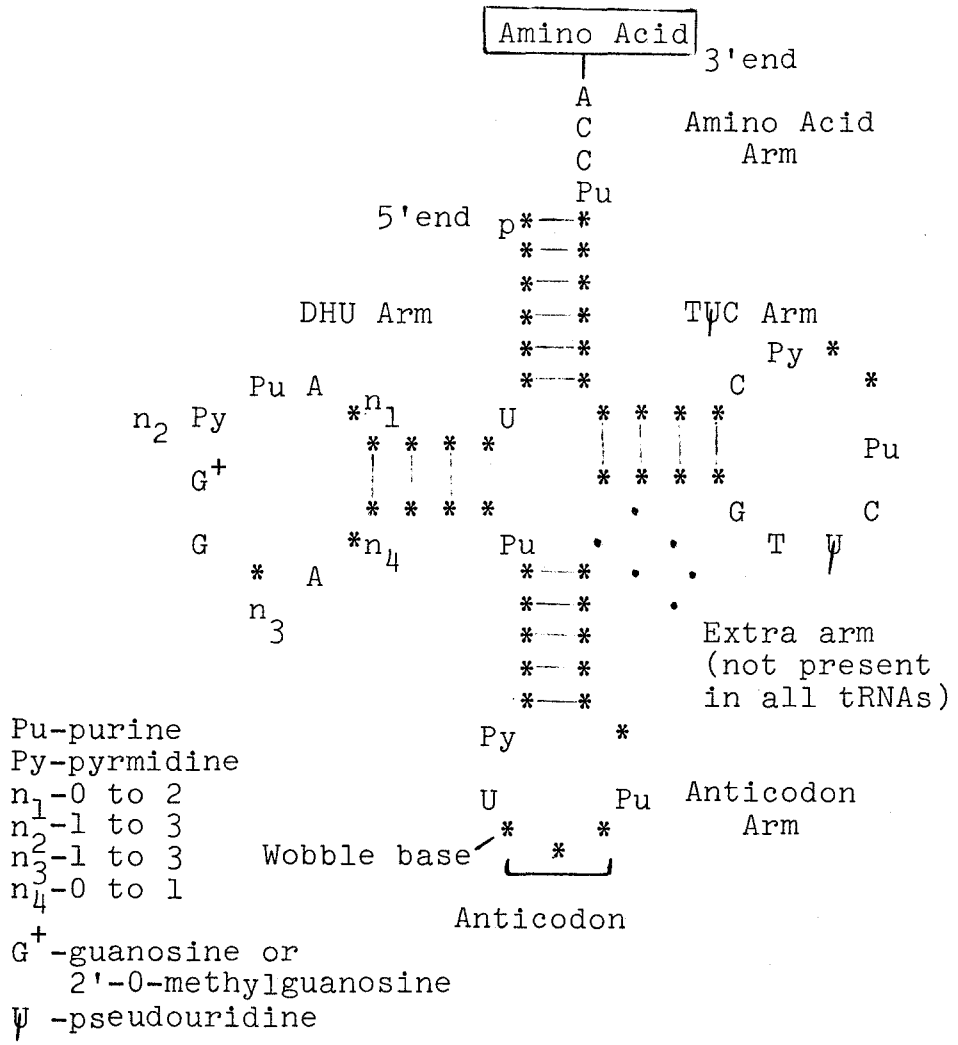


Figure 2. Cloverleaf structure of tRNA. * represent variable nucleosides. Nucleosides common to all tRNAs are indicated.

in position 10 of yeast $tRNA^{Phe}$ (RajBhandary et al., 1967) to such complex structures as 2-methylthio-6-(Δ^2-isopentenyl) adenosine in position 38 of E. coli $tRNA^{Tyr}$ (Harada et al., 1968).

The modified nucleosides can be classified into three groups depending upon their location. In the first group the modified nucleosides occupy the first position of the anticodon (wobble base); in the second group they are located in the position adjacent to the 3' end of the anticodon and in the third group the modified nucleosides are located in other parts of the tRNA. Although the effect of the modified nucleosides on the function of tRNA is not fully understood, it can be assumed (from their location in groups one and two) that they play a role in codon-anticodon interactions (for review see Sen, 1974). However other workers have suggested that modification of nucleosides may be an important process in cell differentiation (for review see Sueoka and Kano-Sueoka, 1970).

1.2 Transfer RNA Biosynthesis

The biosynthesis of tRNA (for review see Smith, 1976) poses many intriguing questions. First, as messenger and ribosomal RNA arise by the sequential cleavage of primary transcription units of nucleotide sequence longer than the mature products, it is not surprising that tRNA also follows this pattern. Secondly, as Watson-Crick base pairing provides insufficient information to specify the modified bases, these bases must arise subsequent to transcription.

The most information on the maturation of a tRNA molecule is provided by the E. coli system for the synthesis of $su^+_{III}tRNA^{Tyr}$. Infection of E. coli with the transducing bacteriophage ϕ80 carrying

mutants of $tRNA^{Tyr}$ leads to the accumulation of a tRNA precursor (Altman, 1971). Apparently a point mutation in the precursor molecule retards maturation (Altman and Smith, 1971). This precursor begins with pppG- and therefore probably represents an initial transcript. There is a stretch of 41 nucleotides to the 5' side of the tRNA and three nucleotides to the 3' side. Further, this precursor contains a -CpCpA- sequence which forms the 3' terminus of the native tRNA. [It is generally considered that -CpCpA- is not present in the precursor of most tRNAs but is added by nucleotidyl transferase during processing (Daniel, 1970). As an example, the T_4-specific tRNAs, $tRNA^{Ser}$ and $tRNA^{Pro}$, are synthesized from a dimeric tRNA precursor which does not contain the sequence -CpCpA- at the 3' end (Barrel et al., 1974)]. It was also observed that there was a low level of base modification in the precursor of $su^+_{III}tRNA^{Tyr}$.

Recently, an in vitro system was developed to allow the identification of the enzymatic activities required for the synthesis of tRNA (Bikoff and Gefter, 1975 and Bikoff et al., 1975). With $\phi 80psu^+_{III}$ DNA as template, DNA-dependent RNA polymerase catalyzed the formation of a large molecular weight transcript that was initiated 41 nucleotides proximal to the 5' end of $su^+_{III}tRNA^{Tyr}$ and terminated at least 100 nucleotides beyond the 3' terminus of the tRNA. An endonuclease containing fraction was required to remove most of the nucleotides from the 3' end of the transcript to give what had been originally termed 'precursor' $su^+_{III}tRNA^{Tyr}$. Apparently the 'precursor' tRNA isolated from the in vivo systems by earlier workers was in fact a cleavage product of the original transcript. A third enzymatic activity (RNase P III) specifically removes the extra nucleotides from the 3' terminus. The fourth activity,

an endonuclease (RNase P) catalyzes the removal of the extra 5' nucleotides (see Figure 3.).

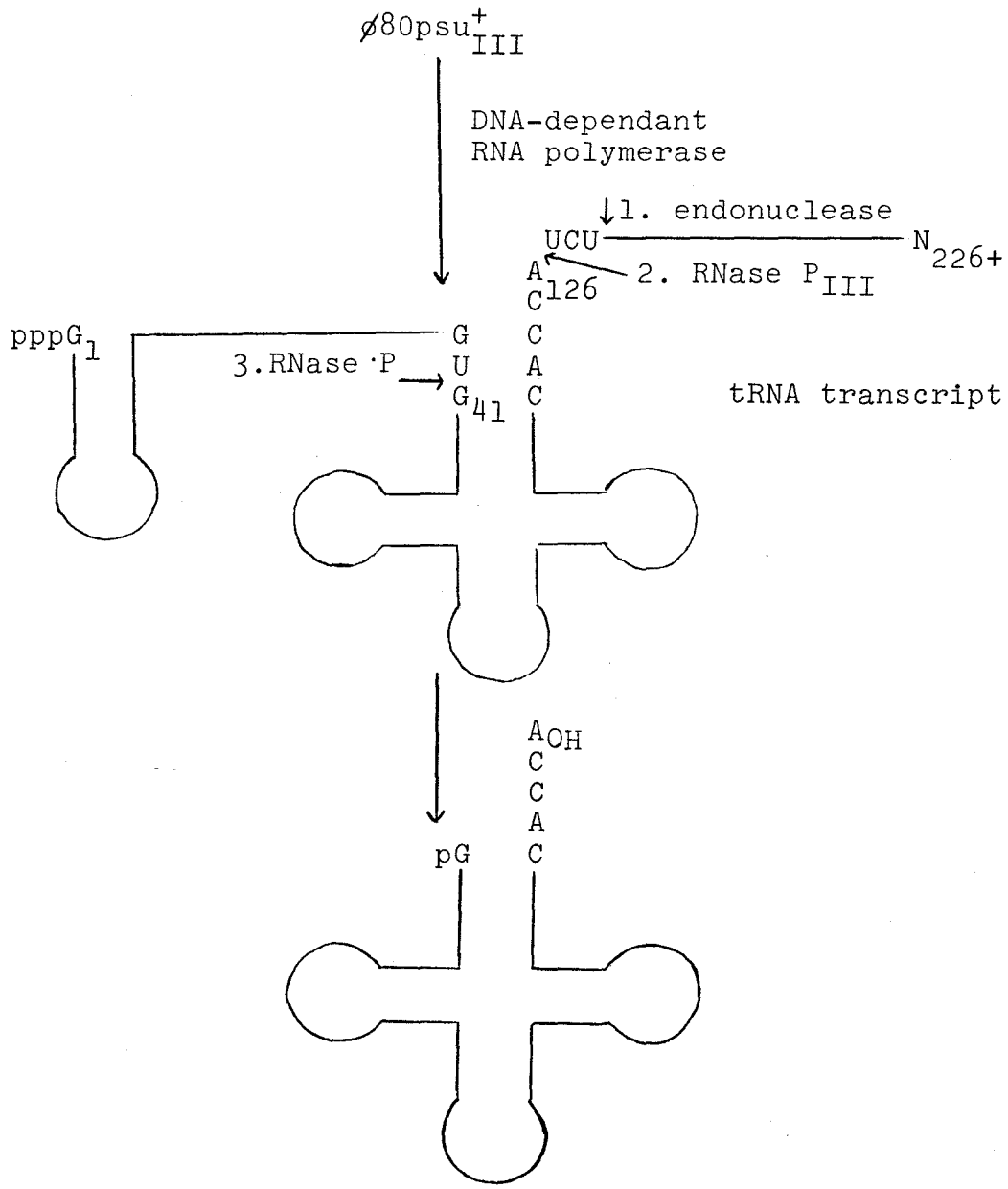
Although many enzymatic activities responsible for base modification have been isolated (for review see Hall, 1971), there is little information available as to what stage in the maturation process of the tRNA these enzymes act. The use of bacteriophages in *in vitro* systems is one technique that should be useful in solving this question.

Recently, an alternate approach with broader application has been developed to determine if base modification is an ordered or random process (Kitchingman *et al.*, 1976). When a mutant strain of *E. coli*. (relaxed control) was deprived of leucine, unique (under-modified) species of $tRNA^{Phe}$ were observed. Following the restoration of protein synthesis by the addition of leucine these unique species were converted to normal forms. They proposed that "some of the enzymes involved in the post-transcriptional modification of precursor tRNAs may be short-lived and thus must be synthesized continually; in the absence of protein synthesis modification-deficient tRNAs accumulate".

1.3 Transfer RNA Structure

In 1953 Watson and Crick proposed the double helix as the structure for DNA. This structure not only accounted for the numerous observations on the chemical and physical properties of DNA but also suggested a mechanism for its replication (Watson and Crick, 1953b). Similarly, there are other examples where detailed knowledge of a macromolecule's three-dimensional structure has permitted a clearer understanding of the molecule's function (eg. the co-operative binding of oxygen by haemoglobin, Perutz, 1970). Perhaps this too will be the case with tRNA.

Figure 3. Biosynthesis of su^{+}_{III} $tRNA^{Tyr}$.



Secondary structure (helix formation) in tRNA was first suggested by temperature-dependent absorption studies (Tissieres, 1959). tRNAs have reversible melting curves which resemble those obtained for other double-helical nucleic acids. The X-ray diffraction patterns of oriented fibres of tRNA also indicated helix formation (Doctor et al., 1969).

From an inspection of the primary sequences of several tRNAs it appeared that there was a common secondary structure, the cloverleaf. Experimental proof of this structure has been obtained from high-resolution Nuclear Magnetic Resonance studies of tRNAs in solution (for review see Kearns and Shulman, 1974). The resonances for the hydrogen-bonded ring NH protons (G_1H and U_3H) of standard Watson-Crick base pairs (Figure 1) occur at very low field (11-15 ppm) and are well resolved from other resonances. As each base pair contains one ring NH proton, each low-field resonance corresponds to one base pair. Knowing the locations of the resonances for the standard (unshifted) base pairs and the magnitude of the ring current shifts exerted by the four different bases on a neighbouring base pair, the spectra of a polynucleotide of known secondary structure can be predicted.

The NMR spectra of several tRNAs have been studied (eg. yeast $tRNA^{Phe}$, E. coli. $tRNA^{Glu}$, and $tRNA_f^{Met}$, Shulman et al., 1973) and the integration of the resonances in the low field was consistent with the number of base pairs predicted by the cloverleaf model. However due to the large number of resonances ($\sim$20) it was not possible to assign resonances to specific base pairs. When fragments of yeast $tRNA^{Phe}$ possessing only part of the total secondary structure were studied, the observed resonances were distinct and could be assigned (Lightfoot et al.,

1973). The positions of these resonances compared favourably with those predicted. The loss of secondary structure with elevated temperature can also be followed by NMR as the resonances are observed to broaden and eventually disappear (eg. yeast $tRNA^{Phe}$, Wilbers et al., 1973).

Although high-resolution NMR studies have confirmed the presence of secondary structure in tRNA they provide little information on possible tertiary (three-dimensional) structure. Tertiary structure would exist in tRNA if the helical elements of the molecule were held in a specific conformation by additional forces. Studies on the physical properties of tRNA, such as small-angle X-ray scattering, have indicated that tRNA is a very compact molecule - much more compact than suggested by the clover-leaf structure. This conclusion is supported by the stability of tRNA to nuclease degradation and the reduced susceptibility of certain bases to chemical modification (for review see Cramer, 1971). Although certain features of the tRNA molecule are suggested by these experiments they do not provide sufficient information to establish a three-dimensional structure. As with proteins, detailed information on the tertiary structure of nucleic acids has been revealed by X-ray crystallography.

The three-dimensional structure of yeast $tRNA^{Phe}$ at 3.0 Å resolution has been reported for the orthorombic (Kim et al., 1974) and the monoclinic (Robertus et al., 1974) crystal forms. The structures reported by these studies are in excellent agreement (average difference 1.0 Å) and a number of common features have been established (for review see Sussman and Kim, 1976). These common structural features can be summarized as follows:

"1. The tRNA molecule has the overall shape of a letter L (see Figure 4).

2. The secondary structure predicted by the cloverleaf model is present.

3. The amino acid arm and TψC arm make one long helix; the dihydrouridine arm and the anticodon arm make the other long helix. These two long helices are related by a pseudo twofold axis and form an L.

4. The 3' terminus is at one end and the anticodon is at the other end of the L. The TψC and the dihydrouridine loops are at the corner of the L.

5. The molecule is about 22 Å thick and the lengths of the two axes of the L are about 73 and 70 Å, respectively.

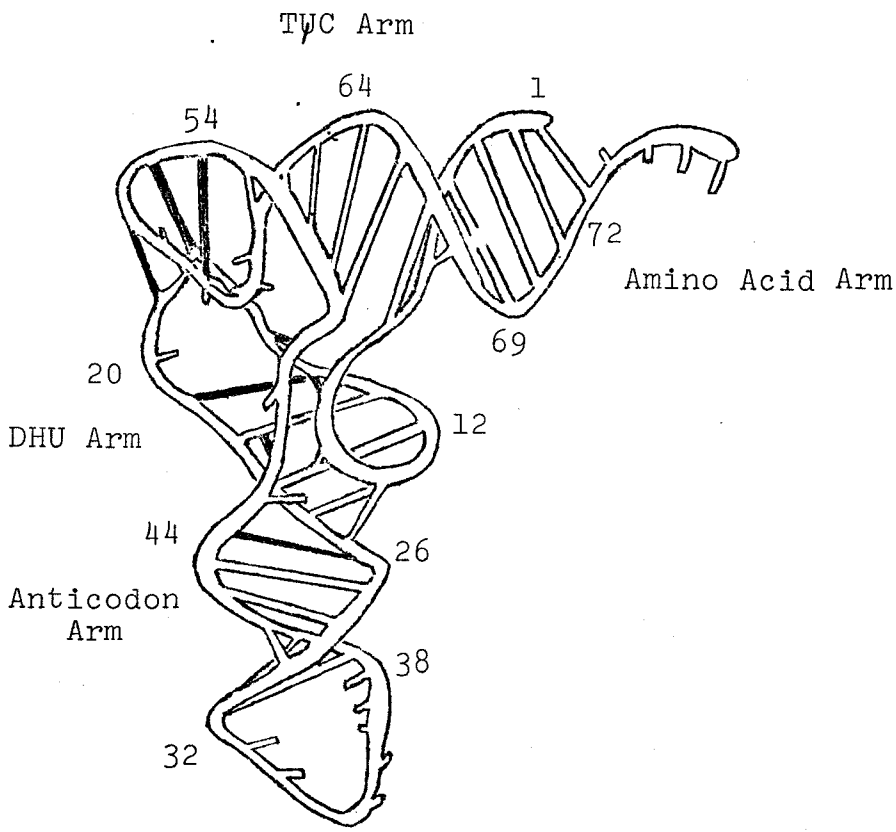
6. All bases except five are stacked along the axes of the molecule. They are D16, D17, G20, U47, and A76.

7. All the double helical stems are of the genus A type.

8. There are eight hydrogen bonded tertiary interactions between bases which are invariant or semi-invariant in most tRNAs. These are U8.A14, G15.C48,G18.ψ55, G19.C56, G26.A44, T54.A58. There are two base triples, U12.A23.A9 and C13.G22.G46" (Sussman and Kim, 1976). [N.B. These tertiary base pairs can be of the Watson-Crick type (eg. G19.C56) or they can involve other interactions (eg. U8.A14 or C13.G22.m^7G46) (see Figure 5).]

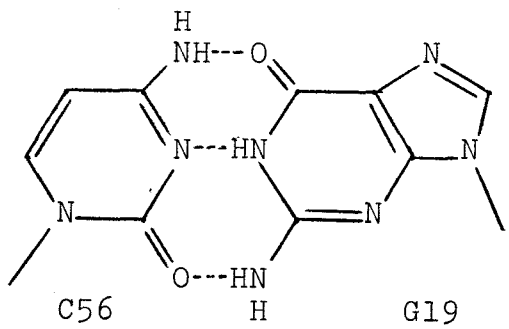
Some doubt has been expressed as to whether the structure of yeast $tRNA^{Phe}$ in crystals is the same as that in solution where the molecule is biologically active. It has been shown that the Raman spectra of this tRNA in the crystalline state and in solution are identical (Chen et al., 1975). As Raman spectra are very sensitive to molecular conformation this study would suggest that their tertiary structures are also identical. Similarly, Langlois and co-workers (1975) have demonstrated that there is no difference in the fluorescence of the Y base of yeast $tRNA^{Phe}$ in crystals and in solution.

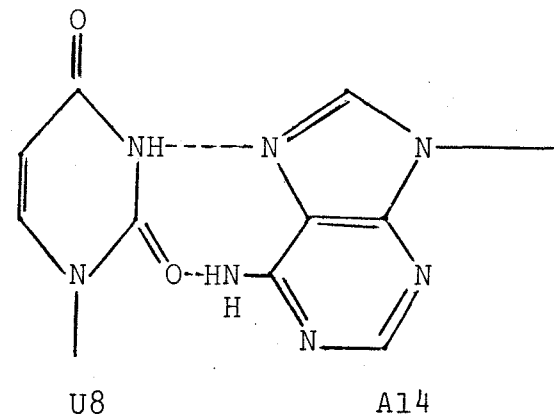
Figure 4. Tertiary Structure of Yeast $tRNA^{Phe}$

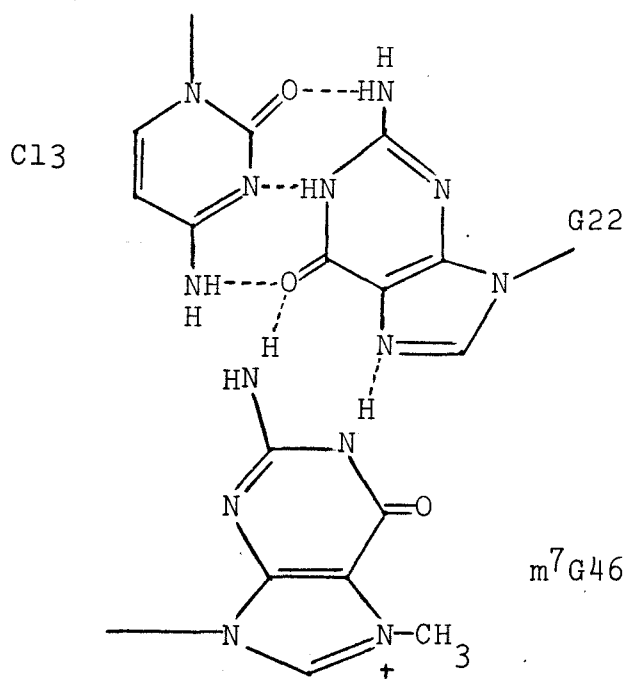


Ribose-phosphate backbone is shown as a continuous cylinder with bars to indicate hydrogen-bonded base pairs. Unpaired bases are represented by shortened rods and solid bars indicate the tertiary base pairs.

Figure 5. Examples of tertiary base pairs in yeast $tRNA^{Phe}$.







Evidence supporting the tertiary structure of yeast $tRNA^{Phe}$ is obtained from high resolution NMR studies. The position of the resonances of the base pairs at the junction of the amino acid arm and the TψC arm indicate that these two arms are aligned to form a continuous helix (Shulman et al., 1973). Although no extra resonances from tertiary base pairs were observed in the original high-resolution NMR studies at 220MHz (Shulman et al., 1973), recently, with the superior resolving power of 360 MHz spectrometers it has become possible to distinguish additional resonances in the low-field region (Reid and Robillard, 1975). Integration of the resonances in the low-field now yields 26 ± 1 protons - 20 from secondary structure and 6 from tertiary base pairs.

Additional information on various features of the three-dimensional structure of tRNA have been obtained from studies on the susceptibility of tRNA to nuclease degradation (eg. Reid et al., 1972), the ability of complementary oligomers to bind to tRNA (eg. Uhlenbeck, 1972), the chemical reactivity of certain bases (eg. Igo-Kemenes and Zachau, 1971), and the photochemically induced cross-linking (eg. Ofengand and Bierbaum, 1973). If the tertiary structure of yeast $tRNA^{Phe}$ is to be universally accepted the structure must be consistent with the experimental results of these studies. Robertus and co-workers (1974) have examined the correlation between the three-dimensional structure and the chemical reactivity of yeast $tRNA^{Phe}$. Their findings support the model; those bases which react chemically lie in exposed positions and those which do not are either in the double helical stem regions or else are involved in maintaining the tertiary structure through pairing or stacking interactions.

The question has been raised as to whether or not there is any

change in the secondary or tertiary structure of a tRNA upon aminoacylation. Reports claiming there is no change (Wong et al., 1973) and reports indicating that there is a change (Thomas et al., 1973) have appeared. A recent electron paramagnetic resonance study of spin-labelled yeast $tRNA^{Phe}$ (Caron et al., 1976) suggested a change in the tertiary structure - possibly exposing the TψC loop. As will be discussed later this could have important consequences on the molecule's function.

Finally, it must be considered whether the tertiary structure of yeast $tRNA^{Phe}$ is common to all tRNAs. The fact that all of the hydrogen-bonded tertiary interactions of yeast $tRNA^{Phe}$ are between invariant or semi-invariant bases of the class of tRNAs that have four base pairs in the DHU arm and five residues in the extra arm (over 50 percent of all known tRNA sequences) strongly suggests a common structure for these tRNAs. As crystals of high quality can be prepared from a mixture of all tRNAs (Blake et al., 1970) it is apparent that whatever differences in conformation exist they must be small.

1.4 Transfer RNA in Protein Synthesis

The transfer RNA molecule plays a central role in the conversion of genetic information into functional proteins. It acts at the interface between polynucleotide information and polypeptide information. In protein synthesis tRNA participates in a number of complex interactions with other species of RNA and with proteins. The fidelity of the protein synthesized is dependent on the specificity of these interactions.

The tRNA is first aminoacylated by its cognate aminoacyl-tRNA ligase. The charged tRNA then enters the ribosome where its anticodon interacts with the messenger RNA while the 3' end of the tRNA, 76 Å away,

serves as the actual site of peptide growth. Although the basic events of protein synthesis are known (for review see Haselkorn and Rothman-Denes, 1973 and refs. therein) there is little detailed information available about the interactions of tRNA with its ligase and the ribosome.

A better understanding of the function of tRNA can be obtained if those interactions that involve other RNA species are distinguished from those that involve proteins. tRNA-RNA interactions can be simply described in terms of Watson-Crick hydrogen bonding. However tRNA-protein interactions are more difficult to describe as no mechanism for highly specific interactions between protein and nucleic acids has been determined. Also, it is helpful to distinguish those interactions that rely on the similarities of sequence and structure in tRNA from those that rely on their differences.

1.4.1 The Interaction of Transfer RNA with Aminoacyl-Transfer RNA Ligase

The aminoacyl-transfer RNA ligases (synthetases) catalyze the first step in protein synthesis. They esterify an amino acid to the ribose hydroxyl group of the 3'-terminal adenosine of the tRNA. For each amino acid there is an aminoacyl-tRNA ligase and a tRNA. The energy required for the formation of the aminoacyl linkage is derived from the hydrolysis of ATP to AMP and PPi. The overall reaction is thus written:

$$\text{ATP} + \text{amino acid} + \text{tRNA} \overset{\text{aminoacyl-tRNA ligase}}{\rightleftarrows} \text{aminoacyl-tRNA} + \text{AMP} + \text{PPi} \qquad \text{(eq. 1)}$$

Experimental evidence suggest that the aminoacylation reaction occurs by a two-step mechanism (for review see Novelli, 1967 and Schimmel,

1973) In the first step (equation 2) aminoacyladenylate is formed. This intermediate remains firmly attached to the enzyme and does not accumulate as free aminoacyladenylate. This reaction can be followed by ATP/PPi isotope exchange, usually in the absence of tRNA. There is a requirement for divalent cations (eg. Mg^{+2}).

aminoacyl-tRNA ligase + amino acid + ATP $\rightleftarrows$

aminoacyl-AMP·aminoacyl-tRNA ligase + PPi (eq. 2)

The second step (equation 3) is the transfer of the activated amino acid from AMP to the tRNA. There is no requirement for divalent cations for this reaction.

aminoacyl-AMP·aminoacyl-tRNA ligase + tRNA $\rightleftarrows$

aminoacyl-tRNA + AMP + aminoacyl-tRNA ligase (eq. 3)

It has been proposed that the rate determining step for the aminoacylation reaction is the release of aminoacyl-tRNA from the enzyme (Yarus and Berg, 1969). This has been demonstrated in the case of E. coli isoleucyl-tRNA ligase (Eldred and Schimmel, 1972). The formation of isoleucyladenylate was also demonstrated to enhance the release of charged tRNA.

However this two-step mechanism for the aminoacylation reaction is not consistent with all of the experimental evidence: (for review see Loftfield, 1972)

1. Some aminoacyl-tRNA ligases are unable to form the enzyme·aminoacyladenylate complex.
2. Aminoacyl-tRNA can be formed under conditions where no aminoacyladenylate is formed. (eg. Spermine can replace Mg^{+2} for the esterification reaction but it does not catalyze the ATP/PPi exchange reaction (equation 2).

3. The transfer of amino acid to tRNA (equation 3) is too slow and too inefficient to account for *in vitro* esterification of tRNA or the rate of *in vivo* protein synthesis.

4. The ATP/PPi exchange reaction may not be physiologically important. The cellular concentration of aminoacyl-tRNA ligase and tRNA, and the high affinity of the enzyme for tRNA (Km $\sim 10^{-7}$M) suggests that all the enzyme is present as enzyme·tRNA complex. As the kinetics of the exchange reaction for tRNA-free and tRNA-complexed enzyme are very different it is difficult to interpret the significance of this reaction.

On the basis of these observations Loftfield (1972) has proposed a concerted mechanism. In this model, all three substrates (amino acid, ATP and tRNA) are present together in the active site and the amino acid is transferred directly to the tRNA. Aminoacyladenylate (equation 2) is formed only in the absence of tRNA. In this reaction the conformation of the active site is thought to be altered so that tRNA is not readily accommodated. This would explain the observed slow rates of amino acid transfer via equation 3.

Recent experiments by Loftfield (Lovgren *et al.*, 1976) have failed to confirm the studies of Eldred and Schimmel which indicated that the rate determining step for charging was the release of aminoacyl-tRNA.

It is difficult to evaluate the two mechanisms proposed for the aminoacylation reaction as they are based on information derived for various ligases from different sources. Part of the problem may also lie in attempting to demonstrate a common mechanism. Although the enzymes catalyze the same esterification reaction, have the same requirement for

ATP, and all have a reactive sulfhydryl group there are some differences in such characteristics as their molecular weight and subunit structure (for review see Kisselev and Favorova, 1974).

By modifying the 3'-terminal adenosine, the actual site of aminoacylation for several tRNAs has been determined. Aminoacylation of yeast $tRNA^{Phe}$ occurs exclusively at the 2' hydroxyl (Sprinzel and Cramer, 1973). However, as the aminoacyl group rapidly migrates between the 2' and 3' position the site of esterification will have little consequence on the aminoacyl-tRNA's function. These studies are nevertheless very informative as they illustrate the heterogeneity of aminoacyl-tRNA ligases. The tRNAs of E. coli could be separated into three classes according to the site of aminoacylation (Fraser and Rich, 1975). The first two classes were charged exclusively at either the 2' or 3' position while the third class showed no specificity. This observation indicates that there must be differences in the mechanism of the aminoacylation reaction for the three classes of ligases.

To ensure that mutant proteins are not synthesized there must be a high degree of fidelity in this reaction. Once an amino acid is attached to the tRNA it makes no contribution to that tRNA's specificity. Only the interaction of the anticodon of the tRNA with the codon of the messenger RNA determines the amino acid that is inserted into the growing polypeptide. This fact has been adequately demonstrated in the experiments of Chapeville and co-workers (1962). The hybrid tRNA, alanyl-$tRNA^{Cys}$, incorporated alanine for cysteine in an in vitro assay for protein synthesis. Clearly the aminoacyl group of the tRNA is not recognized by either the ribosome or the messenger RNA.

A novel reaction of the aminoacyl-tRNA ligase has been recently described. Apparently some enzymes are capable of catalyzing the deacylation of misacylated cognate tRNAs (Yarus, 1972). It was suggested that this 'verification' process may serve an important role in correcting errors in aminoacylation and thereby maintaining the fidelity of protein synthesis. However on closer examination the rates for the deacylation were determined to be 100 to 1000 times slower than the charging reaction (Bonnet and Ebel, 1974). The verification reaction is therefore too slow to have a significant physiological role.

Considerable effort has been directed towards elucidating the mechanisms of the highly specific recognition of a tRNA by the corresponding aminoacyl-tRNA ligase. Most studies have adopted the hypothesis that there is a recognition site on the tRNA which consists of a region(s) of unique primary sequence. As a consequence, there has been much interest in the differences in tRNA structure.

Originally, the anticodon itself was considered to serve as the recognition site. However it has been clearly demonstrated that certain tRNAs can be aminoacylated despite complete loss of codon recognition (Thiebe and Zachau, 1968). Similarly, modified nucleosides with their diversity in structure and location were also thought to be important in recognition (Hall, 1971). As modification-deficient tRNAs are aminoacylated, albeit to a lower extent than normal tRNA (Kitchingman et al., 1976), modified nucleosides can only play a minor role.

In attempting to identify the recognition site three methods have been widely used - chemical modification, enzymatic dissection and structural analysis of isoaccepting species (for review see Chambers, 1972).

In chemical modification studies it is assumed that the modification of bases in the recognition site will lead to a loss in the ability of that tRNA to be aminoacylated. These studies require a reagent that will react specifically and completely with the target bases. Proper analysis of the results demands that loss of activity due to modification be distinguished from that due to denaturation of the tRNA (or some other similar event).

The conversion of cytidine to uridine by bisulphite ion is one example of a common modification procedure. No loss in acceptor activity for yeast $tRNA^{Tyr}$ was observed when cytidines at positions 34, 76, and 77 were modified (Kucan et al., 1971). It was therefore concluded that these residues were not involved in the recognition process of this cognate tRNA/aminoacyl-tRNA ligase system.

The objective of enzymatic dissection is the isolation of tRNA fragments that contain sufficient information for aminoacylation. Thus, those sequences that have been excised can not be responsible for recognition. Perhaps the most successful application of this technique has been the work of Chambers and co-workers. They were able to reconstitute alanine acceptor activity by annealing a 3'-quarter molecule (a nonadecanucleotide) with a 5'-quarter molecule (a hexadecanucleotide) from yeast $tRNA^{Ala}_{II}$ (Imura et al., 1969). On the basis of this experiment and photoinactivation studies (Schulman and Chambers, 1968), Chambers (1969) has proposed that the amino acid acceptor arm is required for the recognition of $tRNA^{Ala}_{II}$ by alanyl-tRNA ligase. As no aminoacylation was observed for fragments containing only the 3' portion of the acceptor arm, this would indicate that secondary structure is also necessary for recognition.

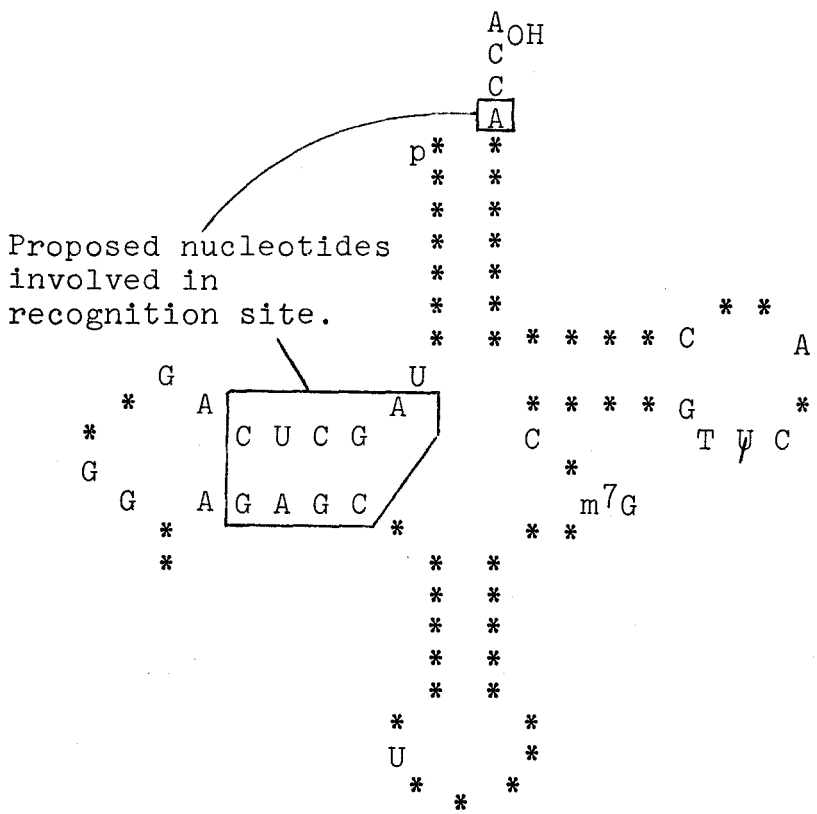
However it cannot be concluded that the recognition sites for all tRNAs are located in the acceptor arm.

Under various reaction conditions certain aminoacyl-tRNA ligases will esterify tRNAs from other species (for review see Jacobson, 1971). This misacylation is not limited to tRNAs specifying the same amino acid. An interesting example of these heterologous charging systems is the yeast phenylalanyl-tRNA ligase. This enzyme aminoacylated $tRNA^{Phe}$ from E. coli and wheat, plus E. coli $tRNA^{Val}_{1,2A,2B}$, $tRNA^{Met}$, $tRNA^{Ile}$, $tRNA^{Ala}_{I,II}$ and $tRNA^{Lys}$ (Dudock et al., 1971 and Roe and Dudock, 1972). When the primary sequence of these tRNAs were compared two regions of constant structure were observed (see Figure 6), - the double-stranded region of the dihydrouridine arm and the fourth nucleotide from the 3' terminal. It was therefore suggested that these two regions of the tRNA molecule form a recognition site for yeast phenylalanyl-tRNA ligase. However this does not imply that these regions are the only parameters involved in recognition. Tertiary structure, base modifications, loop size, etc., may also serve important roles.

Despite the intensive search, the recognition site has remained very elusive. Only in isolated instances have any of the three methods described proved successful. One fact however does stand out - as virtually all regions of the tRNA molecule have been implicated, there can be no common location for the recognition site and quite probably this site is more complex than just a unique sequence of nucleotides.

The elements of recognition have been indirectly studied by an analysis of tRNA·aminoacyl-tRNA ligase complexes. If it is assumed that recognition sites interact intimately with the enzyme, then exposed

Figure 6. Composite tRNA and yeast phenylalanyl- tRNA ligase recognition site.



regions of the tRNA can be excluded. However these exposed regions may be important in maintaining correct conformation.

Dube (1973) has demonstrated that E. coli methionyl-tRNA ligase protects the anticodon loop, the 3' side of the amino acid acceptor arm and the extra loop of $tRNA_f^{Met}$ from digestion with T_1 ribonuclease. Interaction of the enzyme with the anticodon loop is confirmed by the observation that there was a loss of methionine acceptor activity resulting from a cytidine to uridine base change in the anticodon (Schulman and Goddard, 1972). Recognition was therefore proposed to involve a 'three-point attachment' of the tRNA to the enzyme. This model may be applied to all tRNAs as three regions would provide sufficient information to distinguish structures. (This situation is analogous to the triplet genetic code.) Further, the attachment sites need not be located in the same regions for all tRNA·enzyme complexes. Nuclease susceptibility studies on other tRNAs support this view (Horz and Zachau, 1973).

Additional information on the interaction of tRNA with aminoacyl-tRNA ligases has been obtained by photochemically cross-linking tRNA·enzyme complexes. With yeast $tRNA^{Phe}$ the regions that cross-linked to the cognate phenylalanyl-tRNA ligase overlapped with the region proposed as a recognition site from the heterologous charging experiments of Roe and Dudock (Shoemaker et al., 1975).

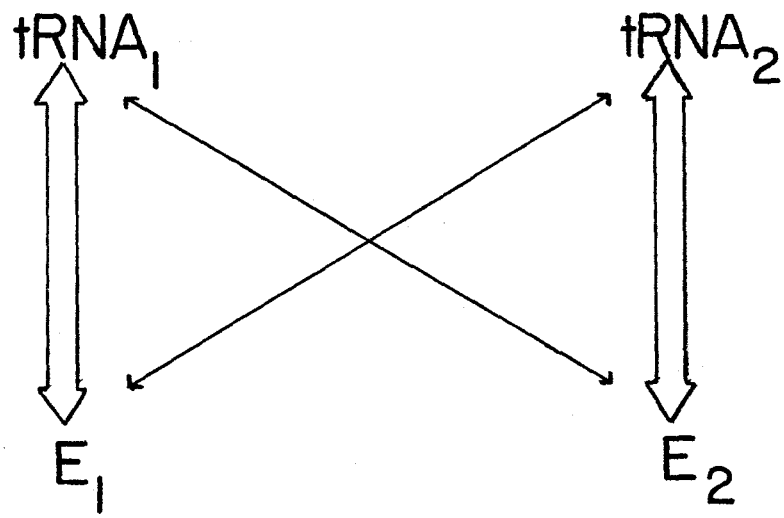
However, the concept that specificity of tRNA aminoacylation is a direct result of an absolute specific recognition between the aminoacyl-tRNA ligase and the cognate tRNA has gradually lost support (Ebel et al., 1973). It would now appear that this recognition is less than absolute. This statement is supported by the following observations:

1. the misacylation of tRNA in homologous systems under normal aminoacylation conditions (Yarus and Mertes, 1973).
2. the inhibition of the correct aminoacylation reaction by non-cognate tRNA (Kern et al., 1972).
3. the lack of specificity of the AMP/PPi independent aminoacyl-tRNA ligase catalyzed deacylation (Yarus, 1973), and
4. the isolation of complexes between aminoacyl-tRNA ligases and non-cognate tRNA species (Ebel et al., 1973).

If the specificity of the recognition process is less than complete how is the fidelity of the aminoacylation reaction maintained? Yarus (1972) has described how parallel systems of ligands could enhance the precision of this reaction. In Figure 7 all the possible interactions between two cognate tRNA/aminoacyl-tRNA ligase systems are illustrated. As the in vivo concentration of tRNA and ligase are approximately equimolar (Calendar and Berg, 1966), the high affinity of $tRNA_1$ and E_1 results in $tRNA_1$ being unavailable for reaction with E_2. Similarly E_2 removes $tRNA_2$ from reaction with E_1. Yarus has calculated that the simultaneous presence of parallel systems should reduce the intrinsic velocities of misacylation reactions by two to three orders of magnitude.

Ebel and co-workers (1973) have suggested that the specificity of tRNA aminoacylation depends more upon the maximal velocity of the reaction (Vmax) than the association between tRNA and aminoacyl-tRNA ligase (Km). They found that the affinities of the ligases for non-cognate tRNAs, as estimated by the Km of the misacylation reaction, were diminished by only one to two orders of magnitude when compared to the correct acylation reaction. However the Vmaxs of the misacylation reaction were diminished

Figure 7. The network of possible interactions between two cognate tRNA/aminoacyl-tRNA ligase systems - $tRNA_1$ plus E_1 and $tRNA_2$ plus E_2.



by three to four orders of magnitude. These authors therefore propose that aminoacylation consists of two discrimination mechanisms. The first acts at the level of recognition and can be measured by the Km. The second, the catalytic process, is more effective in terms of specificity and is measured by the Vmax. They stress that the recognition problem can only be solved if the recognition process is distinguished from the catalytic step. Knoore (1975) has supported this viewpoint. The catalytic step can be considered separately as a final adjustment of the tRNA on the enzyme and probably involves orientating the 3'-terminal adenosine in the catalytic centre.

From an examination of the general three-dimensional structure of tRNA (the structure of yeast $tRNA^{Phe}$) and limited primary sequence and X-ray crystallography data on aminoacyl-tRNA ligase, Kim (1975) has presented a model for the recognition process. This model proceeds through two steps. In the first, the aminoacyl-tRNA ligase recognizes the common tertiary structural features of tRNA molecules. In the second, the specific recognition of unique features selects the cognate tRNA. (However, as indicated above this step can be less than absolute).

Kim has directed his attention to the first step of this model. Two types of symmetry elements relating the two extended helices of the L-shaped tRNA molecule have been identified - a pseudo twofold axis and a pseudo twofold screw axis. Similar symmetry elements in aminoacyl-tRNA ligases are also indicated. Kim's hypothesis is "that the pseudo-symmetric regions of a tRNA are recognized by the pseudo-symmetric regions of an aminoacyl-tRNA ligase...". This symmetry-matching process would align tRNA and enzyme such that most of the symmetric regions of the tRNA would

be in contact with the enzyme. Kim notes that these contact areas correspond to many of the proposed recognition sites. Symmetry recognition has also been observed in other nucleic acid interactions. In the lactose operon of E. coli, twofold symmetry was observed for the catabolite gene activator protein (CAP) binding site in the promoter and for the repressor protein binding site in the operator (for review see Dickson et al., 1975).

In summary, although many regions of the tRNA have been implicated, no region has been conclusively proven to be a recognition site. Further, it has become evident that the interaction of an aminoacyl-tRNA ligase with a tRNA is less than aboslute. There appears to be merit to the proposal of Ebel et al., that the recognition process can be better understood if divided into two steps. In the first, the overall tertiary structure is recognized, possibly on the basis of symmetry as proposed by Kim. This step can be related to the Km of the aminoacylation reaction. In the second step, the selection of the cognate tRNA occurs on the basis of unique structural features. This step probably involves orientating the tRNA on the enzyme such that the 3'terminal adenosine is correctly positioned in the catalytic site. Thus it is related to the Vmax. To date the basis for this specific recognition of cognate tRNAs is unknown.

1.4.2 Transfer RNA in the Initiation of Protein Synthesis

The initiation of protein synthesis has been studied in both prokaryotes and eukaryotes (for review see Lucas-Lenard and Lipmann, 1971 and Haselkorn and Rothman-Denes, 1973). Although the prokaryotes, E. coli in particular, have been better characterized, the eukaryotes are

similar in most aspects.

Methionyl-tRNA initiates translation in response to the codon AUG. This initiator tRNA is structurally distinct from the species that insert methionine internally. In prokaryotes both methionyl-tRNA species are aminoacylated by the same aminoacyl-tRNA ligase, but the initiator tRNA is subsequently formylated in the α-amino group. Only N-formyl-methionyl-tRNA can initiate prokaryote protein synthesis. In eukaryotes the initiator tRNAs are distinguished by the absence of the sequence -GpTpψpC-in the TψC arm (Simsek et al., 1973). There is also no requirement for formylation.

Initiator methionyl-tRNA does not enter the ribosome directly. It forms an initiation complex with the messenger RNA and the small ribosomal subunit. The entry of methionyl-tRNA into this complex is stimulated by an initiation factor - IF-2 and M2 in prokaryotes and eukaryotes respectively. The functional ribosome is then formed by the association of the large ribosomal subunit with the initiation complex. The initiation factors dissociate from the complex and GTP is hydrolyzed. The initiator tRNA is now located in the peptidyl or P site of the ribosome and protein synthesis can proceed.

1.4.3 Transfer RNA in the Elongation Cycle of Protein Synthesis

The process of peptide chain elongation can be divided into three distinct and consecutive steps - the codon-directed binding of aminoacyl-tRNA, the formation of the peptide bond and the translocation of the peptidyl-tRNA (for review see Lucas-Lenard and Lipmann, 1971 and Haselkorn and Rothman-Denes, 1973.)

The aminoacyl-tRNA does not enter the ribosome directly but rather forms a complex with an elongation factor that stimulates its binding to the messenger RNA. In E. coli the aminoacyl-tRNA forms a complex with GTP and EF-Tu (elongation factor - transfer, unstable). The aa-tRNA·EF-Tu·GTP complex then binds the ribosome at the aminoacyl or A site. This binding is directed by specific, Watson-Crick base pairing between the anticodon of the tRNA and the codon of the mRNA. EF-Tu is released from the ribosome by the hydrolysis of GTP and active EF-Tu is regenerated from the EF-Tu·GDP complex by EF-Ts (stable). A similar series of events also occurs in eukaryotes.

In E. coli all aminoacyl-tRNAs except the initiator tRNA form a complex with EF-Tu·GTP. The formylation of the initiator tRNA is not necessary for this discrimination. This is probably the mechanism whereby this tRNA is prevented from coding methionine internally. It has also been demonstrated that uncharged tRNAs do not associate with the EF-Tu·GTP complex (Skoultchi et al., 1968).

It would appear that the elongation factor recognizes some common feature of the aa-tRNA, possibly the common ester linkage to the 3'-terminal sequence -CpCpA. Recognition of the 3'-terminal of tRNA by the EF-Tu·GTP complex is indicated by the slow rate of deacylation for complexed aminoacyl-tRNA relative to free (Beres and Lucas-Lenard, 1973). However,as the initiator tRNAs have similar 3' terminals, other features of the aa-tRNA must also be recognized. It is interesting to note that conformational changes have been suggested for the aminoacylated tRNA (Caron et al., 1976). Thus different conformations could also be a basis for the distinction of charged and uncharged tRNAs by the elongation factor.

In the interaction of the tRNA with the mRNA, the antiparallel base pairing of the first and second bases of the codon (from the 5' end) with the third and second bases of the anticodon respectively are strictly Watson-Crick (A with U and G with C). However the third base of the codon can 'wobble' and pair with non-complementary bases in the first position of the anticodon (Crick, 1966). Thus the following 'wobble' base pairings are possible:

Anticodon	Codon
U	A, G
C	G
A	U
G	C, U
I	U, C, A

In part, this explains the observed degeneracy of the genetic code.

On first inspection, the interaction of codon and anticodon does not appear to be sufficient to explain the high degree of precision observed in protein synthesis. As complementary trinucleotides do not form stable helices (Jaskunas et al., 1968) it may be concluded that tRNA will not bind firmly to the mRNA. However the anticodon and the two bases to the 3'side of it are stacked on to the helix formed by the anti-codon arm and the DHU arm. This holds the anticodon in a conformation that is more suited to base pairing (Fuller and Hodgson, 1967). (N.B. The stacking of the anticodon can also account for 'wobble' base pairing.)

The modified nucleosides adjacent to the 3'end of the anticodon can also influence the base pairing properties of tRNA. The removal or alteration of this nucleoside affects codon recognition, possibly through

some change in the conformation of the anticodon (Ghosh and Ghosh, 1972).

Measurements of the affinity of trinucleotides for their complementary sequences in the anticodon have indicated that the interactions are quite weak (Keq. $10^{3}M^{-1}$ at 0°). However the most surprising result was that the affinity constant for the interaction of a tRNA with the cognate triplet was only one order of magnitude greater than that for the interaction of the same tRNA with a related, non-cognate triplet (Uhlenbeck et al., 1970). Surely the simple interaction of a tRNA with a complementary triplet cannot account for the fidelity of tRNA selection.

As may be expected, the ribosome may provide additional binding sites to enhance the affinity between tRNA and mRNA. As the sequence -TpψpCpG- in position 1 to 4 of the TψC loop is common to all tRNAs, this sequence may represent a binding site on the tRNA for the ribosome (Ofengand and Henes, 1969). The finding of the complementary sequence, -CpGpApA-, in the 5S RNA of the 50S ribosomal subunit of E. coli supports this proposal (Brownlee et al., 1967). It has also been demonstrated that the tetramer IpψpCpG binds strongly to the ribosome and that the -CpGpApA- sequence of 5S RNA is exposed in the ribosome (Erdmann et al., 1973). Further, TpψpCpG inhibits the enzymatic binding of aminoacyl-tRNA to the ribosome (Grummt et al., 1974).

However the TψC loop of tRNA is buried and not available for base pairing (Pongs et al., 1973). Therefore a conformational change in the tRNA that exposes the TψC loop for interaction with the 5S RNA must occur. Although certain studies suggest that the aminoacylation of tRNA exposes this loop (Caron et al., 1976), a more interesting model proposes that codon-anticodon recognition induces the necessary conformational changes

(Shwarz et al., 1974). Kurland and co-workers (1975) have adopted this latter hypothesis to account for tRNA selection. They propose that the codon-anticodon interaction of cognate tRNA induces conformational changes that unmask additional binding sites to maximize the interactions with the ribosome. These interactions with the ribosome increase the energy difference between cognate and noncognate tRNA binding. Thus the codon is thought to function as an allosteric effector.

Once the A and P site of the ribosome are occupied peptide bond formation can proceed. A peptidyltransferase activity associated with the large ribosomal subunit is responsible for the transfer of the peptide attached to the tRNA in the P site to the α-amino of the aminoacyl-tRNA in the A site. The uncharged tRNA in the P site is then released and the new peptidyl tRNA·mRNA complex in the A site is translocated to the P site by G factor in prokaryotes and EF-2 in eukaryotes. The translocation step requires the hydrolysis of GTP. The next codon reading in the 5' → 3' direction is now located in the A site and another round of the elongation cycle may proceed.

One of the conceptual problems in protein synthesis is visualizing the presence of two tRNA molecules on adjacent codons (the A and P site). Fuller and Hodgson (1967) have examined this problem by model building and have concluded that by placing a 'kink' between the codons of the mRNA, two tRNAs can be accommodated. However it is not clear if this 'kink' can fulfill the requirement of the peptidyl transfer reaction that the 3' ends of both tRNAs be in close proximity. There is no evidence for a peptidyl·peptidyltransferase intermediate. Rich (1974) has suggested that peptidyl transfer could be accomplished if the L-shaped

tRNA molecule undergoes some kind of rotatory motion. Such a movement would involve the TψC loop at the corner of the molecule. This region has already been implicated in conformational changes on the ribosome.

The peptidyltransferase activity has been extensively studied in terms of the amount of tRNA-like structure that is required for reaction. It is evident that only the common -CpCpA terminal of tRNA is recognized. Puromycin, an aminoacyl-adenosine analog, can replace aminoacyl-tRNA as an acceptor in the A site and formylmethionyl-CpCpA can replace formylmethionyl-tRNA as a donor in the P site (Monro et al., 1968). Although little of the tRNA structure is recognized by peptidyltransferase, the correct positioning of the 3' terminal in the catalytic centre of the enzyme is required for optimal activity. This statement is supported by the observation that E. coli $tRNA^{Phe}_{(CCCA)}$ (additional CMP in the acceptor arm) was bound as efficiently to the A and P site as native $tRNA^{Phe}$, but was not as active in peptide formation (Thang et al., 1974).

It is not known how the G Factor (EF-2) interacts with the ribosome, mRNA, and peptidyl tRNA to cause translocation. However the translocation step does appear to be coupled with the release of the uncharged tRNA in the P site. Possibly the movement of the new peptidyl tRNA from the A to P site forces the uncharged tRNA from the ribosome. Without the additional stabilization of the ribosome binding sites, the uncharged tRNA probably diffuses from its weak association with the mRNA. This diffusion could be aided by the high affinity of the tRNA for its cognate aminoacyl-tRNA ligase.

1.4.4 Transfer RNA in the Termination of Protein Synthesis

The mechanics of the recognition of the termination signal and the release of protein from peptidyl-tRNA are unknown. However two activities necessary for termination, a release factor and peptidyl-transferase,have been identified. tRNA does not appear to serve any function in this process as no species of tRNA has been demonstrated to interact with the termination codons (Fox and Ganoza, 1968).

1.5 Summary and Evaluation

The transfer RNA molecule proceeds through a complex series of interactions with the various components of the protein synthesizing machinery. With most of these interactions only the basic events are understood.

The interaction of tRNA with mRNA and 5S RNA is adequately described by Watson-Crick base pairing. The 'wobble' base pairing is the only unusual feature. The interaction of the TψC loop and the 5S RNA is common to all tRNAs, while the codon-anticodon interaction is selective.

The interactions of tRNA with proteins can be divided into two groups. In the first, the interaction depends on the recognition of common structural features. The elongation factors and peptidyltransferase belong to this group. In the second, the interaction depends on the recognition of unique features in the tRNA molecule. The aminoacyl-tRNA ligases and the initiation factor responsible for the binding of methionyl-tRNA belong to this group. No mechanisms for the specific recognition of nucleic acids by protein are known.

Of all the interactions of tRNA, the selection of cognate tRNAs by aminoacyl-tRNA ligases probably represents the most interesting and challenging problem. Despite intensive investigations the basis for the specificity of this interaction has not been defined.

The traditional approach to the identification of the elements of recognition has been to study the requirements for the aminoacylation reaction. Schimmel (1973) has critized this approach. He suggests, "One obvious tactic is to study the interaction of tRNA fragments or modified tRNAs with the ligase. If a strongly binding fragment is found, for example, it is logical to conclude that it must contain a significant portion of the locus (or loci) for the ligase interactions." The preparation of tRNA fragments has relied on limited digests of tRNA that attack exposed regions (eg. Harbers et al., 1972) or chemical scission at reactive bases (eg. Wintermeyer and Zachau, 1970). However only a limited number of fragments can be produced by this method and the contribution of individual nucleosides cannot be determined. This technique also requires that sufficient quantities of pure tRNA are available and that the fragments can be efficiently separated. It was therefore decided that the synthesis of desired sequences would provide a more satisfactory approach. The work described in thesis explores this possibility.

2. THE SYNTHESIS OF OLIGONUCLEOTIDES

Three strategies have been developed for the synthesis of oligonucleotides of defined sequence:

1. a chemical approach
2. an enzymatic approach and
3. a combined chemical/enzymatic approach

Chemical synthesis has been quite successful for the preparation of oligonucleotides of both the ribose and deoxyribose series. However the relatively low efficiency for the stepwise addition of nucleoside (or nucleotide) units to a growing polymer or for the joining of oligomer blocks has placed rather strict limits on the length of sequence that can be synthesized (n=20) (Agarwal et al., 1970).

Few enzymes have proved suitable for the task of oligonucleotide synthesis. To obtain the desired product, elaborate procedures are often required to manipulate the enzyme's specificity.

The combination of the chemical and enzymatic approach has produced some of the most impressive synthetic results. The short oligomers (n=5 to 10) efficiently synthesized by chemical means, can be joined together by certain enzymes. By this method sequences of more than one hundred nucleotide units can be produced.

As these approaches to oligonucleotide synthesis have been adequately reviewed in recent years (Narang and Wightman, 1973; Kossel and Seliger, 1975), only a brief description of what this author feels are the major achievements will be described. As the synthesis of tRNA

fragments is planned, particular attention will be paid to the synthesis of oligoribonucleotides.

2.1 Chemical Synthesis

The objective of oligonucleotide synthesis is the formation of a 3'-5' internucleotide phosphodiester bond. Chemically this is a difficult task due to the number of reactive sites - the 3' and 5' hydroxyl of the carbohydrate moiety (plus the 2' hydroxyl in the ribo series), the amino of the aromatic bases (A, G and C) and the phosphate. Those sites which are not intended for reaction must therefore be suitably protected. The successful solution of this synthetic problem requires a knowledge of the organic chemistry of carbohydrates, nitrogen hetrocycles and phosphate esters.

The additional 2'-hydroxyl group of the carbohydrate moiety of the ribo series makes the chemical synthesis of ribooligonucleotides a more difficult task than the synthesis of deoxyribooligonucleotides for the following reasons:

1. Selective protection of the 2'-OH that leaves the 3'-OH unprotected requires lengthy pathways.

2. Migration of the phosphate between the 3' and 2'-hydroxyl groups occurs readily by either acid or base catalysis, and

3. The 2'-OH protecting groups can sterically hinder the condensation of nucleotide units thereby decreasing yields. (Ikehara, 1974).

Narang and Wightman (1973) have identified the areas where the major portion of the synthetic work has been devoted. They include:

1. the improvement of methods for the activation of phosphate esters so as to permit attack by the weakly nucleophilic hydroxyl groups

of the nucleosides,

2. the design of protecting groups that can be quantitatively introduced and readily but selectively removed,

3. the development of methods for the joining of oligomer blocks,

4. the development of condensation procedures that are either quantitative or free of contaminating side products, and

5. the development of effective techniques for the purification and characterization of the resulting polynucleotides.

Rather than attempting to condense the large body of information available on the synthesis of oligonucleotides, two procedures that have been successfully employed in the synthesis of oligoribonucleotides corresponding to sequences in tRNA molecules will be described. Special reference will be made to the relative strengths or weaknesses of the procedures.

The first procedure to be discussed is the synthesis of the terminal 3'-nonanucleotide and the 5'-hexanucleotide of yeast $tRNA^{Ala}$ by Ohtsuka and co-workers (1973). The second is the synthesis of a nonanucleotide corresponding to the anticodon loop of E. coli $tRNA_f^{Met}$ by Neilson and Westiuk (1974). The two procedures differ basically in the protection of the phosphate. The phosphate is unprotected in the first and it is therefore referred to as a phosphodiester synthesis. 2,2,2,-trichloroethyl protects the phosphate in the second procedure and the method is referred to as a phosphotriester synthesis.

2.1.1 Phosphodiester Synthesis

The two sequences required to form the amino acid acceptor arm of yeast $tRNA^{Ala}$ are the hexanucleotide, GpGpGpCpGpU, and the nonanucleotide, CpGpUpCpCpApCpCpA (Figure 8a). The synthesis of these sequences was accomplished by the condensation of oligomer blocks (Figures 8b and 8c).

Ikehara and co-workers utilized the following protecting groups:

1. Benzoyl for the amino functions of C and A and for the 2' hydroxyl of C,A and U.

2. Either isobutyryl or acetyl for the amino function and the 2' hydroxyl of G.

3. Anisidate for the phosphate of the 'incoming' nucleotide of a condensation reaction, and

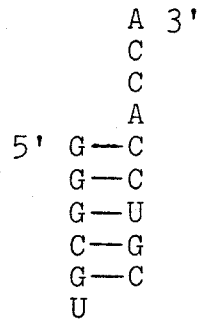
4. Monomethoxytrityl for the 5' hydroxyl of the nucleotide destined to occupy the 5' end of the sequence.

Figure 9 outlines the stepwise synthesis of the trinucleotide CpGpUp. This synthesis proceeds in the 5' to 3' direction. The 3' phosphate of the 5'-terminal nucleotide (8) was activated with either dicyclohexylcarbodiimide or triisopropylbenzenesulfonyl chloride. The activated nucleotide was then condensed with the 5' hydroxyl of the incoming nucleotide (9). Anisidate was removed specifically by treatment with isoamyl nitrite before the dinucleotide (10) was activated and condensed with (11) to produce (12).

This trinucleotide was then used in a 'block' coupling reaction (12 + 6a, Figure 8c) to form the nonanucleotide (7). In this block coupling the 3' phosphate of (12) was condensed with the 5' hydroxyl of another oligomer (6a), rather than a nucleotide. 6a was prepared by the

Figure 8. Phosphodiester synthesis of the amino acid acceptor arm of yeast alanine tRNA.

a.



b.

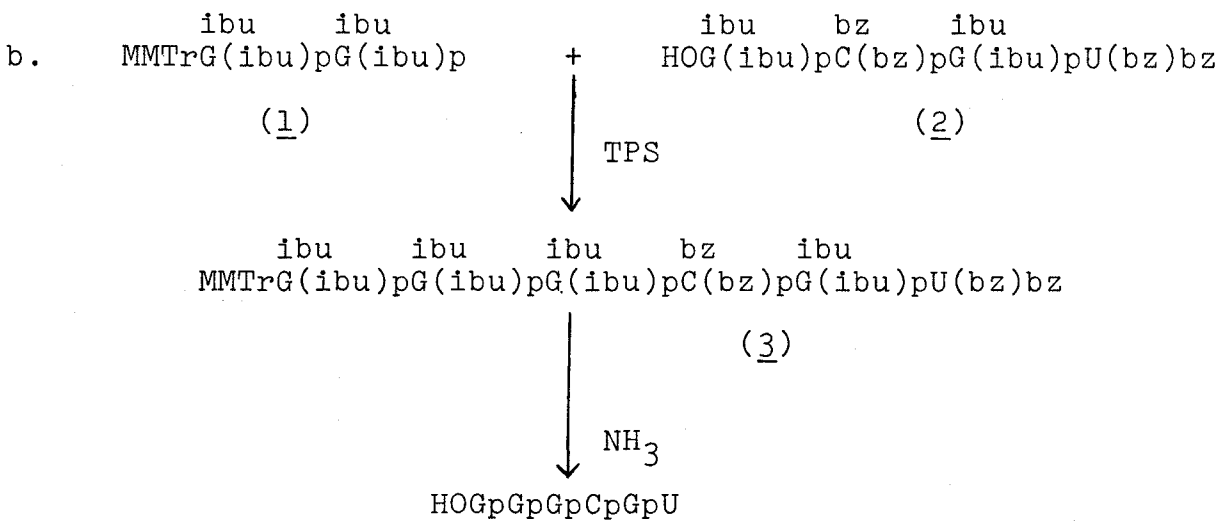
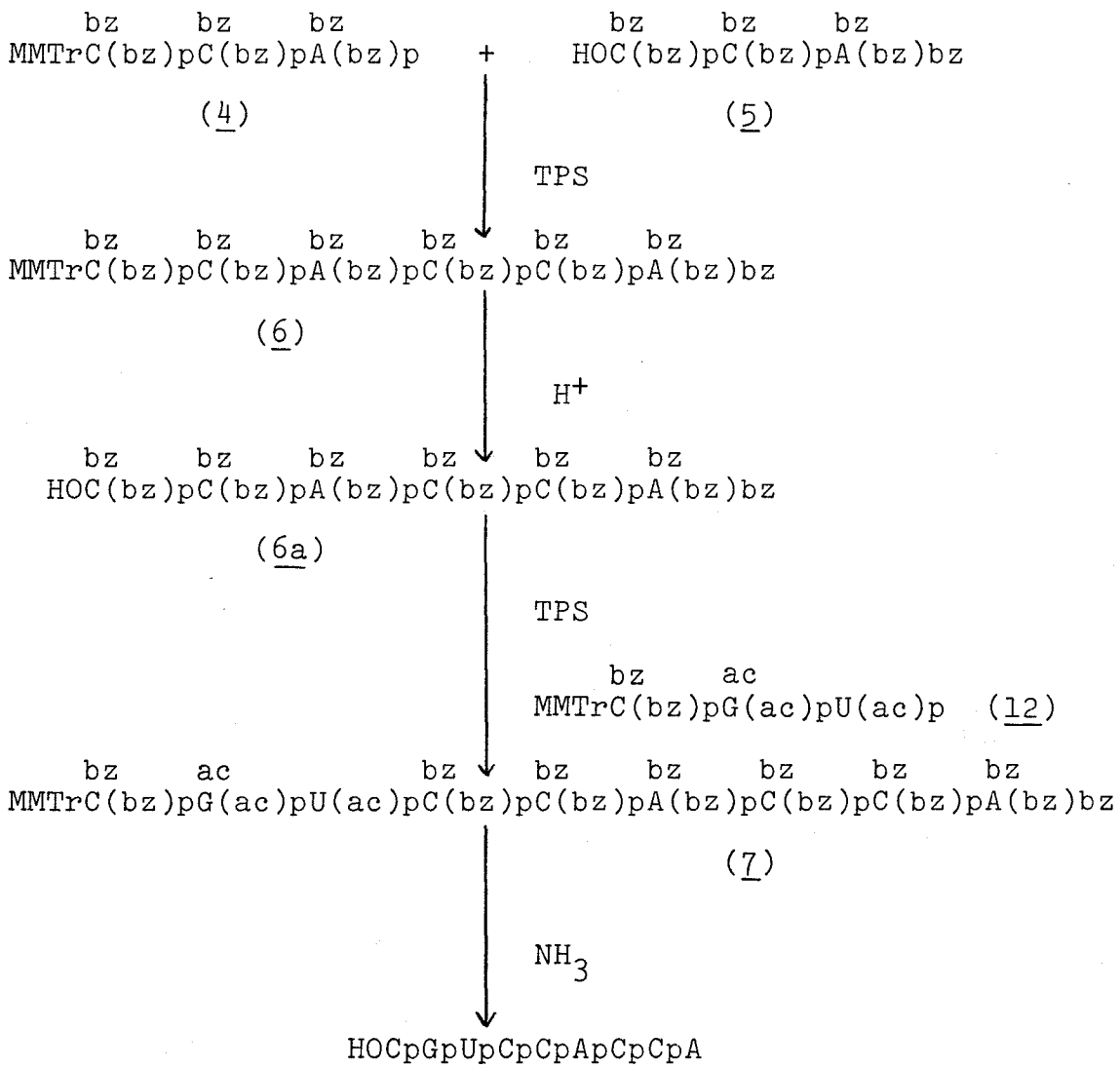


Figure 8c.


bz bz bz
MMTrC(bz)pC(bz)pA(bz)p
(4)
+
bz bz bz
HOC(bz)pC(bz)pA(bz)bz
(5)
TPS
bz bz bz bz bz bz
MMTrC(bz)pC(bz)pA(bz)pC(bz)pC(bz)pA(bz)bz
(6)
H+
bz bz bz bz bz bz
HOC(bz)pC(bz)pA(bz)pC(bz)pC(bz)pA(bz)bz
(6a)
TPS
bz ac
MMTrC(bz)pG(ac)pU(ac)p (12)
bz ac bz bz bz bz bz bz
MMTrC(bz)pG(ac)pU(ac)pC(bz)pC(bz)pA(bz)pC(bz)pC(bz)pA(bz)bz
(7)
NH3
HOCpGpUpCpCpApCpCpA

acidic hydrolysis of the 5'-MMTr group of 6. To prevent the incorrect 3'-3' internucleotide linkage, the 3' terminal residue of 6a was protected at both the 2' and 3' position with benzoyl. Treatment of the protected nonanucleotide 7 with acid removed the 5'-MMTr group and subsequent treatment with methanolic ammonia removed the amino and 2'-hydroxyl protecting groups to yield the free oligomer.

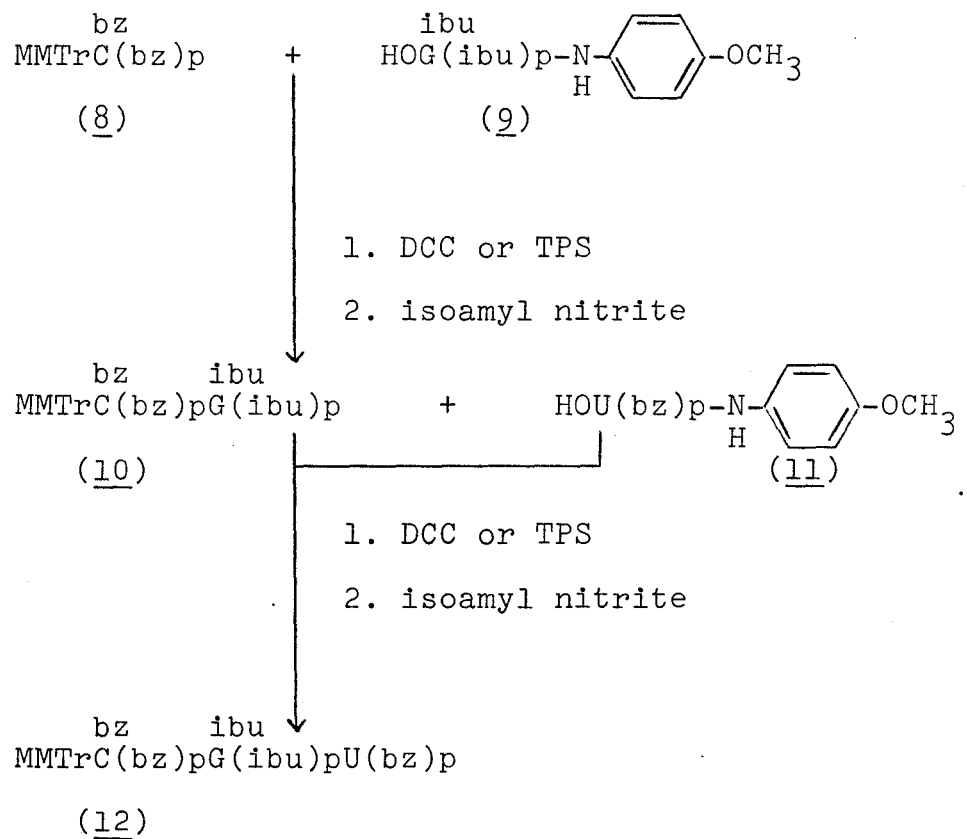
The yields obtained in the step-wise synthesis were in the 20 to 40% range. As an example, MMTrC(bz)p (8) (0.3 mmol) was activated with a ten-fold excess of DCC (3 mmol) and condensed with HOG(ibu)p-anisidate (9) (0.2 mmol) to yield 32% of protected dinucleotide (10).

The yields obtained for block couplings however were much lower. The trinucleotide MMTrC(bz)pG(ibu)pU(bz)p (12) (44 umol) was activated with an excess of TPS (70 umol) and condensed with the hexanucleotide HOC(bz)pC(bz)p A(bz)pC(bz)pC(bz)pA(bz)bz (6a) (2.7 umol). After deprotection the yield of nonanucleotide was only 8%. This was in spite of the sixteen fold excess of 12.

After a condensation reaction, partial purification was performed by gel filtration in Sephadex LH-20. Column chromatography on anion exchange resins DEAE or TEAE-cellulose was employed for the final purification. As the intermediates of this phosphodiester synthesis contained charged phosphates they were insoluble in organic solvents. However, their solubility in aqueous media was reduced by the amino and hydroxyl protecting groups. It was therefore necessary to use mixed solvents such as 80% ethanol or 90% dimethylformamide in the purification.

Completely deprotected hexanucleotide and nonanucleotide were purified by column chromatography on DEAE-cellulose in 7M urea.

Figure 9. Phosophodiester synthesis. Step-wise synthesis of a trinucleotide.



The columns used in the purification procedures are tedious as they are of low capacity and require several hours to run. The presence of various side products in this synthesis further complicated the purification. As an example, there were more than 9 major peaks in the elution profile of the hexanucleotide GpGpGpCpGpU and the DEAE-cellulose column took over 60 hours to run.

The low capacity of the columns and the low yields of the condensation reactions permitted only small scale (umol) synthesis.

2.1.2 Phosphotriester Synthesis

In the phosphotriester method of oligoribonucleotide synthesis of Neilson and co-workers the nucleosides were protected in the following manner:

1. The amino functions of A,G, and C were protected by the base-labile benzoyl group.

2. The 2' hydroxyls were protected by the acid-labile group, tetrahydropyranyl.

3. The phosphates were protected by 2,2,2-trichloroethyl. This group was removed under neutral conditions by reductive cleavage with Zn/Cu couple. And

4. The nucleoside destined to occupy the 5'-terminal position was also protected by trityloxyacetyl in the 5' hydroxyl. Trityloxyacetyl was cleaved by mild alkali. It was not necessary to protect the 3' hydroxyl in this synthesis as the 'bulky' tetrahydropyranyl group sterically hinders the formation of the undesired, 3'-3' internucleotide linkage (Neilson, 1969).

The direction of synthesis was 5' to 3' and the coupling was carried out in a two-step procedure. Typically, TPS (4 equiv.) was used to activate the pyridinium salt of mono-2,2,2-trichloroethyl phosphate (2 equiv.). The activated phosphate was then condensed with the protected 5'-trityloxyacetyl nucleoside or oligomer (1 equiv.) to yield the 3'-phosphate derivative. This derivative was isolated by solvent extraction, reactivated with TPS (2 equiv.) and condensed with the 5' hydroxyl of the next nucleoside or oligomer (1 to 1.5 equiv.). It is important to note that a large excess of one sequence was not necessary in the coupling of oligomer blocks. After each coupling reaction a sample was completely deprotected and characterized by nuclease degradation to check the fidelity of the internucleotide linkage.

The phosphorylation and coupling reactions were conveniently monitored by the change in R_F on silica gel tlc in 10% methanol-methylene chloride. The trityl group allowed the detection of minute quantities as a yellow colouration by acidic ceric sulfate spray.

As the phosphates of the oligomers were protected, these oligomers were readily soluble in organic solvents. They were therefore purified by silica gel column chromatography in methylene chloride using increasing concentrations of methanol as eluent. These columns efficiently separated product from unreacted materials as the sequence bearing the 3' phosphate and the incoming nucleoside (oligomer), lacking a 5'-trityloxyacetyl group, were both retained longer than the condensation product. Occasionally, the incoming nucleoside co-eluted with product. 'Back-tritylation' of the unprotected 5' hydroxyl of incoming nucleoside was used to alter its mobility and thereby affect the separation (Neilson

and Werstiuk, 1971b). Silica gel columns are also advantageous as they can be run quickly (<8 hr) and have high capacities.

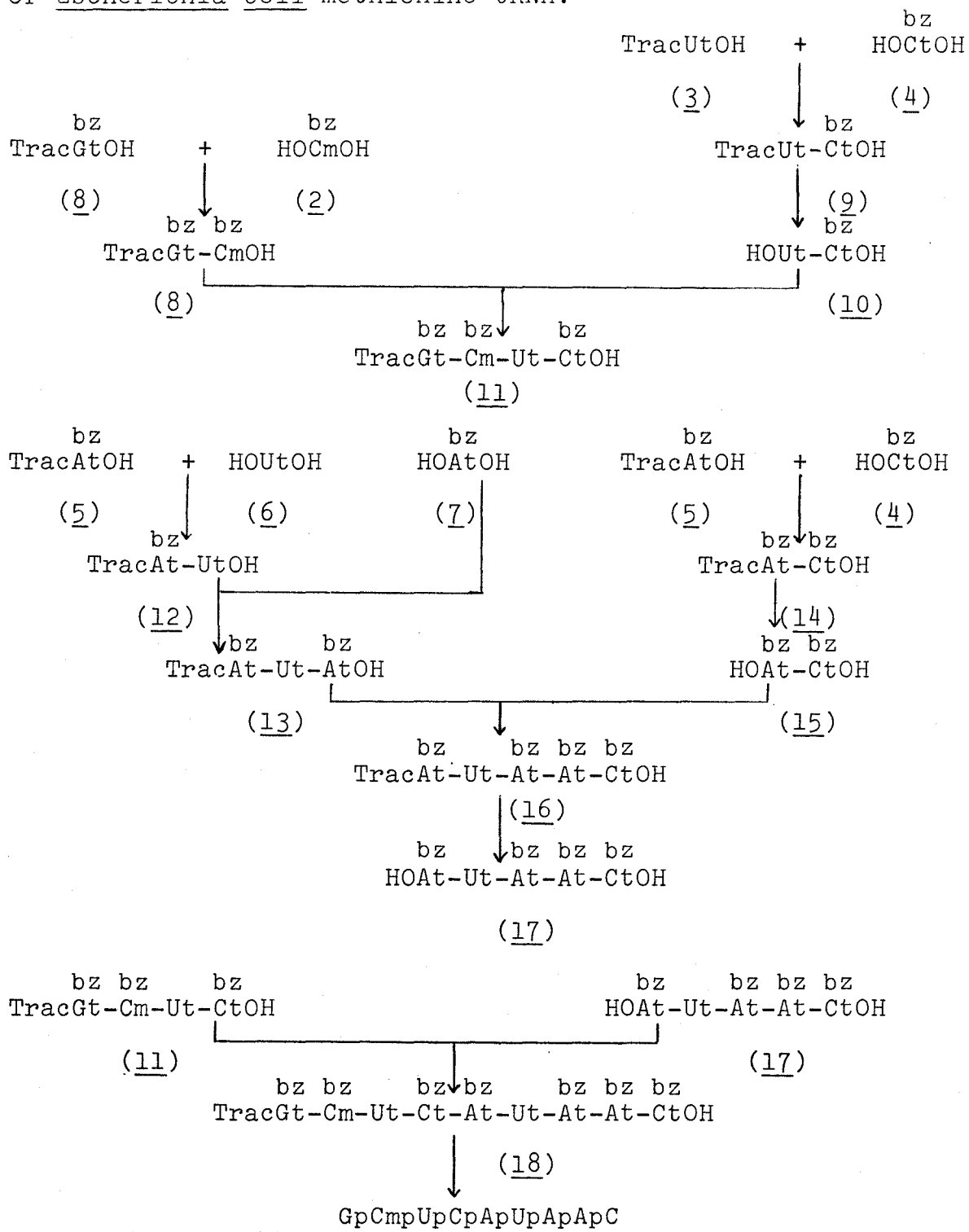
Figure 10 outlines the scheme for the synthesis of the non-anucleotide, GpCmpUpCpApUpApApC, corresponding to the anticodon loop of *E. coli* $tRNA_f^{Met}$. The protected tetranucleotide, TracGt-Cm-Ut-CtOH (11) was prepared by condensation of dinucleotides 8 and 10. The protected pentanucleotide, TracAt-Ut-At-At-CtOH (16), was prepared by condensation of 13 with 15. 16 was converted to 17 by mild alkali which selectively removes 5'-trityloxyacetyl groups (Werstiuk and Neilson, 1972) and 17 was condensed with the 3'-phosphate derivative of 11 to yield the final protected nonanucleotide (18). Deprotection of 18 by a three-step procedure (Neilson and Werstiuk, 1971) and purification by descending paper chromatography gave the desired, free oligomer.

The yields for the condensation reactions were above 60% for the coupling of single nucleosides. As an example, TracAtOH (1) (1.46 mmol) was phosphorylated and condensed with HOUtOH (2) (2.19 mmol) to yield TracAt-UtOH (12), 75%.

The coupling of oligomer blocks gave lower yields, in the 20 to 40% range. TracGt-CmOH (8) (0.083 mmol) was phosphorylated and condensed with HOUt-CtOH (10) (0.125 mmol) to give 11, 24%.

The good yields obtained for the condensation reactions plus the high capacity of the silica gel columns permitted relatively large scale (mmol) synthesis.

Figure10. Phosphotriester synthesis of the anticodon loop of Escherichia coli methionine tRNA.



Abbreviations: Trac is 5'-O-trityloxyacetyl, t is 2'-O-tetrahydropyranyl, bz represents benzoyl modification of base amino function, and hyphen between two characters eg. At-Ut, indicates a 2,2,2-trichloroethyl phosphotriester internucleotide linkage.

2.1.3 Evaluation of Phosphodiester and Phosphotriester Synthesis

When the two methods of oligoribonucleotide synthesis were compared, the phosphotriester synthesis of Neilson and co-workers was found far superior to the phosphodiester synthesis of Ikehara's group in regards to the yields for the condensation reactions, the scale of these couplings and the ease of purification. There is only one aspect in which the phosphodiester synthesis distinguishes itself - the time required for the condensation and deprotection reactions.

With TPS as an activating agent, the phosphodiester condensations were complete within 24 hours. DCC activated reactions were somewhat longer, normally 3 to 4 days. On the other hand, the phosphotriester synthesis required 1 to 2 days for the phosphorylation reaction and 2 to 5 days for the coupling reaction. Similarly, the deprotection of phosphotriester intermediates required longer time intervals than did the intermediates of the phosphodiester synthesis. Phosphodiester intermediates were deprotected by treatment in 80% acetic acid for 1 hour and 15N methanolic ammonia for 16 hours. The intermediates of the phosphotriester synthesis were deprotected by a three-step procedure: 1. 1 day Zn/Cu couple, 2. 2 day methanolic ammonia and 3. 1 day pH 2.

However, despite the slower reactions of the phosphotriester synthesis, the higher yields etc., made it the most practical method for the chemical synthesis of oligoribonucleotides. (There is a hidden advantage in the longer reaction times as more sequences can be synthesized concurrently.)

It is interesting to note that Letsinger and co-workers (1975) have examined methods for the rapid formation of internucleotide phosphotriester linkages. They have developed a synthesis that is based on the high reactivity of phosphorocholoridites ($ROPCl_2$ and ROP(OR')Cl) towards alcohols in tetrahydrofuran at low temperature and the facile oxidation of phosphites to phosphates by iodine and water. In a preliminary communication o-chlorophenyl ester derivatives of thymidylylthymidine (3'-3', 5'-5' and 3'-5' internucleotide linkages) were prepared from o-chlorophenyl phosphorodichloridite and suitably protected thymidines. The reactions proceeded to completion rapidly (seconds to minutes) and afforded the desired phosphotriester in 65 to 75% yields. Successful extension of this procedure to the coupling of the other nucleosides and to the ribose series could facilitate an efficient general synthesis of polynucleotides.

2.2 Enzymatic Synthesis

The advantage of enzyme-catalyzed synthetic reactions stems from the specificity guaranteed by the respective enzymes. The protection of the various functional groups is therefore unnecessary. However, only small quantities of material may be prepared as large scale reactions require prohibitive amounts of enzyme.

The enzymes that have been studied for their usefulness in polynucleotide/oligonucleotide synthesis can be divided into three main classes: (for review see Kossel and Seliger, 1975)

I. Polymerizing enzymes
   a. primer dependent
   b. primer-template dependent

II. Polynucleotide ligases

III. Ribonuclease

The primer dependent polymerizing enzymes add activated nucleotide units to the 3' ends of short oligonucleotide primers. Depending on the availability of these activated nucleotides, either homopolymers or random copolymers are generated.

Polynucleotide phosphorylase has been used extensively in the synthesis of ribopolynucleotides. This enzyme catalyzes the polymerization of nucleotide-5'-diphosphate to the 3' end of a dinucleotide monophosphate, or longer oligoribonucleotide primer -

$$N_1pN_2 + ppN \xrightleftharpoons{\text{PD PNPase}} N_1pN_2pN_3(pN)_n + nPi$$

At high concentrations of inorganic phosphate this reaction is reversed towards phosphorolysis of ribopolynucleotides.

Polynucleotide phosphorylase has also been employed for the synthesis of ribooligonucleotides (for review see Thach, 1966). High salt (0.4M NaCl) inhibits the polymerization reaction and the addition of only a few nucleotides (1 to 3) to a dinucleotide monophosphate can be accomplished with good yields. The product of this reaction can be conveniently isolated by paper chromatography. However, there are problems in using a primer of greater length than a dinucleotide as phosphorolysis will cause randomization. Theoretically this can be avoided by maintaining a high NDP to Pi ratio.

Recently, 2'-O-(α-methoxyethyl)nucleoside 5'-diphosphates were developed as 'single-addition' substrates for the step-wise synthesis of specific ribooligonucleotides with polynucleotide phosphorylase (Bennet et al., 1973). The O-(α-methoxyethyl) fulfills all of the following

requirements:

1. The group is stable under the conditions of the enzyme reaction.

2. It permits the addition of a single nucleotide to an acceptor oligonucleotide.

3. It prevents the phosphorolysis reaction.

4. The group is readily removed under conditions that do not affect the structure of the ribooligonucleotide product, and

5. After the removal of the blocking group the ribooligonucleotide is available for a second, single addition reaction.

By two consecutive addition reactions, the pentanucleotide pApApApUpA was synthesized from the trinucleotide pApApA in excellent yield. (MacKey and Gilham, 1971).

Similarly, Walker and Uhlenbeck (1975) have used 2'(3')-O-isovaleryl ribonucleoside 5'-diphosphates as 'single addition' substrates. They have reported the synthesis of several tetranucleotides in 30 to 70% yields in umole scale reactions.

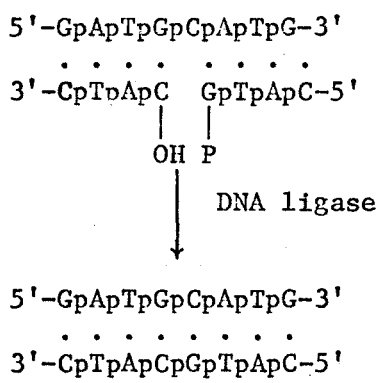
The synthesis of deoxyribopolynucleotides has been accomplished with terminal deoxynucleotide transferase. This enzyme catalyzes the polymerization of deoxynucleotide-5'-triphosphates onto the 3' end of a deoxytrinucleotide diphosphate, or longer, primer -

$$dN_1pdN_2pdN_3 + dNTP \rightleftharpoons dN_1pdN_2pdN_3pdN_4(pdN)n + nPPi$$

It has not been possible however to synthesize deoxyribooligonucleotides with this enzyme by either inhibition of the polymerization reaction or by the development of 'single' addition nucleotides.

DNA-dependent DNA polymerase and DNA-dependent RNA polymerase are examples of primer-template dependent polymerizing enzymes. As these enzymes can only synthesize a polynucleotide that is complementary to the template, their usefulness in the synthesis of defined sequences is limited. However if small quantities of the desired sequence are available these enzymes may be used for their amplification.

Polynucleotide ligases catalyze the joining of the 3'-terminal hydroxyl of one sequence with the 5'-terminal phosphate of another. Two enzymes with this activity have been described - DNA ligase (Weiss and Richardson, 1967) and RNA ligase (Silbur et al., 1972). These enzymes differ not only in their substrate specificity but also in their requirement for a template. To ensure DNA ligase activity the two sequences to be joined must be held in adjacent position by means of base pairing with an overlapping template -



There is no template requirement for RNA ligase.

The use of polynucleotide ligases to join sequences together is analogous to the development of block coupling procedures in chemical synthesis. They both overcome the limitations of step-wise synthesis.

Ribonucleases make up the third class of enzymes employed in the synthesis of ribooligonucleotides. The 2',3'-cyclicphosphates that are

formed in the first step of the degradation of RNA are hydrolyzed to 3'-monophosphates in the second. Although the second step is irreversible, under certain conditions it proceeds at a slower rate than the transesterification step. Thus if high concentrations of the 5'-hydroxyl component are present, the equilibrium may be shifted towards internucleotide bond formation (see Figure 11).

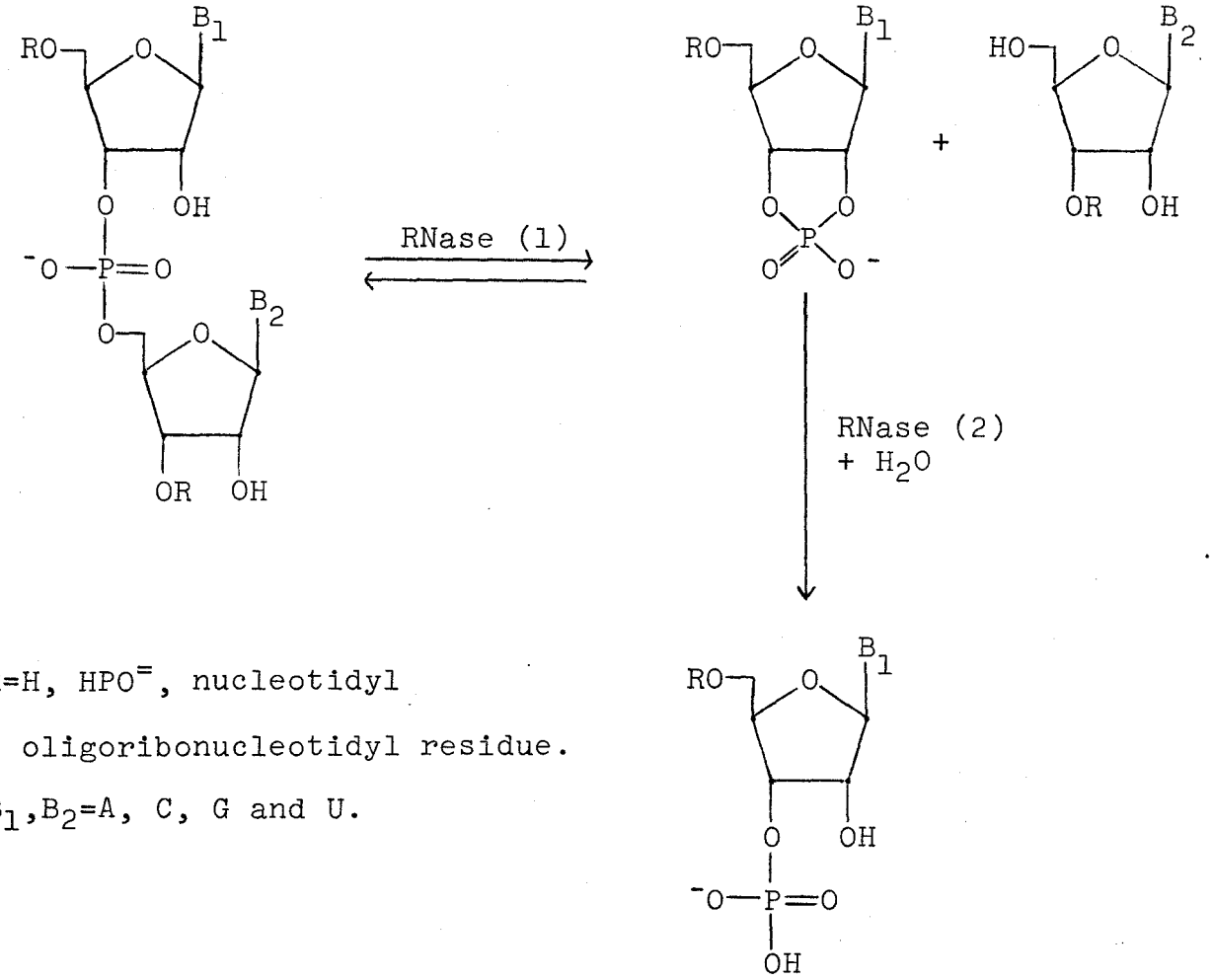
Ribooligonucleotides ranging from dinucleotides to octanucleotides have been successfully synthesized by the ribonuclease method. However, this approach has a number of disadvantages, namely:

1. low yields, 5 to 30%
2. low scale
3. inability to join oligonucleotides containing susceptible bonds. (eg. T_1 ribonuclease can only be used for the joining of oligomer blocks which do not contain any internal or 5'-terminal guanosine residues.) and
4. incomplete removal or denaturation of ribonuclease will lead to degradation during workup or purification.

2.2.1 Evaluation

Primer dependent polynucleotide phosphorylase is the only enzyme that has been sufficiently characterized to be suitable for the preparation of oligoribonucleotides corresponding to sequences in tRNA. By inhibition of the polymerization reaction, model oligoribonucleotides of defined sequence have been prepared for thermodynamic (Martin et al., 1971) and oligomer binding studies (Uhlenbeck, 1972). Although this method originally suffered from low scale and low yields - especially for the addition of a purine NDP to a pyrimidine dinucleotide, Borer and co-workers

Figure 11. Ribonuclease synthesis of oligoribonucleotides.



(1975) have developed methods for the synthesis of a hexanucleotide, ApApGpCpUpU, on a 50 mg scale (see Figure 12).

Similarly, the single addition of 2'-protected ribonucleoside 5'-diphosphates to oligoribonucleotides by PD PNPase has been scaled up to produce mg quantities of a tetranucleotide, CpCpGpG, for an NMR study (Arter et al., 1974).

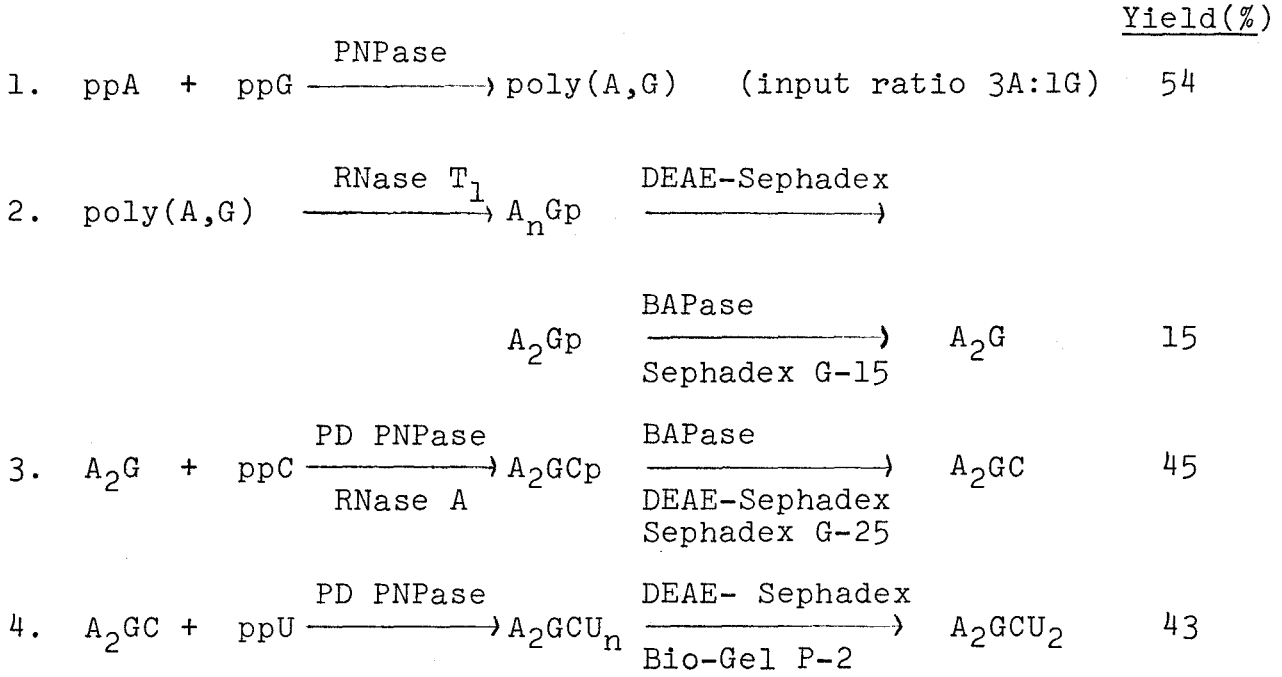
Both of these step-wise techniques are limited however, by the length of sequence that can be efficiently synthesized. The utilization of RNA ligase could extend this limit. RNA ligase normally catalyzes the intra-molecular cyclization of a sequence bearing a 5' phosphate and a 3' hydroxyl. However if the sequence with a 5' phosphate is kept shorter than the minimum length required for the cyclization reaction (an octanucleotide) and a high concentration of the sequence with a free 5' and 3' hydroxyl is maintained, the intermolecular reaction is catalyzed (Walker et al., 1975). Alternately it may be possible to prevent the intramolecular reaction by protecting the 3' hydroxyl of the sequence bearing the 5' phosphate.

2.3 Chemical Enzymatic Synthesis

The total synthesis of the gene for yeast $tRNA^{Ala}$ (Agarwal et al., 1970) and the synthesis of the structural gene for the precursor of E. coli $tRNA^{Tyr}_{su^+_{III}}$ (Khorana, 1976) represent the most impressive achievements in polynucleotide synthesis. The former is a double-stranded DNA, 77 nucleotides in length and the latter is a 126-nucleotide-long DNA duplex.

These genes were designed primarily to study structure-function relationships in tRNA. By the introduction of point mutations, deletions, and additions, the role of the various loci in the molecule could be

Figure 12. Enzymatic synthesis of ApApGpCpUpU.



studied. Further, the events of tRNA maturation could also be investigated.

The yeast $tRNA^{Ala}$ gene synthesis was initiated in 1965 before the concept of mature RNA species arising from longer transcripts was established. The transcription of this gene has therefore not been possible as it does not contain the appropriate initiation and termination signals. The switch to the E. coli $tRNA^{Tyr}_{su^+III}$ was initiated by the sequencing of the tRNA precursor (Altman and Smith, 1971). As the precursor contained a 5' triphosphate it was considered to be an initial transcript. However, it now appears the termination signal for the gene is at least 100 nucleotides beyond the 3' terminal of the 'precursor' tRNA of Altman and Smith (Bikoff et al., 1975). The synthesis of the structural gene plus the appropriate initiation and termination signals for transcription would present a formidable task.

The following plan was designed for the gene synthesis:

1. Chemical synthesis by diester method of deoxyribooligonucleotides 8 to 12 units in length and containing free 3' and 5' hydroxyls. These segments would represent both strands of the DNA duplex. Further, these segments are selected to allow overlaps of four to seven base pairs.

2. Enzymatic phosphorylation of the 5' ends by polynucleotide kinase, and

3. Ligation of the segments in the appropriate duplex by DNA ligase.

These strategies have been recently refined for optimized yields and reduced time investments for both the enzymatic and chemical synthetic steps (Powers, 1975).

A similar strategy employing T4 RNA ligase could be envisioned for the synthesis of polyribonucleotides.

2.4 Summary

To date, the only methods that appear appropriate for the efficient synthesis of oligoribonucleotides are the phosphotriester chemical synthesis of Neilson and co-workers and the step-wise enzymatic synthesis with primer dependent polynucleotide phosphorylase (Walker and Uhlenbeck, 1975 and Borer et al., 1975). Two factors enter in the choice of method - scale and time. The phosphotriester method has the advantage of scale but the coupling reaction is much slower than the enzyme catalyzed addition.

However when this research project was initiated (1972) only the phosphotriester synthesis had been described. It was therefore the method that was employed. It was also planned that this work would extend the previous synthesis of a few simple tri- and tetranucleotides (Neilson and Werstiuk, 1971b and Werstiuk and Neilson, 1972) and also improve the incorporation of guanosine into the synthetic scheme (Neilson et al., 1973).

3. THE CHEMICAL SYNTHESIS OF OLIGORIBONUCLEOTIDES CORRESPONDING TO SEQUENCES IN TRANSFER RNA MOLECULES

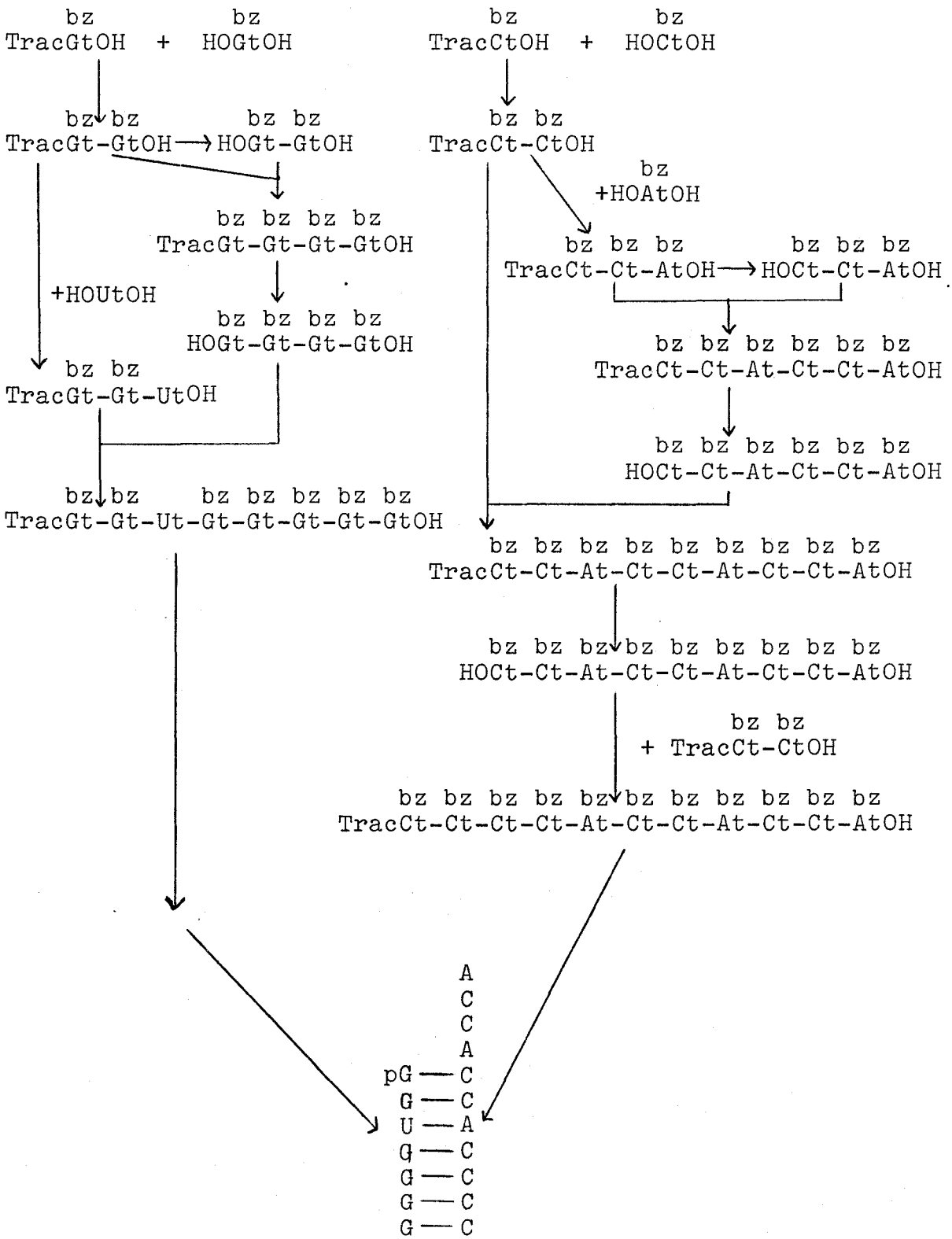
3.1 An Amino Acid Acceptor Arm

Although no specific recognition sites for the interaction of an aminoacyl-tRNA ligase with its cognate tRNA have been identified, certain loci in the tRNA are prime candidates for involvement in this process. When this study was initiated (1972) much of the attention was focused on the amino acid acceptor arm.

On the basis of the photoinactivation of amino acid acceptor activity for yeast $tRNA^{Ala}_{Iab}$, Shulman and Chambers (1968), concluded that the residues in positions 5,6 and 7 (from the 3' end) were part of the specific recognition site. As the residues in these positions in other tRNAs were unique, a hypothesis was proposed that "the specific recognition site for the aminoacyl-tRNA ligase involves the first three base pairs of tRNA.... but the entire stem region plays a role in maintaining the stereochemical integrity of the recognition site." The reconstitution of alanine acceptor activity from fragments of yeast $tRNA^{Ala}_{II}$ (Imura *et al.*, 1969), further implicated the acceptor arm as a recognition site.

The synthesis of oligoribonucleotides corresponding to the 5' and 3' strands of the acceptor arm of a tRNA was therefore planned. A survey of the known primary structures of several tRNAs (Dirheimer *et al.*, 1972) indicated that the synthesis of the acceptor arm of *E. coli* $tRNA^{Tyr}_{II}$ would be the simplest task (Figure 13). The highly repetitive nature of the sequences would permit the use of the recently developed, block condensation reaction (Werstiuk and Neilson, 1972). Further, the preparation of

Figure 13. Scheme for the synthesis of the amino acid acceptor arm of E. coli tyrosine tRNA.



necessary guanosine derivatives and their utilization in the phosphotriester method of Neilson and co-workers had been described (Neilson et al., 1973).

A common feature of all tRNA molecules is the terminal 5' phosphate. As the phosphotriester synthesis yields oligomers with free 5'-hydroxyl groups, it was necessary to devise a mechanism for the insertion of a 5' phosphate on the heptanucleotide, GpGpUpGpGpGpG. Although 5' phosphorylation with polynucleotide kinase can be conveniently achieved (eg. Beltchev and Grunberg-Manago, 1970a), chemical insertion by an extension of the general phosphotriester method of oligoribonucleotide synthesis was investigated (Neilson et al., 1974).

The choice of the amino acid acceptor arm of E. coli $tRNA_{II}^{Tyr}$ was not based solely on synthetic considerations. A combination of the 5' fragment (a pentadecanucleotide) and the 3'-half molecule competitively inhibited the aminoacylation of the native tRNA (Beltchev and Grunberg-Manago, 1970a). In support of the hypothesis of Shulman and Chambers, it was therefore suggested that the acceptor arm of E. coli $tRNA_{II}^{Tyr}$ contained a specific recognition site. This assumption was strengthened by the demonstration that a combination of the 3' fragment (a nonadecanucleotide) and oligo G (10-12 nucleotide units) also competitively inhibited aminoacylation (Beltchev and Grunberg-Manago, 1970b). On the basis of these results, the synthetic amino acid acceptor arm of E. coli $tRNA_{II}^{Tyr}$ was expected to have similar activity. Unlike the isolated acceptor arm of E. coli $tRNA_{II}^{Ala}$, this acceptor arm would probably not be aminoacylated (Nishimura, 1973).

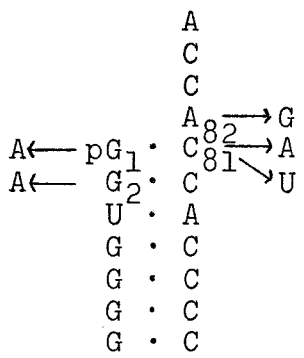
It is interesting to note that the combination of the 3' fragment and oligo G cannot form the A_3=U_{79} base pair. This base pair was therefore not considered to be essential for the specific recognition of the acceptor arm. Chemical synthesis of acceptor arms incorporating base substitutions at these positions would provide excellent models for the examination of this possibility.

Additional evidence that the acceptor stem contains a specific recognition site was obtained from studies of mutants of the su^+_{III} tyrosine suppressor tRNA gene (Celis et al., 1973). Single base substitutions near the -CpCpA terminus caused mischarging with glutamine (Figure 14). Thus, the two terminal base pairs and the base in position 82 were also prime candidates for investigation.

However genetic analysis provides a limited approach to the study of the relationship of structure and function in tRNA. Only tRNA species that are defective in suppressor activity are detected. Base substitutions that do not affect aminoacylation or codon recognition may not be observed. Also if a single base change in a double-stranded region leads to defective function it may be wrongly concluded that the base is part of a recognition site. A complementary base change in the opposite strand could restore normal activity. Finally, as base substitutions are rare and random events the task of examining all the possible substitutions in a specific position becomes very difficult. Chemical synthesis provides an attractive alternate approach as a specific position can be studied directly and more than one substitution can be introduced.

A comparison of the ability of native and mutant amino acid acceptor arms to inhibit aminoacylation may not be sufficient to locate

Figure 14. Base substitutions in amino acid acceptor arm of glutamine mischarging tyrosine tRNAs.



a recognition site. As suggested by Schimmel (1973), studies which quantitate the association constants of tRNA fragments for the cognate ligase may be more informative. It was therefore proposed that either the membrane filter assay of Yarus and Berg (1967) or gel filtration of the enzyme·acceptor arm complex (eg. Fasiolo et al., 1974) be used to study the affinity of E. coli tyrosyl-tRNA ligase for the amino acid acceptor arm. These studies would also investigate the role of the acceptor arm in the interaction between the aminoacyl-tRNA ligase and the native tRNAs. Although the acceptor arm is clearly not the only site of interaction with the ligase, there is some confusion as to the contribution of this region to tRNA binding (Bonnet et al., 1975).

Unfortunately, synthetic problems forced the abandonment of the project. The following aspects of the project were, however, realized:

1. The synthesis of protected oligoribonucleotide blocks, bz bz bz bz bz bz TracGt-GtOH, TracGt-Gt-UtOH and TracCt-CtOH, and

2. The chemical insertion of a 5' phosphate in oligoribonucleotide pGpG, a model of the 5' terminus of the acceptor arm (Neilson et al., 1975).

3.2 The Double-Stranded Region of the Dihydrouridine Arm

A comparison of the primary structures of several tRNA species that were aminoacylated by yeast phenylalanyl-tRNA ligase indicated the sequence of the double-stranded region of the dihydrouridine arm was common (Dudock et al., 1971). It was proposed therefore, that this region was a specific recognition site for the enzyme. A more extensive survey (Roe and Dudock, 1972), not only supported this hypothesis but indicated that a second site, the fourth nucleotide from the 3' end, was also

involved (see Figure 6). There was an absolute requirement for adenosine at this position.

The kinetic parameters of the homologous and heterologous aminoacylation reactions have also been investigated (Roe et al., 1973). The tRNA species were observed to fall into three distinct classes (based on differences in Vmax) - fast, intermediate and slow. The slow class had a nine-member DHU loop instead of the normal, eight-member loop. The intermediate and fast classes were distinguished by the substitution of N^2-methylguanosine for guanosine at position 10 in the latter. Methylation of this residue had a considerable effect on the Vmax (200-fold difference), but only a negligible effect on the Km (10-fold). N^2-methylguanosine cannot be considered essential for recognition, but it is clear that the modification of this base can influence the function of the tRNA by altering its kinetic parameters. This observation would tend to support the hypothesis of Ebel and co-workers (1973) that the recognition of a tRNA is less than absolute and that the specificity of the aminoacylation reaction is governed by kinetic parameters (ie. Vmax).

Whether all nine nucleotides of the neck of the DHU arm are required for recognition is not known. However, it has been suggested that the upper strand (-ApGpCpUpC-) may serve a primary role (Roe et al., 1973). This assumption was based on the effect of the methylation of G_{10} on the kinetics of the aminoacylation reaction and on the charging of E. coli $tRNA^{Arg}$ with yeast phenylalanyl-tRNA ligase. This tRNA has an identical upper strand but the bottom strand has U instead of C at position 25.

Nevertheless, the heterologous aminoacylation reaction of yeast phenylalanyl-tRNA ligase has been so well characterized that based on the charging of E. coli $tRNA_I^{Ala}$ and the kinetics of the reaction, the molecule was correctly predicted to have:

1. the specific nucleotides of the neck of the DHU arm
2. adenosine in the fourth position from the 3' terminus
3. a DHU loop of 8 nucleotides, and
4. unmethylated guanosine at position 10 (Williams et al., 1974).

It is interesting to note that phenylalanine tRNAs from both prokaryotes (eg. Bacillus stearothermophilus, Guerrier-Takada et al., 1975) and eukaryotes (eg. calf liver, Keith et al., 1974) have the same DHU neck and fourth nucleotide from the 3' end. The same structures have also been found in nearly 25% of sequenced tRNAs. These observations have lead to the suggestion that these tRNAs might have been derived from a common ancestral gene (Jukes and Holmquist, 1972) and that during evolution this region has been maintained as an important recognition site for the aminoacyl-tRNA ligase (Roe et al., 1973).

Another approach that has been used in the identification of regions or bases that are essential for enzyme recognition is the study of the interaction of aminoacyl-tRNA ligase with split or fragmented tRNAs. It has been demonstrated that the integrity of the DHU loop (Samuelson and Keller, 1970), the anticodon loop (Thiebe and Zachau, 1969), and the TψC loop (Schmidt et al., 1970) were not necessary for amino-acylation of yeast $tRNA^{Phe}$. Furthermore, removal of the anticodon arm and the extra arm did not abolish acceptor activity (Thiebe et al., 1972). However an intact DHU neck and TψC neck were required. This last

observation is consistent with the hypothesis that the neck of the DHU arm functions as a recognition site.

Alternately, the recognition process has been studied by attempting to map the general topology of the enzyme-tRNA complex. Nuclease digestion of the complex of yeast phenylalanyl-tRNA ligase and $tRNA^{Phe}$ has indicated that both nuclease-sensitive sites, the DHU loop and the anticodon loop, were strongly protected (Horz and Zachau, 1973). It was also observed that the ligase afforded some protection to the 5'-half molecule. As the only secondary structure in this fragment is the DHU arm it would suggest that the proposed recognition site is an enzyme binding site. Supportive evidence for this statement is obtained from photochemical cross-linking of enzyme-tRNA complexes. Three regions of $tRNA^{Phe}$ were found to cross-link with yeast phenylalanyl-tRNA ligase -

1. a region extending from the middle of the 5' side of the acceptor arm to the DHU arm

2. a region extending from the 3' side of the extra arm into the TψC arm, and

3. a region extending from the middle of the TψC loop to the acceptor arm (Shoemaker et al., 1975).

These regions must therefore have been in close contact with the ligase.

A number of aspects of the photochemical cross-linking studies require additional comment:

1. The demonstration that three regions of yeast $tRNA^{Phe}$ make close contact with the ligase is similar to the 'three-point attachment' observed for E. coli $tRNA_f^{Met}$ by Dube (1973).

2. Although well apart in the primary and secondary structure, these regions are quite close in the tertiary structure. They are located at the corner of the L-shaped molecule. The importance of tertiary structure in the formation of the enzyme tRNA complex has been well established (Horz and Zachau, 1973).

3. The finding that the region extending from the 5' side of the acceptor arm to the DHU arm makes close contact with the ligase not only supports the hypothesis that the double-stranded region of the DHU arm is a recognition (binding) site but also supports the proposal that only the upper strand is recognized by the ligase (Roe et al., 1973) and

4. No interaction with the terminal portion of the acceptor arm, especially in the region of the fourth nucleotide from the 3' end, was observed. In fact, in the nuclease digestion studies of enzyme·tRNA complexes it was found that removal of the terminal 7 residues from the 3' end of yeast $tRNA^{Phe}$ had only a minimal effect on the stability of the complex (Horz and Zachau, 1973). These observations do not support the proposal that the fourth nucleotide is a recognition site but rather support the proposal that the role of the acceptor arm is the alignment of the 3'-terminal adenosine in the catalytic centre (Bonnet et al., 1975).

In summary, it would appear that the partial recognition site for yeast phenylalanyl-tRNA ligase, the double-stranded region of the DHU arm, is involved in the interaction between the tRNA and the enzyme. However no attempt has been made to measure the contribution of this region to the binding of the native tRNA. A study of the affinity of the ligase for the isolated DHU neck was therefore proposed to provide important information about this interaction. The effect of base

substitutions on the binding affinity could then determine which of the nine nucleotides of the DHU neck were required for specific recognition. The phosphotriester method of oligoribonucleotide synthesis of Neilson and co-workers was the only method that was suitable for the preparation of sufficient quantities of the desired sequence.

In this thesis the synthesis of sequences which correspond to the common DHU neck of the tRNAs aminoacylated by yeast phenylalanyl-tRNA ligase is described (England and Neilson, 1976). Oligoribonucleotides GpApGpC and ApGpCpUpC, synthesized by the step-wise method (Figures 15 and 16 respectively), were expected to form duplex I (Figure 17). However, as ApGpCpUpC contains a self-complementary sequence, it may form duplex II which would compete with the formation of the desired duplex I. Oligoribonucleotide, GpCpUpC, was also synthesized (Figure 18) to permit formation of duplex III which is free from such complications and still approximates the recognition site.

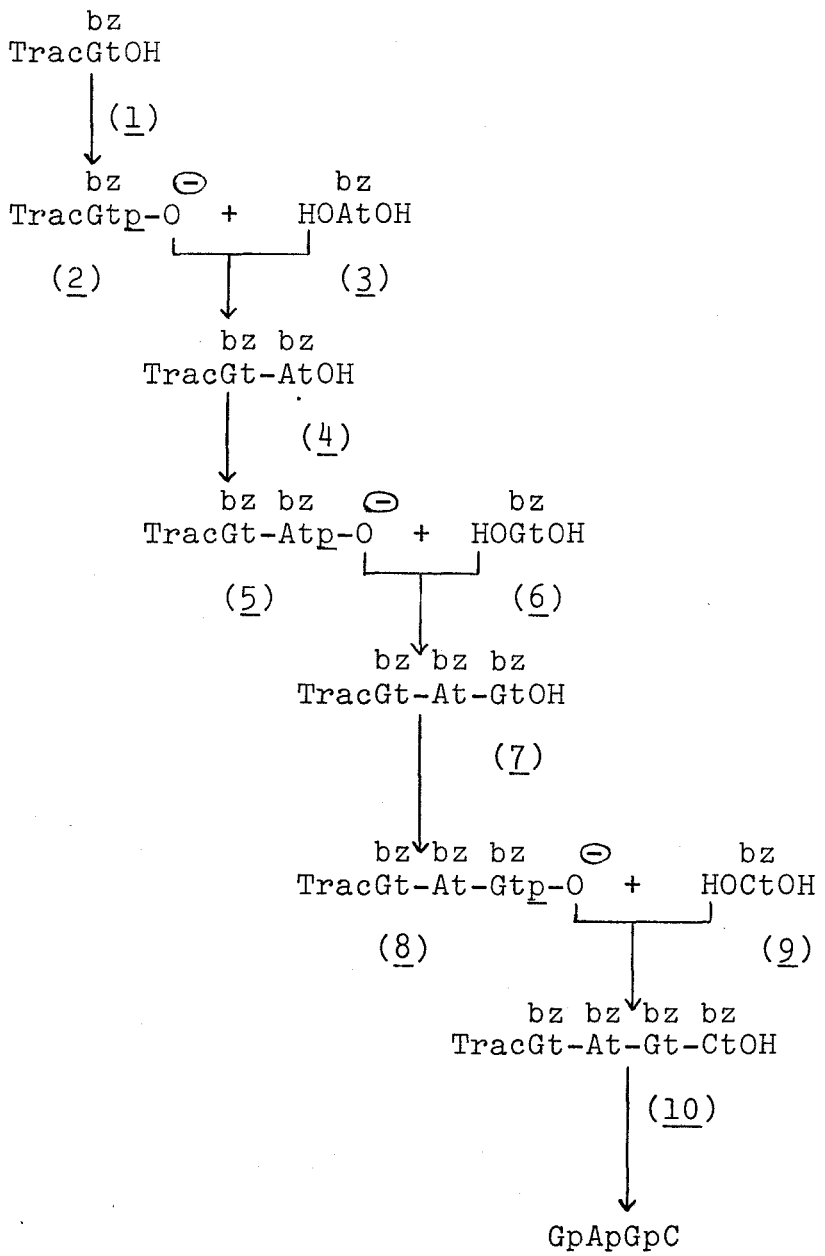
In the cognate tRNA, the sequence of the upper strand of the DHU neck is $Apm^2GpCpUpC$ (RajBhandary et al., 1967). However N^2-methylguanosine was not incorporated into the sequence as -

1. The cost of purchasing or synthesizing (Yamazaki et al., 1967) sufficient quantities was prohibitive.

2. Procedures for the preparation of the protected derivative would have to be developed, and

3. N^2-methylguanosine has only a small effect on the affinity of yeast phenylalanyl-tRNA ligase for the native tRNA (Roe et al., 1973). [Recently, a pentanucleotide containing N^2-methylguanosine was synthesized by a phosphodiester method (Ohtsuka et al., 1976)].

Figure 15. Scheme for the step-wise phosphotriester synthesis of GpApGpC.



Abbreviations. p-O$^{\ominus}$ is 3'-O-(2,2,2-trichloroethyl) phosphate.

Figure 16. Scheme for the step-wise phosphotriester synthesis of ApGpCpUpC.

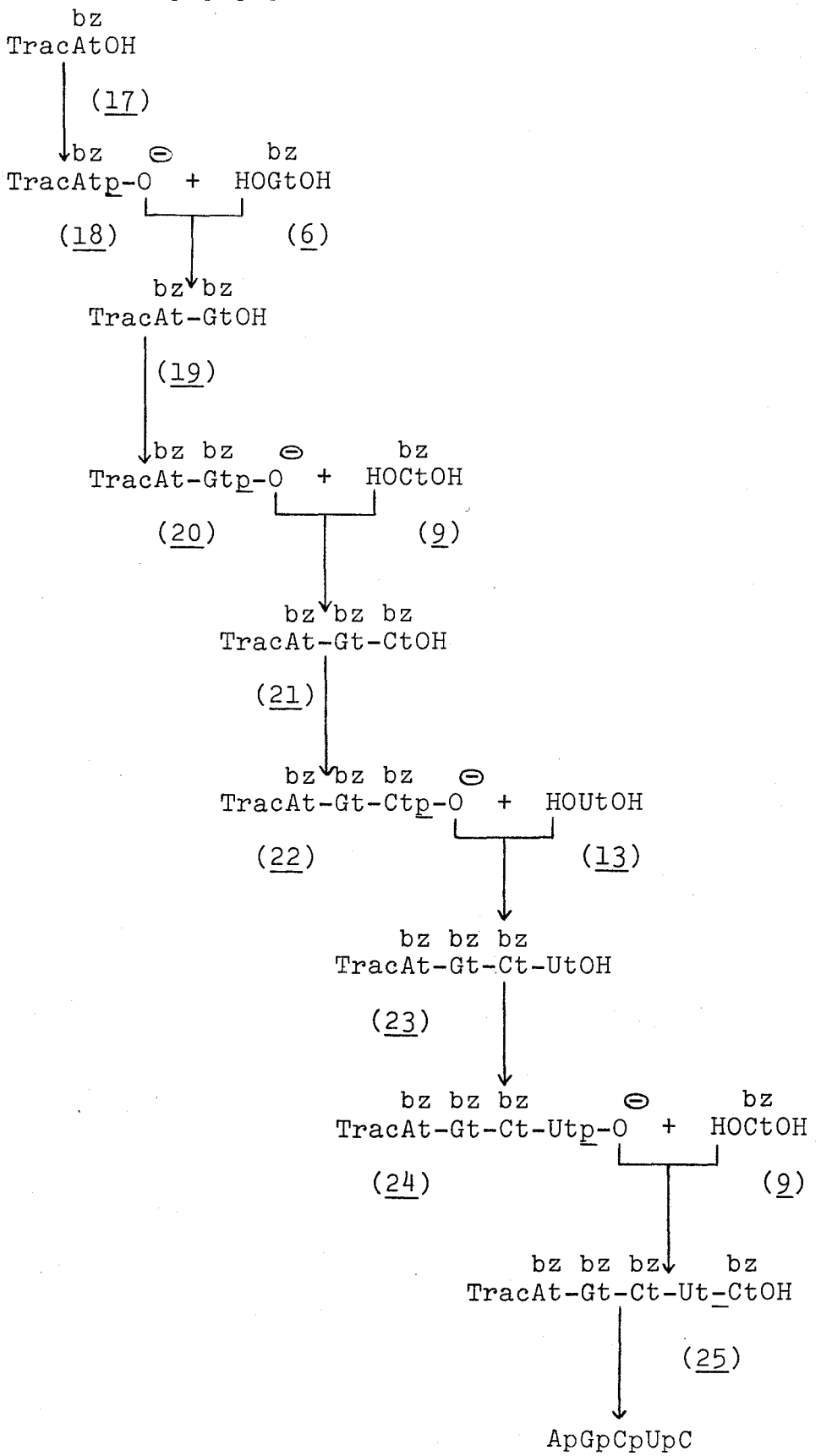
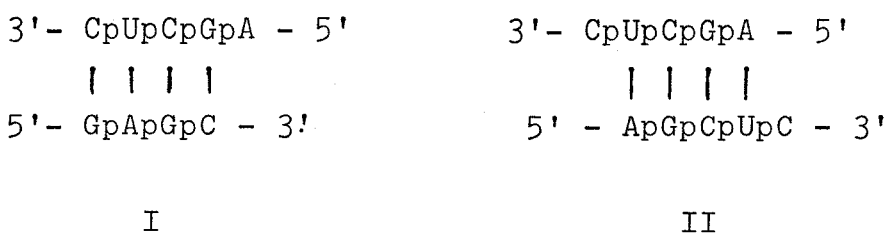


Figure 17. Duplexes formed by synthetic oligoribonucleotides.



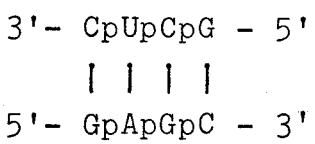
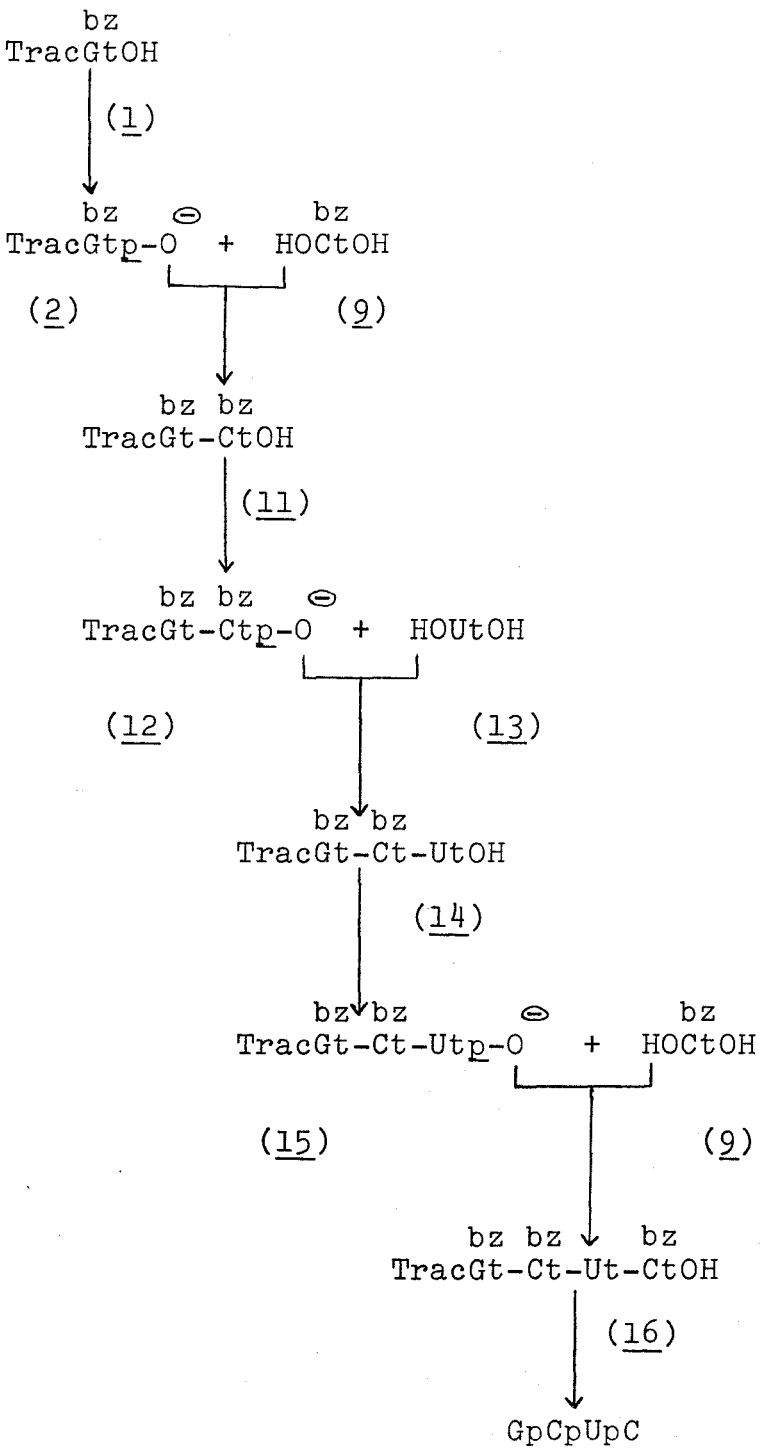


Figure 18. Scheme for the step-wise phosphotriester synthesis of GpCpUpC.



Unlike the synthesis of the amino acid acceptor arm of E. coli $tRNA^{Tyr}_{II}$, no difficulties were anticipated for the synthesis of the DHU neck. As the longest sequence was a pentanucleotide, the sequences could be conveniently obtained by a step-wise procedure. Further, as none of the sequences necessitated a G to G coupling, poor yields were not expected for any of the condensation reactions. However it was found that the reactions rarely went to completion, even with the addition of excess equivalents of the activating agent, TPS. The decreasing quantities of material with each step of the synthesis had serious consequences on the final yield of desired product as the condensation reactions were observed to be dependent on both scale and oligomer length.

The deprotection of the synthetic sequences was also unsatisfactory as low yields were obtained and often the material was seriously contaminated with side-products. A number of modifications were introduced into the three-step deprotection procedure (Neilson and Werstiuk, 1971b) in an attempt to both improve the yields and to reduce the extent of contamination. With this modified procedure it was possible to deprotect and characterize the dinucleotides ApG, GpC, and GpA and the trinucleotides ApGpC, GpCpU, and GpApG. However tetranucleotides ApGpCpU and GpCpUpC and the pentanucleotide ApGpCpUpC were still contaminated as characterization by nuclease degradation gave ambiguous results.

Attempts to purify the longer oligomers were only partially successful. When a homogeneous tetranucleotide (as judged by its chromatographic behaviour on paper) was chromatographed on a DEAE-cellulose column in 7M urea numerous peaks were observed. When the peak correspond-

ing to the correct number of charges was desalted by gel filtration on Biogel P-2 it was found to contain a mixture of oligomers. The first peak eluted from the Biogel P-2 column usually had the correct nucleoside ratio for the desired product. However the final yield of this deprotected sequence was very low (<10%).

Three explanations were considered for the large number of side-products observed in the deprotection procedure:

1. nuclease degradation
2. chemical degradation
3. impure protected oligomers.

Nuclease degradation was eliminated as it was observed that the material eluted from a Biogel P-2 column re-chromatographed on DEAE-cellulose as a single peak at the same salt concentration as in the original DEAE-cellulose column. Nuclease degradation prior to the DEAE-cellulose column was also ruled out as nucleases would not be active under the conditions of the deprotection.

If degradation was a result of the deprotection procedure, it was thought to have occurred during the reductive removal of the phosphate protecting group, 2,2,2-trichloroethyl, by Zn/Cu couple. The conditions for both the aminolysis of the base labile protecting groups and the hydrolysis of the acid labile groups are too mild for backbone cleavage. Further,2'-O-tetrahydropyranyl protection prevents the participation of 2' hydroxyls in the alkaline hydrolysis of the internucleotide bond. Although degradation of protected oligoribonucleotides by Zn/Cu couple has been suggested by another investigator in this laboratory (Deugau, 1975), this possibility has not been fully examined.

The final explanation, impure protected oligomers, was considered the most plausible. The final protected sequences may have been contaminated with prematurely terminated and/or chemically modified sequences.

The phosphorylation of certain oligomers (especially the longer sequences) were relatively slow and often failed to reach completion despite the addition of further equivalents of activated mono-2,2,2-trichloroethyl phosphate. This incomplete reaction may have been due to residual cyclohexylamine* or it may have been the result of some steric factor wherein the conformation of the terminal nucleoside reduces its reactivity. While the separation of a protected dinucleotide from its precursor nucleoside by silica gel column chromatography was relatively simple, the separation of a protected tetranucleotide from its precursor trinucleotide was a more difficult task as they eluted at similar methanol-methylene chloride concentrations. Contamination of the desired oligomer by its precursors could have been a source of the numerous products observed in the deprotection procedure.

Alternatively, the product may have been contaminated by sequences that were chemically modified during synthesis. Apparently arenesulfonyl chlorides react with the N-7 position of guanosine derivatives. Such modification would have serious consequences upon the synthesis as during the deprotection procedure these guanosines would be lost. In alkali the imidiazole ring of modified guanosines would open and during subsequent

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* The bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate was converted to the pyridinium salt by repetitive evaporation in vacuo from anhydrous pyridine before activation with TPS. Residual cyclohexylamine would inhibit phosphorylation and condensation reactions as TPS reacts with primary amines.

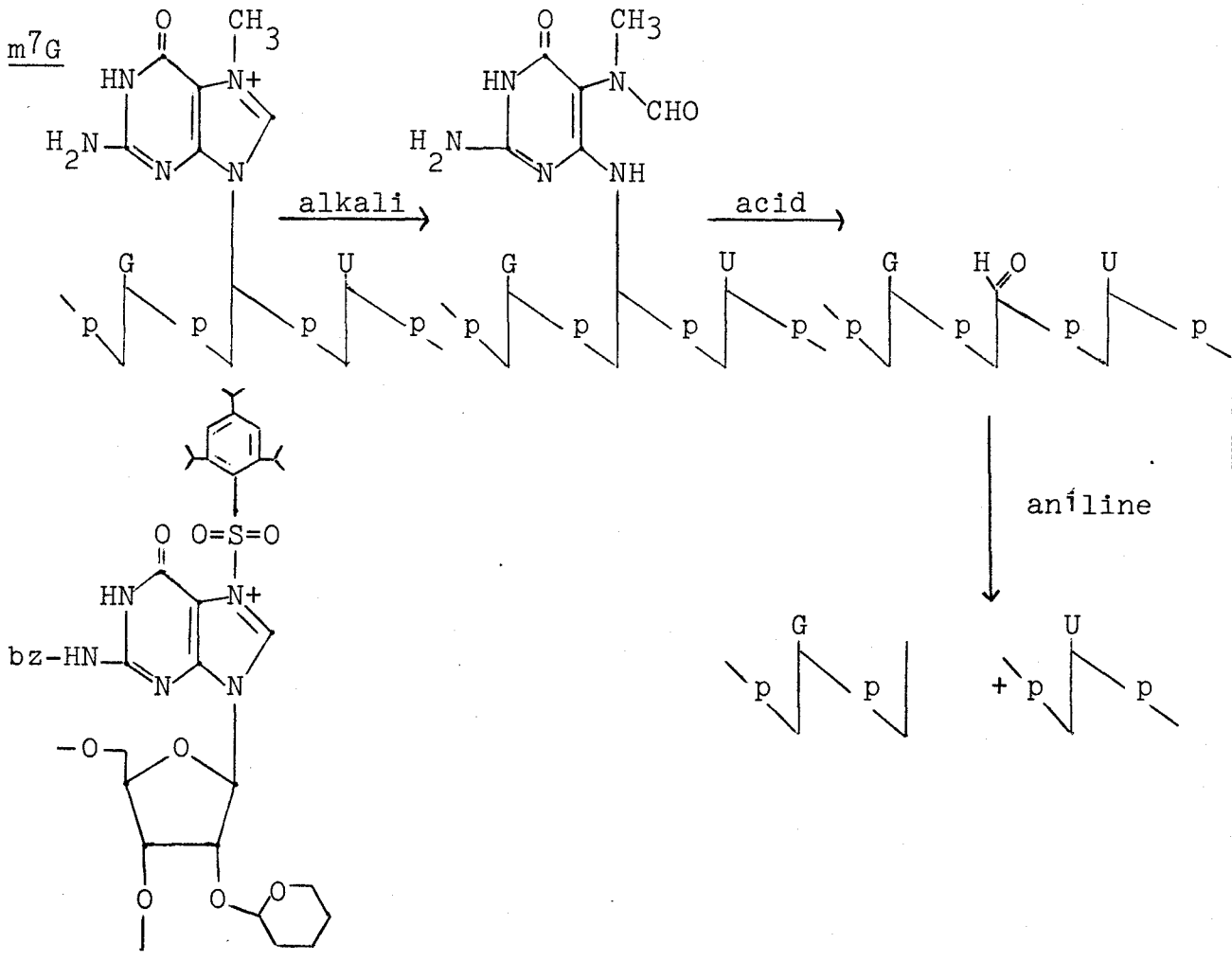
treatment at pH 2 the N-glycosidic bond to the 4-ribosylamino pyridimine would be split. This situation is analogous to the reactions of m^7G (see Figure 19) in the specific chain cleavage of yeast $tRNA^{Phe}$ (Wintermeyer and Zachau, 1970).

The liberation of HCl by arenesulfonyl chlorides during activation may have serious consequences. Pyridine hydrochloride cannot be considered a passive side product, especially towards acid-labile tetrahydropyranyl groups. The loss of 2'-hydroxyl protection not only introduces a new reactive site but can lead to chain cleavage during the deprotection procedure. The phosphodiester bond is easily cleaved in alkaline conditions by the participation of the neighbouring 2'-hydroxyl.

Both the random modification of guanosine residues and the loss of tetrahydropyranyls could pass undetected during the synthesis of longer oligomers if they were not extensive. These modifications would have a negligible effect on the chromatographic behaviour of the compounds. Furthermore, a single modification would be sufficient to destroy a sequence.

In order to avoid the possible pitfalls in the synthetic scheme associated with the use of the bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate and the activating agent, TPS, two modifications were introduced. First, the acid salt of the phosphate derivative replaced the cyclohexylammonium salt. The acid salt was readily converted to the pyridinium form by evaporation in vacuo from anhydrous pyridine. Second, a new activating agent, mesitylenesulfonyl-1,2,4-trizole (Katagiri et al., 1974) was employed. MST was an attractive condensing reagent as it does not liberate HCl and it had been successfully employed

Figure 19. Reaction scheme for conversion of m^7G and scission of polynucleotide chain.



in a phosphotriester synthesis of oligodeoxyribonucleotides.

MST was also investigated as a condensing reagent for the selective esterification of the 5'-hydroxyl group of protected nucleosides with triphenylmethoxyacetyl. This would have eliminated TPS entirely from the synthesis.

The oligomers prepared by the modified synthetic procedure were deprotected in improved yields without serious contamination. The simplicity of these deprotections was attributed directly to the purity of the protected oligomers.

However the yields for the deprotection did not approach those expected from the pioneering studies on the removal of 2,2,2-trichloroethyl groups from protected dideoxyribonucleotides (Eckstein and Rizk, 1969). As other investigators have also reported low yields for the deprotection of similarly protected phosphotriesters (Smrt, 1972 and Catlin and Cramer, 1973), alternate procedures to the reductive cleavage with Zn/Cu couple were investigated.

Letsinger has reported the use of the napthalene radical anion in the removal of o-methoxytrityl from nucleoside derivatives (Greene and Letsinger, 1975) and has also indicated that this procedure would remove benzoyls and trichloroethyl groups (Letsinger, 1975). It was therefore hoped that the napthalene radical anion could replace both the Zn/Cu couple step and the methanolic ammonia step of the three-step procedure. This would both simplify and shorten the deprotection.

Methanolic ammonia was investigated as an alternate to reductive cleavage as trichloroethyl groups can be removed by alkaline hydrolysis [0.4 N NaOH (0.5 ml) in dioxane/water mixture (4:1, 3.5 ml) (Eckstein

and Rizk, 1969)]. This would reduce the deprotection to a two-step procedure as benzoyls and triphenylmethoxyacetyls are removed under the same conditions.

3.3 Formation of Duplexes Corresponding to the Double-Stranded Region of the Dihydrouridine Arm

Once the desired sequences were synthesized a most important question had to be answered. Could these complementary sequences form a stable duplex of only four base pairs? It was clear that the interaction of trinucleotides is too weak to form short helices (Jaskunas et al., 1968), but there was some uncertainty as to whether the contribution of an additional base pair would be sufficient to overcome this difference. The fact that the DHU arm is formed in the native yeast $tRNA^{Phe}$ does not imply that the isolated DHU neck will also base pair. In the native tRNA the DHU arm is part of an extended helix (Sussman and Kim, 1976 and refs. therein).

Secondary structure has also been demonstrated for 5' half-molecules of yeast $tRNA^{Phe}$ (Romer et al., 1969 and Lightfoot et al., 1973). However the intramolecular hydrogen-bonding of the half-molecule cannot be compared with the intermolecular bonding of the duplexes. Uhlenbeck and co-workers (1973) have demonstrated that the stability of a helix forming the stem of a hairpin loop is considerably greater than an intermolecular helix with the same length and composition.

Despite numerous thermodynamic studies on short RNA helices (eg. Martin et al, 1971; Porschke et al., 1973; Ravetch et al., 1974; etc.) there have been only two instances where a stable duplex of only four base pairs was formed - the interaction of GpCpCpC and GpGpGpC (Podder,

1971) and the self-complementation of CpCpGpG (Arter et al., 1974). Unlike duplex I and III neither of these duplexes contained an A=U base pair. This A=U pair was expected to decrease the stability of duplex I and III relative to the above duplexes.

Initial studies on the helix-coil transition of short duplexes were based on temperature-dependent changes in the ultraviolet absorption (eg. Martin et al., 1971) and circular dichroism spectra (Borer et al., 1973). Recently proton magnetic resonance studies have revealed further information on helical structure (Arter et al., 1974 and Borer et al., 1975). These three techniques were employed to establish duplex formation.

3.4 Interaction of Duplexes with Yeast Phenylalanyl-Transfer RNA Ligase

After establishing the formation of stable duplexes corresponding to the DHU neck of yeast $tRNA^{Phe}$, the major question raised in this thesis was explored. Would yeast phenylalanyl-tRNA ligase interact with a duplex which resembles a partial recognition site in the native tRNA molecule?

Although the subunit structure of this enzyme (Fasiolo et al., 1970) and its interactions with the substrates of the aminoacylation reaction (Fasiolo et al., 1974) were extensively examined, few attempts to study the interaction of yeast phenylalanyl-tRNA ligase with fragments of the cognate tRNA have been made.

Thiebe and co-workers (1972) studied these interactions by measuring the inhibition of $tRNA^{Phe}$ charging by fragments or fragment mixtures which had no acceptor activity themselves. In a similar manner, the interactions of duplexes I and III with the ligase were also studied. As no inhibition of the aminoacylation reaction was detected, it was

concluded that the affinity of the enzyme for the duplexes was probably too small relative to the high affinity for the native tRNA. In accordance with Schimmel's suggestion (1973), the value of this type of approach was considered limited and more direct means of studying the recognition of tRNA fragments must be investigated. One possible procedures is presented in the discussion of this thesis.

4. EXPERIMENTAL PROCEDURES

4.1 Materials and Reagents

All reagents and solvents were commercial reagent grade and were used without further purification except for the following: 2,4,6-triisopropylbenzenesulfonyl chloride (Aldrich Chemical Co.) was recrystallized from hot petroleum ether (30-60°); urea (Baker Analyzed) was deionized by passage of a fresh 10M solution over a mixed base ion exchanger (MB-3, Wm. Anderson and Co.); pyridine, dimethyl formamide, and p-dioxane (all Baker Analyzed reagents) were stored over Fisher 4Å molecular seives to render them anhydrous.

It was essential that moisture be excluded from all condensation reactions. After evaporation in vacuo of the pyridine solution, normal pressure was restored with dry nitrogen and not atmospheric air. All such evaporations were carried out in the temperature range 30-40°C.

Emulsions, frequently obtained during methylene chloride extractions of aqueous pyridine solutions, were broken by addition of a few millilitres of saturated sodium chloride solution.

Thin layer chromatograms of protected nucleosides and oligomers were run on Analtech prescored Silica Gel G plates (250 microns thickness) with 10% methanol in methylene chloride. Detection was accomplished by spraying with 1% $H_4Ce(SO_4)_4$ in 10% sulfuric acid and heating to 100°. Compounds containing trityl groupings appeared as bright yellow spots and those without appeared as brown spots.

Silica gel columns were prepared from 40-140 mesh Baker Analyzed silica gel (dried at 80° over NaOH for 2-4 days before use) in methylene chloride and eluted with step gradients of methanol in methylene chloride.

Melting points were recorded uncorrected from a Fischer-Johns stage type apparatus.

Completely deprotected sequences and enzymatic digests thereof were characterized by tlc on Avicel or Avicel F cellulose plates or by descending paper chromatography on Whatman 3MM, #1 or #40 paper, developed in the following solvents:

A. ethanol/NH_4OAc (1M, pH 7.3), 7:3 (v/v)

B. ethanol/NH_4OAc (1M, pH 7.3), 1:1 (v/v)

C. 1-propanol/c.NH_3/water, 55/10/35, (v/v)

D. isobutyric acid/0.5N NH_4OH, 50/30, (v/v), pH 4.3.

A short wave ultraviolet lamp was used for detection of nucleotide material.

Paper electrophoresis was performed on a Savant flat-bed apparatus, Whatman #1, 0.05M triethylammonium bicarbonate (pH 8.0): 1400V/95cm.

Ultraviolet spectra were recorded on a Cary 118 spectrophotometer.

Extinction coefficients (ε_{260nm}) were calculated from those of parent nucleoside and nucleotides [A=14.9x10 ^{3}, C=8.6x10 ^{3}, G=11.8x10 ^{3}, U=7.1x10 ^{3}, pA=15.4x10 ^{3}, pC=7.5x10 ^{3}, pG=11.6x10 ^{3} and pU=10.0x10 ^{3} $M^{-1}cm^{-1}$ (Sober, 1968)] assuming the following hypochromicity factors at 20°C PypPy, 95%: PupPy, 90%; and PupPu, 85%. As an example the ε_{260} of ApUpG was calculated:

$$(14.9 + 10.0 + 11.6) \times 10^3 \times .90 \times .90 = 29.6 \times 10^3\ M^{-1}cm^{-1}.$$

DEAE-cellulose (DE23) was purchased from Whatman; Sephadex G-10, Sephadex G-200 and DEAE-Sephadex (A-25) were purchased from Pharmacia;

and Chelex 100, Bio-Gel P-2, and Bio-Gel HTP were purchased from Bio-Rad.

Bulk tRNA from brewer's yeast, phenylalanine-specific tRNA (957 pmole/A260 unit), ATP trisodium salt, cytidine-5'-diphosphate (H^+ salt) and phenylalanine were all products of Boehringer-Mannheim. L-Phenylalanine [ALANINE-3-^{3}H(N)] (16.1 Cl/mmol) was purchased from New England Nuclear. Highly purified snake venom phosphodiesterase, spleen phosphodiesterase, bacterial alkaline phosphatase and pancreatic ribonuclease were purchased from Worthington and stored at -20°. Primer dependent polynucleotide phosphorylase (M. lysodeikticus) was purchased from Miles Research.

4.1.1 Preparation of DEAE-cellulose

DEAE-cellulose for column chromatography was prepared in the following manner; the ion exchanger was

1. suspended in 0.1N NaCl (15vols) for 1h,
2. filtered and washed well with water,
3. resuspended in 0.1N NaOH (15 vols) for 30 min.,
4. filtered and washed with water until filtrate pH was neutral,
5. resuspended in 0.1N HCl (15 vols) for 30 min.,
6. filtered and washed with water until filtrate pH was neutral,
7. resuspended in tris buffer (0.02M, pH 7.5, 15 vols) for 30 min.,
8. filtered and washed with
   a) 0.02M tris buffer, pH 7.5, 7M urea, 1.0M NaCl (15 vols),
   b) 0.02M tris buffer, pH 7.5, 7M urea (15 vols),
9. resuspended in 0.02M tris buffer, pH 7.5, 7M urea (15 vols),
10. degassed under reduced pressure,

11. allowed to settle for ca. 30 min.

The fines were decanted and a volume of tris buffer (0.02M, pH 7.5, 7M urea) equal to the settled volume was added. Upon resuspension of the ion exchanger, the column was poured. The column was packed by pumping buffer through until the column bed height was constant.

4.1.2 Nucleoside Composition of Oligoribonucleotides

The nucleoside composition of the oligoribonucleotides were determined by the method of Sen and Ghosh (1974). Oligoribonucleotides were degraded to their constituent nucleosides by digestion with snake venom phosphodiesterase and bacterial alkaline phosphotase. Typically, 1-2 A_{260} units of oligomer were incubated with 0.6 units of alkaline phosphotase and 40 ug of snake venom phosphodiesterase in 0.10ml of 0.2M sodium acetate (pH 8.8) containing 0.02M magnesium acetate for 4h at 37°. An aliquot (0.005-0.010ml) of the hydrolyzed oligomer was applied to an Aminex A-7 column (30 x 0.3 cm) of a Varian Aerograph LCS-1000 liquid chromatograph. The column was eluted at a column pressure of ca. 1000 psi with 0.2M ammonium formate (pH 4.55) at 58°. For quantitation the area under the appropriate peak was determined by making a photocopy of the profile and cutting and weighing the peak. Comparative molar ratios of the nucleosides were obtained by dividing these weights by the respective molar extinction coefficients at 254 nm in the eluting buffer.

4.1.3 Protected Nucleosides

The nucleoside derivatives were synthesized and characterized by published procedures: HOUtOH (Griffin, Jarman and Reese, 1968); $HOC^{bz}tOH$ and $HOA^{bz}tOH$ (Neilson and Werstiuk, 1971a); $HOG^{bz}tOH$ and $Trac^{bz}GtOH$ (Neilson, Wastrodowski and Werstiuk, 1973); $Trac^{bz}AtOH$ (Neilson et al., 1975).

4.1.4 Acid Salt of Mono-2,2,2-trichloroethylphosphate (England and Neilson, 1976)

The acid salt of mono-2,2,2-trichloroethyl phosphate was prepared by a modification of Eckstein's procedure for the preparation of the bis (cyclohexylammonium) salt (Eckstein, 1967). Instead of adding cyclohexylamine, the material was acidified with a 2N sulfuric acid solution, extracted with diethyl ether and evaporated *in vacuo* to a light yellow oil. During evaporation *in vacuo* from toluene, white crystals separated, m.p. 119-121°C (120-121°C)*.

4.2 Synthesis of the Amino Acid Acceptor Arm of *E. coli* $tRNA^{Tyr}_{II}$

4.2.1 N^2-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanylyl (3'-2,2,2-trichloroethyl-5') N^2-benzoyl-2'-O-tetrahydropyranylguanosine

The bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate (Eckstein, 1967) (900 mg, 2.0 mmol, 2.9 equiv.) was dissolved in anhydrous pyridine (50 ml) with warming and converted to the corresponding pyridinium salt by repetitive evaporation *in vacuo* and solution in pyridine (5 x 50 ml). TPS (1.210 g, 4.0 mmol, 5.8 equiv.) was added to the above pyridine solution (*ca.* 25 ml) and the solution was left to stand at room temperature under a nitrogen atmosphere. After 1h a pyridine solution (*ca.* 5 ml) of $TracGt^{bz}OH$ (530 mg, 0.69 mmol, 1 equiv.) was added to the deep yellow solution of activated phosphate. Tlc indicated the phosphorylation was *ca.* 80% complete (R_F 0.70 → 0.30) after 1 day. Additional TPS (100 mg. 0.30 mmol) was added to complete the reaction in 3 days. Ice (*ca.* 2g) was added to quench the reaction.

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*C.B. Reese, Personnal Communication.

After stirring for 30 min. the reaction was poured into ice-water (ca. 100 ml) and extracted with methylene chloride (4 x 50 ml). The combined organic fractions were washed with water (2 x 50 ml) and evaporated in vacuo to a brown foam.

^{bz}TracGtp-O$^-$ (ca. 0.69 mmol) was repetitively evaporated from anhydrous pyridine (3 x 50 ml, final volume ca. 20 ml) and TPS (300 mg, 1mmol, 1.45 equiv.) was then added. After 1h a pyridine solution (ca. 5 ml) of the high R_F isomer of ^{bz}HOGtOH (450 mg, 0.95 mmol, 1.38 equiv.) was added and the reaction was stored at room temperature under nitrogen. Additional portions of TPS (3 x 100 mg) were added on days 2, 4 and 6. After 8 days tlc indicated ca. 80% reaction (R_F 0.30 → 0.65). The reaction was quenched with ice (ca. 2g), poured into ice-water (ca. 100 ml) and extracted with methylene chloride (4 x 50 ml). The combined organic fractions were washed with water (2 x 50 ml) and evaporated in vacuo to dryness. The last traces of pyridine were removed from the residue by co-distillation with toluene (2 x 20 ml) to give a brown foam which was purified by silica gel column chromatography. Elution with 2.5% methanol-methylene chloride yielded a mixture of the diastereoisomers of ^{bz}TracGt-^{bz}GtOH (375 mg, 0.26 mmol, 38%).

4.2.2 N^2-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanylyl (3'-2,2,2-trichloroethyl-5')N^2-benzoyl-2'-O-tetrahydropyranylguanylyl (3'-2,2,2-trichloroethyl-5') 2'-O-tetrahydropyranyluridine

As above the bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate (300 mg, 0.68 mmol, 2.7 equiv.) was converted to the corresponding pyridinium salt by repetitive evaporation in vacuo from anhydrous pyridine (5 x 25 ml) and activated with TPS (410 mg, 1.36 mmol,

5.4 equiv.). After 1h a pyridine solution of TracGt-GtOH (360 mg, 0.25 mmol, 1 equiv.) was added to the deep yellow solution of activated phosphate. Additional portions of TPS (3 x 100 mg) were added on days 2, 4 and 6. Tlc indicated the reaction was ca. 80% complete (R_F 0.65 → 0.25) after 7 days. The reaction was quenched, poured into ice-water (ca. 50 ml) and extracted with methylene chloride (4 x 25 ml). The combined organic fractions were washed with water (2 x 25 ml) and evaporated in vacuo to a brown foam.

TracGt-Gtp-O$^-$ (ca. 0.25 mmol) was repetitively evaporated in vacuo from anhydrous pyridine (3 x 20 ml, final volume ca. 10 ml and TPS (100 mg, 0.33 mmol, 1.32 equiv.) was then added. Additional portions of TPS (2 x 50 mg) were added on days 2 and 4. After 10 days tlc indicated ca. 90% reaction (R_F 0.25 → 0.60). The reaction was quenched with ice (ca. 2g), poured into ice-water (ca. 40 ml), and extracted with methylene-chloride (4 x 25 ml). Combined organic fractions were washed with water (25 ml) and evaporated in vacuo to dryness. The last traces of pyridine were removed by co-distillation with toluene (2 x 20 ml) to give a brown foam which was purified by silica gel column chromatography. Elution with 3.5% methanol-methylene chloride yielded a pure fraction of TracGt-Gt-UtOH (250 mg, 0.13 mmol, 52%).

4.2.3 N^4-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-cytidylyl (3'-2,2,2-trichloroethyl-5') N^4-benzoyl-2'-O-tetrahydropyranylcytidine

The bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate (450 mg, 1.01 mmol, 2 equiv.) was converted to the pyridinium salt by repetitive evaporation in vacuo from anhydrous pyridine and activated with TPS (600 mg, 1.98 mmol, 3.9 equiv.). After 1h a pyridine

solution of $TracCt^{bz}OH$ (370 mg, 0.51 mmol, 1 equiv.) was added. Additional TPS (100 mg) was added on day 2 and after 3 days tlc indicated the reaction was complete (R_F 0.75 → 0.35). The reaction was quenched and worked-up in the normal manner to yield a yellow foam.

$TracCt^{bz}p\text{-}O^-$ (ca. 0.51 mmol) was repetitively evaporated in vacuo from anhydrous pyridine (3 x 20 ml, final volume ca. 10 ml) and activated with TPS (180 mg, 0.59 mmol, 1.16 equiv.). After 1h a pyridine solution (ca. 5 ml) of the high R_F isomer of $HOCt^{bz}OH$ (350 mg, 0.81 mmol, 1.59 equiv.) was added. Additional TPS (4 x 100 mg) was added on days 2, 4, 6 and 8. After 10 days tlc indicated ca. 80% reaction (R_F 0.35 → 0.80). On purification by silica gel column chromatography a pure fraction of $TracCt^{bz}\text{-}Ct^{bz}OH$ (360 mg, 0.265 mmol, 52%) was eluted with 2% methanol-methylene chloride.

4.2.4 N^2-benzoyl-2'-O-tetrahydropyranyl-5'-(2,2,2-trichloroethylphosphoryl) guanylyl (3'-2,2,2-trichloroethyl-5') N^2-benzoyl-2'-O-tetrahydropyranylguanosine

The scheme for the insertion of a 5'-phosphate group in the protected dinucleotide of GpG is outlined in Figure 20.

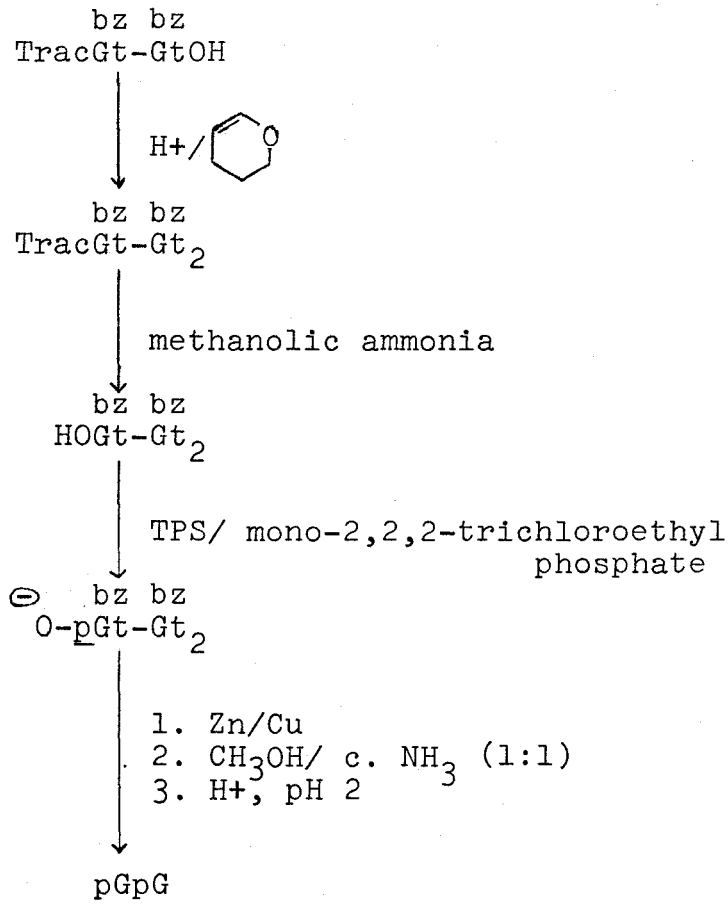
A solution of p-toluenesulfonic acid monohydrate (32.5 mg, 0.170 mmol) in dry dioxan (1.5 ml) containing molecular sieves was cooled in ice till solidified. Dihydropyran (32 mg, 3.85 mmol) was then added with stirring, followed by a dioxan solution (1 ml) of $TracGt^{bz}\text{-}Gt^{bz}OH$ (250 mg, 0.173 mmol). The reaction temperature was allowed to warm to room temperature after 30 min. and stirring was continued a further 1 h. Tlc indicated complete conversion to a product of higher R_F (R_F 0.40 → 0.50 in 5% $CH_3OH\text{-}CH_2Cl_2$). The reaction was cooled, neutralized with dilute

methanolic ammonia, filtered and washed well with methylene chloride. The filtrate was evaporated *in vacuo* to a yellow glass. $^{bz\ bz}$TracGt-Gt$_2$ (206 mg, 0.135 mmol, 78%) was purified by silica gel column chromatography (elution with 2% methanol-methylene chloride).

$^{bz\ bz}$TracGt-Gt$_2$ (195 mg, 0.127 mmol) was stirred in 0.15N methanolic ammonia (20 ml) for 6h. Tlc indicated almost complete removal of 5' triphenylmethoxyacetyl (R_F 0.70 → 0.50). The reaction was stopped by evaporation *in vacuo* to dryness and $^{bz\ bz}$HOGt-Gt$_2$ (65 mg, 0.052 mmol, 41%) was purified by silica gel column chromatography (elution with 3.5% methanol-methylene chloride).

The bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate (36 mg, 0.081 mmol) was converted to the pyridinium form by repetitive evaporation *in vacuo* from anhydrous pyridine. TPS (50 mg, 0.165 mmol) was added to the pyridine solution (*ca.* 2 ml) and activation was allowed to proceed for 1h under nitrogen. A pyridine solution (1 ml) of $^{bz\ bz}$HOGt-Gt$_2$ (55 mg, 0.045 mmol) was then added. Tlc indicated the phosphorylation was complete after 4h (R_F 0.50 → 0.33). The reaction was quenched with ice (*ca.* 1g), poured into ice-water (*ca.* 10 ml) and extracted with methylene chloride (6 x 10 ml). The organic fractions were washed with water (10 ml) and evaporated *in vacuo* to dryness. $^{bz\ bz}$O-pGt-Gt$_2$ (75 mg) was not purified further as this material eluted at high percentages of methanol-methylene chloride (5-6%) and was contaminated with silica gel.

Figure 20. Scheme for the chemical insertion of a terminal 5'-phosphate grouping.



4.2.5 Deprotection of Protected Oligoribonucleotides

bz bz bz bz bz bz bz bz bz bz
TracGt-GtOH, HOGt-Gt_2, $^{-}$O-pGt-Gt_2, TracGt-Gt-UtOH and TracCt-CtOH

were completely deprotected by an identical procedure (Neilson and Werstiuk, 1971b).

Protected oligomer (10 mg) was dissolved in DMF (1 ml) and Zn/Cu couple (Eckstein, 1967) (ca. 10 mg) was added. After stirring 24 to 48 hours at 50°C, tlc indicated loss of 2,2,2-trichloroethyl groups ($R_F \rightarrow 0.0$). Methanolic ammonia (CH_3OH/c.NH_3, 1:1)(1 ml) was added and stirring was continued for a further 2 days. Zn/Cu couple was filtered off and washed well with 1N ammonia. Careful evaporation in vacuo of the filtrate and washings removed methanol and reduced the pH to 8-10. Dowex 50 W (NH_4^+ form) was added (ca. 1 g) and the mixture was stirred for 30 min. The resin was filtered off, washed well with water, and the filtrate and washings were evaporated in vacuo to a small volume (ca. 5 ml). The solution was adjusted to pH 2 with 1N hydrochloric acid and the loss of tetrahydropyranyls was followed by the change in R_F on Avicel F cellulose plates in solvent A. After 2 days the solution was neutralized with dilute ammonia and evaporated in vacuo to dryness. The deprotected oligomer was purified by descending paper chromatography on Whatman #1 or #40 in solvent A. The desired band was cut out, desalted by soaking in absolute ethanol (1h) and diethyl ether (15 min) (Thach, 1966), and eluted with water. Table 4.1 provides the yields for the deprotection of the oligoribonucleotides and their chromatographic behaviour in various systems.

Table 4.1 Experimental data of deprotected oligoribonucleotides (acceptor arm).

Compound	Deprotected product	Yield (%)*	R_{Gp} (pc in solent A)	R_{Gp} (tlc in solvent C)	R_{Gp} (pe)
bz bz TracGt-GtOH	GpG	30	1.27	0.85	0.93
$HOGt\text{-}Gt_2$	GpG	20	1.27	0.85	0.93
bz bz O-pGt-Gt_2	pGpG	20	0.45	0.74	0.96
bz bz TracGt-Gt-UtOH	GpGpU	35	1.00	1.08	-
bz bz TracCt-CtOH	CpC	65	2.74	1.60	-

* calculated from spectrophotometric data.

Oligoribonucleotides were further characterized by nuclease degradation. Snake venom phosphodiesterase and bacterial alkaline phosphatase digestions were performed in tris buffer (0.1M, pH 8.0) and spleen phosphodiesterase digestions were performed in sodium succinate (0.1M, pH 6.5). Typically, 2 to 3 A_{260} units were dissolved in the enzyme-buffer solution (1 mg/ml, 0.10 ml) and incubated at 37°C for 4h. Products of the digestion were identified by comparison of their R_Fs on Avicel F tlc in solvent A with standard nucleotides and nucleosides.

4.3 Synthesis of the Double-Stranded Region of the Dihydrouridine Arm

The schemes for the step-wise synthesis of the oligoribonucleotides GpApGpC, ApGpCpUpC and GpCpUpC are illustrated in Figures 15, 16 and 18. As the conditions for the phosphorylation and the coupling reactions were very similar, only the synthesis of TracGt-AtOH (4) is described. Tables 4.2 and 4.3 summarize the preparation of the protected oligoribonucleotides with TPS and MST, respectively, as condensing reagents. [N.B. Protected sequences longer than trinucleotides did not require 'back tritylation' (Neilson and Werstiuk, 1971b) as these sequences eluted at higher percentages of methanolmethylene chloride than the incoming nucleoside.]

4.3.1 N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine-3'-2,2,2-trichloroethyl phosphate (2)

(a) The bis-cyclohexylammonium salt of mono-2,2,2-trichloroethylphosphate (720 mg, 1.62 mmol) was dissolved in anhydrous pyridine (30 ml) with warming and converted to the corresponding pyridinium salt by repetitive evaporation in vacuo and solution in pyridine (5 x 30 ml). TPS (980 mg, 3.23 mmol) was added to the above pyridine solution (ca. 15 ml)

and the solution was then left to stand at room temperature under nitrogen atmosphere. After 1 hour a pyridine solution (ca. 5 ml) of ^{bz}TracGtOH (1) (400 mg, 0.52 mmol) was added to the deep yellow solution of activated phosphate. Tlc indicated that the phosphorylation was ca. 80% complete (R_F 0.70 → 0.30 in 10% CH_3OH - CH_2Cl_2) after 1 day. Additional TPS (50 mg, 0.17 mmol) was added to complete the reaction in two days. Ice (ca. 2 g) was added to quench the reaction. After stirring for 30 min. the reaction was poured into ice-water (ca. 75 ml) and extracted with methylene chloride (4 x 30 ml). The combined organic fractions were washed with water (2 x 30 ml) and evaporated in vacuo to a brown foam. ^{bz}TracGtp-O^- (2) was used without further purification.

(b) The acid salt of mono-2,2,2-trichloroethylphosphate (300 mg, 1.3 mmol) was converted to the pyridinium salt by solution in anhydrous pyridine and repetitive evaporation in vacuo (5 x 30 ml). MST (650 mg, 2.59 mmol) (Katagiri et al., 1974) was added to this pyridine solution (ca. 10 ml) and then left to stand under nitrogen for one hour. Gentle heating 40°) was required to redissolve pyridinium trichloroethylphosphate which frequently separated. Pyridine solution (ca. 5 ml) containing ^{bz}TracGtOH (1) (500 mg, 0.65 mmol) was added to the clear solution of activated phosphate. Tlc indicated that the phosphorylation was complete (R_F 0.70 → 0.30 in 10% CH_3OH - CH_2Cl_2) in 3 days. Reaction was quenched with ice (ca. 2 g), poured into ice-water (ca. 50 ml) and extracted with methylene chloride (5 x 25 ml). The combined organic extracts were washed with water (2 x 40 ml) and evaporated in vacuo to a light yellow foam which was used immediately.

4.3.2 N^2-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanylyl (3'-2,2,2-trichloroethyl-5')-N^6-benzoyl-2'-O-tetrahydropyranyl adenosine (4)

(a) TracGtp-O$^-$ (2) (ca. 0.52 mmol) prepared above using TPS was repetitively evaporated from anhydrous pyridine (3 x 40 ml, final volume ca. 10 ml) and TPS (180 mg, 0.59 mmol) was then added. After 1 hour a pyridine solution (5 ml) of the high R_F isomer of HOAtOH (3) (345 mg, 0.76 mmol) was added. Additional portions of TPS (50 mg, 0.17 mmol) were added on days 3, 4 and 5. After 8 days tlc indicated ca. 60% reaction (R_F 0.30 → 0.75 in 10% CH_3OH - CH_2Cl_2). Reaction was quenched with ice (ca. 2 g), poured into ice-water (ca. 75 ml) and extracted with methylene chloride (4 x 30 ml). Combined organic fractions were washed with water (30 ml) and evaporated in vacuo to dryness. The last traces of pyridine were removed from the residue by co-distillation with toluene (2 x 20 ml) to give a brown foam which was purified by silica gel column chromatography. Elution with 3.0% methanol-methylene chloride yielded a pure fraction of TracGt-AtOH (4) (202 mg) plus a 50% mixed fraction containing TracGt-AtOH (4) and HOAtOH (3) (325 mg).

This mixed fraction was dissolved in anhydrous pyridine and repetitively evaporated in vacuo (3 x 20 ml). Excess p-anisylchlorodiphenylmethane (150 mg, 0.45 mmoles) was added to the pyridine solution. After 2 hours, contaminating 3 was converted to its 5'-O-p-methoxytrityl derivative (R_F 0.65 → 0.95 in 10% CH_3OH - CH_2Cl_2). Reaction was quenched with ice (ca. 2 g), poured into ice-water (40 ml) and extracted with methylene chloride (3 x 25 ml). Combined organic extracts were washed with water (2 x 40 ml) and evaporated in vacuo to dryness. Subsequent

column chromatography on silica gel yielded further TracGt-AtOH (4) (60 mg). Trityl derivative of 3 was pre-eluted with 1% methanol-methylene chloride.

The total isolated yield of TracGt-AtOH (4) (262 mg, 0.18 mmol) was 35%.

(b) TracGtp-O$^-$ (2) (ca. 0.65 mmol) prepared above using MST was repetitively evaporated *in vacuo* from anhydrous pyridine (4 x 30 ml, final volume ca. 5 ml) and MST (195 mg, 0.78 mmol) was added. After 1 hour a pyridine solution (ca. 5 ml) of the high R_F isomer of HOAtOH (3) (440 mg, 0.97 mmol) was added. Tlc indicated the coupling reaction was complete (R_F 0.30 → 0.75 in 10% CH_3OH - CH_2Cl_2) after 2 days. Reaction was quenched and worked-up in the usual manner to give a light yellow foam which was purified by silica gel chromatography. Elution with 3% methanol-methylene chloride gave a pure fraction of TracGt-AtOH (4) (360 mg) and a mixed fraction (475 mg) of 4 and 3. 'Back tritylation' of the mixed fraction and subsequent silica gel chromatography gave further quantities (195 mg) of 4.

Total yield of TracGt-AtOH (4) (555 mg) was 57%.

Table 4.2 Summary of preparation of protected oligoribonucleotides (TPS).

Phosphorylation reactions

	Reactants				Products	
No.	Quantity (mmol)	Quantity(mmol) mono-2,2,2-trichloroethyl phosphate*	Quantity(mmol) TPS activation	additional	No.	Days to completion
1	400mg (0.52)	+ 720mg (1.62)	+ 980mg (3.23)	1x50mg →	2	2
4	200mg (0.14)	+ 125mg (0.28)	+ 175mg (0.58)	- →	5	2
7	-					
17	500mg (0.66)	+ 590mg (1.33)	+ 800mg (2.64)	2x100mg →	18	4
19	400mg (0.28)	+ 250mg (0.56)	+ 350mg (1.16)	1x50mg →	20	3
21	200mg (0.098)	+ 90mg (0.20)	+ 120mg (0.40)	3x25mg →	22	7
23	75mg (0.029)	+ 39mg (0.088)	+ 53mg (0.175)	1x10mg →	24	4
1	400mg (0.52)	+ 720mg (1.62)	+ 980mg (3.23)	- →	2	2
11	500mg (0.36)	+ 325mg (0.73)	+ 450mg (1.49)	2x100mg →	12	5
14	300mg (0.16)	+ 280mg (0.63)	+ 380mg (1.25)	3x25mg →	15	5

* bis(cyclohexylammonium) salt.

Table 4.2 contd.

Coupling reactions

Reactants						Product		
			Quantity (mmol)					Days
No. (ca. mmol)		Incoming nucleoside	Quantity (mmol)	TPS activation	TPS additional	No.	Quantity (%)	to completion
2 (0.52)	+	3	345mg(0.76)	+ 180mg(0.59)	3x50mg →	4	262mg(35)	8
5 (0.14)	+	6	105mg(0.22)	+ 50mg(0.16)	2x25mg →	7	67mg(17)	6
8	-							
18 (0.66)	+	6	375mg(0.80)	+ 200mg(0.66)	2x100mg →	19	300mg(33)	10
20 (0.28)	+	9	180mg(0.42)	+ 100mg(0.33)	1x50mg →	21	222mg(40)	6
22 (0.098)	+	13	55mg(0.17)	+ 35mg(0.12)	3x15mg →	23	85mg(33)	8
24 (0.029)	+	9	19mg(0.044)	+ 10mg(0.033)	3x10mg →	25	31mg(33)	7
2 (0.52)	+	9	350mg(0.81)	+ 180mg(0.59)	2x100mg →	11	285mg(40)	10
12 (0.36)	+	13	175mg(0.53)	+ 125mg(0.41)	2x50mg →	14	346mg(50)	12
15 (0.16)	+	9	100mg(0.23)	+ 50mg(0.17)	2x20mg →	16	103mg(25)	7

Table 4.3 Summary of preparation of protected oligoribonucleotides (MST)

Phosphorylation reactions

	Reactants					Products	
No.	Quantity (mmol)		Quantity (mmol) mono-2,2,2-trichloroethyl phosphate+		Quantity (mmol) MST	No.	Days to Completion
1.	500mg(0.65)	+	300mg (1.30)	+	650mg (2.59) →	2	3
17	500mg (0.68)	+	305mg (1.30)	+	665mg (2.60) →	18	2
4	525mg (0.37)	+	170mg (0.74)	+	370mg (1.48) →	5	4
19	500mg (0.35)	+	160mg (0.70)	+	350mg (1.41) →	20	2
14	450mg (0.23)	+	160mg (0.70)	+	160mg (1.41) →	15	5
7	300mg (0.14)	+	65mg (0.29)	+	145mg (0.58) →	8	5
23	220mg (0.09)	+	80mg (0.34)	+	172mg (0.69) →	24	6
11	345mg (0.25)	+	115mg (0.50)	+	250mg (1.00) →	12	<80% after 6 days
11	450mg (0.32)	+	220mg (0.97)	+	485mg (1.94) →	12	2
21	400mg (0.20)	+	90mg (0.39)	+	195mg (0.78) →	22	<75% after* 6 days
bz TracCtOH	180mg (0.25)	+	115mg (0.50)	+	250mg (1.00) →	bz TracCtp-O$^-$	2

+acid salt; *Additional MST activated mono-2,2,2-trichloroethyl (0.30mmol) on day 5 drove the reaction to completion by day 7.

Table 4.3 (Cont.)

Coupling Reactions

Reactants							Products		
No. (ca. mmol)		Incoming Nucleoside	Quantity (mmol)		Quantity (MST) (mmol)		No.	Quantity (%)	Days to Completion
2 (0.65)	+	3	440mg (0.97)	+	195mg (0.78)	→	4	555mg (57)	3
2 (0.65)	+	9	420mg (0.97)	+	195mg (0.78)	→	11	660mg (73)	3
18 (0.68)	+	6	470mg (0.99)	+	180mg (0.73)	→	19	680mg (73)	3
5 (0.37)	+	6	260mg (0.56)	+	110mg (0.44)	→	7	450mg (58)	3
12 (0.32)	+	13	160mg (0.48)	+	100mg (0.39)	→	14	475mg (77)	3
20 (0.35)	+	9	195mg (0.46)	+	100mg (0.39)	→	21	370mg (60)	4
8 (0.14)	+	9	95mg (0.22)	+	45mg (0.17)	→	10	175mg (45)	4
15 (0.23)	+	9	150mg (0.35)	+	70mg (0.28)	→	16	250mg (42)	4
22 (0.20)	+	13	95mg (0.29)	+	55mg (0.22)	→	23	275mg (55)	5
24 (0.09)	+	9	75mg (0.17)	+	75mg (0.31)	→	25	125mg (45)	10

4.4 MST Activation of Sodium Triphenylmethoxyacetate

4.4.1 2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyluridine

Sodium triphenylmethoxyacetate (390 mg, 1.15 mmol) (Werstiuk and Neilson, 1972) was evaporated *in vacuo* from anhydrous pyridine (3 x 25 ml) to a final volume of *ca.* 10 ml. MST (290 mg, 1.15 mmol) was added and the solution was let stand at room temperature under N_2 for 2 hrs. A pyridine solution (*ca.* 2 ml) of the low R_F isomer of HOUtOH (250 mg, 0.76 mmol) was then added. After 5 days, silica gel tlc indicated conversion of HOUtOH to two trityl containing compounds (R_F 0.25 → 0.60 and 0.66). The reaction was quenched with ice (*ca.* 2g), poured into ice-water (*ca* 50 ml) and extracted with methylene chloride (5 x 25 ml). The organic fractions were washed with water (2 x 25 ml) and evaporated *in vacuo* to dryness. Purification by silica gel column chromatography gave compound I (195 mg, R_F 0.66) which eluted at 1.5% methanol-methylene chloride and compound II (80 mg, R_F 0.60) which eluted at 2-2.5% methanol-methylene chloride. Compound I was identified as TracUtOH as 1. it had the same R_F on silica gel tlc as an authentic sample and 2. upon mild methanolic ammonia treatment (0.15N) it was converted directly to low R_F HOUtOH. Compound II was identified as HOUt,Trac as upon mild methanolic ammonia treatment it was converted directly to low R_F HOUtOH. Compound II could not have been the disubstituted derivative as 1. TracUt,Trac has a higher R_F (R_F 0.85) on silica gel tlc than compound I and 2. no intermediates were observed in the specific deprotection. The isolated yield of TracUtOH was 41%.

4.4.2 N^2-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetylguanosine

As above the sodium salt of triphenylmethoxyacetate (360 mg, 1.06 mmol) was repetitively evaporated in vacuo from anhydrous pyridine and activated with MST (265 mg, 1.06 mmol). A pyridine solution of the low R_F isomer of ^{bz}HOGtOH (250 mg, 0.53 mmol) was added and after 6 days tlc indicated conversion to two major trityl containing derivatives of similar R_F (0.33 → 0.65 and 0.70). The reaction was worked-up and applied to a silica gel column. It was not possible to separate these compounds as they co-eluted. It was assumed that these compounds represented the 3'- and 5'-monosubstituted derivative as 1. the disubstituted derivative has a higher R_F (R_F 0.83) and 2. upon mild methanolic ammonia treatment both were converted directly to low R_F ^{bz}HOGtOH with no evidence of intermediates.

4.5 Deprotection of Protected Oligoribonucleotides

4.5.1 Zn/Cu Couple

The deprotection and purification of sequences synthesized with TPS was very unsatisfactory due to the low yields and extensive contamination. Although this contamination was considered to be a result of the accumulation of various side-products during the synthesis, the deprotection procedure itself was examined for possible degradation of the sequences. The following modifications were therefore introduced into the three-step deprotection procedure:

1. The length of treatment with Zn/Cu couple was reduced from 24h or longer to ca. 2h. It was observed that a small amount (10 mg) of freshly prepared Zn/Cu couple was sufficient to remove all 2,2,2-tri-

chloroethyl groups in this time period. Any chain cleavage due to prolonged treatment would thus be avoided.

2. To ensure complete removal of the N^2-benzoyl of guanosine derivatives, the length of the treatment with methanolic ammonia was increased from 48 h to 72h. Studies in which the hydrolysis was followed by the change in the ultraviolet spectra indicated that the longer treatment was warranted.

3. Dowex 50W-X8 was replaced by Chelex 100 as the latter is a more powerful resin for the removal of Zn and Cu ions. These ions could interfere with biological assays and could also 'poison' the cation exchange resin of the nucleoside analyzer.

4. Partially deprotected oligomers with only 2'-O-tetrahydropyranyl protection were purified by chromatography on Whatman 3MM in solvent B. This procedure was introduced to remove sequences that had suffered backbone cleavage and other UV absorbing materials (eg. Trac). Oligomers with 2'-hydroxyl protection were also examined for their resistance to nucleases.

Despite this modified deprotection procedure, oligoribonucleotides synthesized with TPS were still seriously contaminated. As an example, the deprotection and purification of TracAt-Gt-Ct-Ut-CtOH (25) is described.

4.5.1.1 Adenyl(3'-5')guanylyl(3'-5')cytidylyl(3'-5')uridylyl(3'-5')cytidine (ApGpCpUpC) (TPS Synthesis)

TracAt-Gt-Ct-Ut-CtOH (25) (20 mg) was dissolved in anhydrous N,N-dimethylformamide (2 ml). Zn/Cu couple (ca. 20 mg) was added and stirred for 2h at 50°. Tlc indicated complete removal of 2,2,2-trichloroethyl

(R_F 0.72 → 0.0 in 10% CH_3OH - CH_2Cl_2). Methanolic ammonia (CH_3OH/conc. NH_3 1:1) (5 ml) was added and stirring was continued for 72 h at room temperature. Reaction was filtered to remove Zn/Cu couple which was washed well with dilute aqueous ammonia. The filtrate and washings were carefully evaporated in vacuo to reduce the pH to ca. 9. Chelex 100 (ca. 1g, 100-200 mesh, Na+ form) was then added to remove Zn and Cu ions. After stirring for 30 mins the resin was filtered off and washed well with water. The filtrate was evaporated in vacuo to a small volume (< 1 ml) and applied to Whatman #40.Descending paper chromatography in system B yielded a major UV absorbing band (R_F 0.76). This band was cut out, desalted by soaking in absolute ethanol (1h) and diethyl ether (10 min), and eluted from the paper with water. Eluate (ca. 115 A_{260} units) was adjusted to pH 2 with a few drops of 2N HCl and stirred at room temperature. Removal of tetrahydropyranyl was followed by Avicel F tlc in solvent A (R_F 0.66 → 0.05). After 48h the solution was neutralized with dilute ammonia, evaporated in vacuo to a small volume (1 ml) and applied to Whatman #40 paper. Descending paper chromatography in solvent A gave a UV absorbing band (R_F 0.20) which was desalted and eluted with water to yield ApGpCpUpC (83 A_{260} units). However, upon paper electrophoresis of ApGpCpUpC three UV absorbing spots were observed.

ApGpCpUpC (80 A_{260} units) was purified by anion exchange column chromatography on DEAE-cellulose (Tomlinson and Tener, 1963). The column was eluted with a linear sodium chloride gradient (Figure 21a). Fractions 22 to 26 (peak II) were combined (ca. 26 A_{260} units) and desalted by passage through a Bio-Gel P-2 column (Figure 21b). Fractions 8 and 9 were combined and lyopholyzed. The final yield of ApGpCpUpC was 8% (18 A_{260}

units). A single spot was observed on paper electrophoresis (R_{PG} 1.08) and the nucleoside ratio was acceptable (obs. A/G/U/C 1.1/0.8/0.7/2.0, theor. 1/1/1/2.

4.5.1.2 Oligoribonucleotides by MST Synthesis

The procedure for the deprotection of the protected oligoribonucleotides 4, 7, 10, 11, 14, 16, 19, 21, 23, and 25, synthesized by the MST method, was identical to that described above for ApGpCpUpC. However, purification by DEAE-cellulose column chromatography was not required. Table 4.4 summarizes the experimental data for the deprotected sequences.

These sequences were characterized by nuclease degradation with snake venom phosphodiesterase and bovine spleen phosphodiesterase. SPDE and SVD digestion gave the 3'- and 5'- terminal residues, respectively, of the oligomers as a free nucleoside which was easily identified by Avicel F tlc in solvent A. The nucleotides were not well resolved in this system and Avicel tlc in solvent D was therefore utilized. It is important to note that all sequences were degraded completely by nuclease. This observation confirms the fidelity of the internucleotide linkage. Table 4.5 summarizes the results of the nuclease degradation of the principal oligoribonucleotides.

4.5.2 Napthalene Radical Anion

In the original procedure (Greene and Letsinger, 1975), a solution of the napthalene radical anion was prepared by adding napthalene (1.2 mmol) to a suspension of sodium chips (1 mmol) in hexamethylphosphoric-triamide (5 ml). Protected nucleosides or oligomers (5 to 10 mg) in anhydrous pyridine (0.2 ml) were then added to the dark green solution and stirred (30 min to 4h.). The reaction was quenched, poured into water

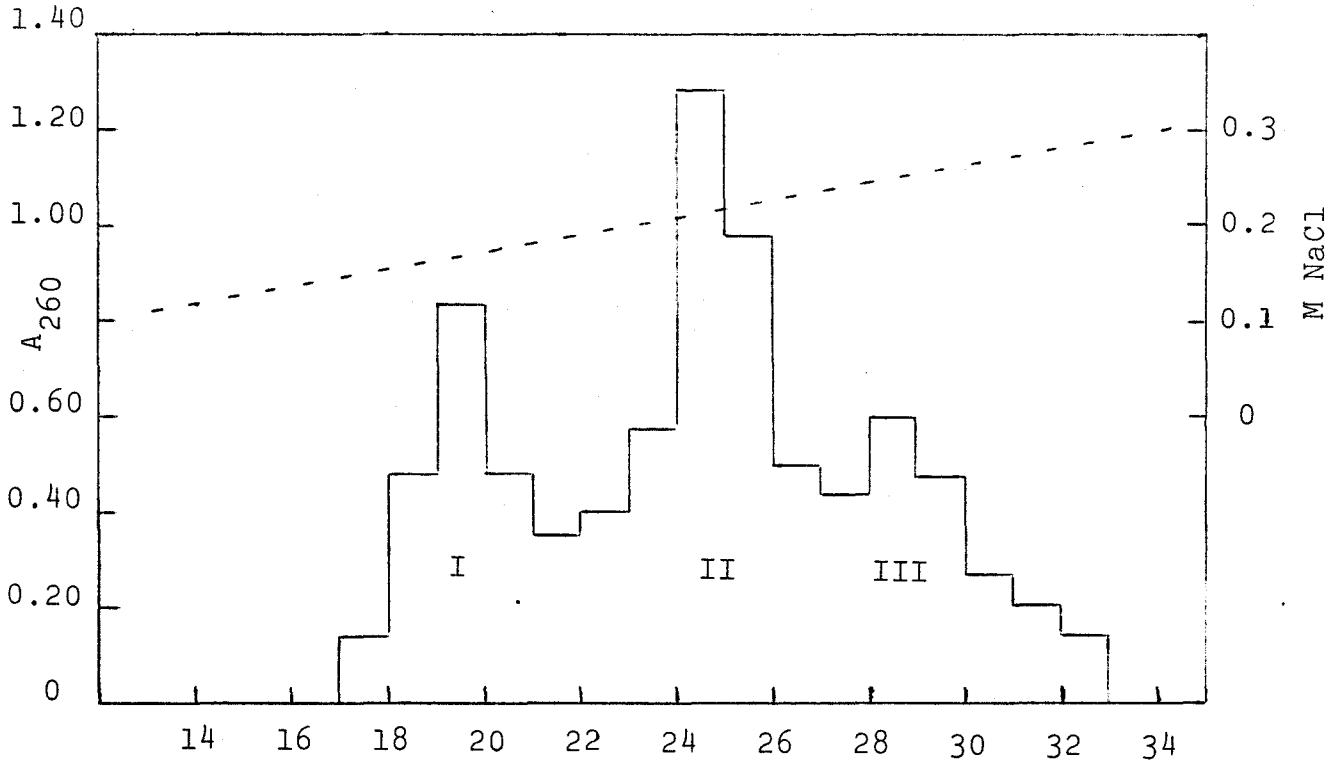


Figure 21a. ApGpCpUpC (80A_{260} units), DEAE-cellulose column (DE-23, 1.2×24cm) eluted with a linear gradient of NaCl (0.02M tris,pH7.5,7M urea,160ml and 0.02M tris,pH7.5,7M urea,0.3M NaCl,160ml). Flow rate ca. 1 ml/min. Fractions 9ml.

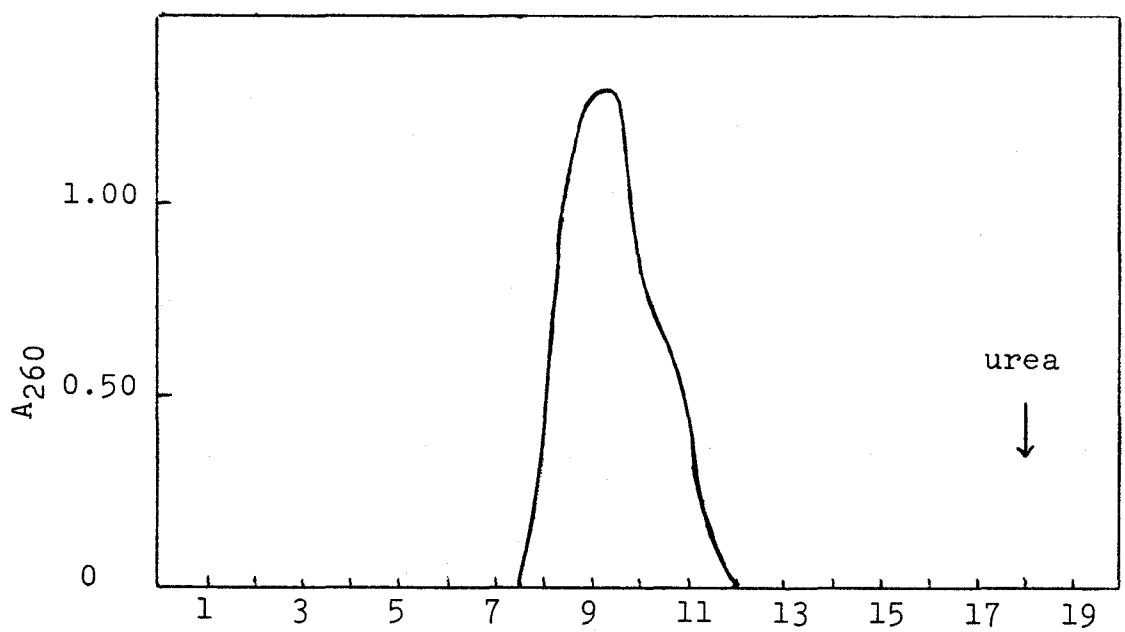


Figure 21b. ApGpCpUpC (26A_{260} units), Bio-Gel P-2 column (2.4×50cm). Flow rate ca. 150 ml/h. Fractios 10ml.

(40 ml), neutralized with 1N HCl and extracted with chloroform (4 x 30 ml) to remove napthalene and HMPTA. The aqueous fraction was evaporated *in vacuo* to a small volume and applied to Whatman 3MM paper which was eluted with solvent B. However HMPTA was not completely removed in the extraction and it was very difficult to remove this high boiling solvent (bp 230-232°) by evaporation *in vacuo*. As organophosphates are also suspected carcinogens, another solvent was investigated.

Tetrahydrofuran has been used as a solvent in the debenzylation of N^3-benzyluridine and N^2-benzylguanosine by reductive cleavage with the napthalene radical anion (Philips and Horwitz, 1975). The low boiling point of THF (bp. 66°) made it an attractive choice. In the alternate procedure, napthalenide was formed by the addition of napthalene (1.2 mmol) to a suspension of sodium chips (11mol) in THF (5 ml) under nitrogen atmosphere. The protected sequence (5 to 10 mg) was then added in anhydrous pyridine (0.2 ml) and stirred (30 min to 4h). The reaction was quenched by exposure to air and then evaporated *in vacuo* to dryness. Napthalene was removed by washing with diethyl ether (3 x 15 ml).

Preliminary studies (in HMPTA) on protected nucleosides indicated that 2'-O-tetrahydropyranyls and the N^2-benzoyl of guanosine were stable under the conditions of the reductive cleavage but that the N^6-benzoyl of adenosine, the N^4-benzoyl of cytidine and triphenylmethoxyacetyls were removed. However as these groups are base labile they may have been cleaved by alkaline hydrolysis during the work-up and not by napthalenide. TracUt-UtOH was chosen as a model for the removal of the phosphate protecting group.

Table 4.4

Experimental data of deprotected sequences

Sequence	R_{Gp} (pc solvent A)	R_{Gp} (tlc solvent A)	R_{Gp} (pe)	Yield (%) for deprotection
GpA	1.53	2.75	0.43	28
GpApG	0.67	0.50	0.56	25
GpApGpC	0.42	0.25	0.80	10
GpC	1.71	3.00	0.54	22
GpCpU	1.33	1.00	0.88	26
GpCpUpC	0.78	0.50	0.96	18
ApG	1.39	2.75	0.44	18
ApGpC	0.92	0.75	0.69	17
ApGpCpU	0.60	0.50	0.90	8
ApGpCpUpC	0.29	0.25	1.08	12

Table 4.5

Nuclease degradation of principal oligoribonucleotides*

Sequence	SVD		SPDE	
GpApGpC	G/pA/pG/pC	1.0/0.9/1.1/1.0	Gp/Ap/C	1.8/1.1/1.0
GpCpUpC	G/pC/pU	1.0/2.1/1.1	Gp/Cp/Up/C	1.0/0.8/1.1/1.0
ApGpCpUpC	A/pG/pC/pU	1.0/1.0/2.2/0.9	Ap/Gp/Cp/Up/C	0.9/1.1/0.8/1.0/1.0

*Degradation products were separated by pc on Whatman #40 in solvent A.

4.5.2.1 Uridylyl(3'-5')uridine

Napthalene (155 mg, 1.2 mmol) was added to a suspension of sodium chips (23 mg, 1mmol) in HMPTA (5 ml) and stirred at room temperature until all the sodium had dissolved (ca. 16h). TracUt-UtOH (10 mg, 0.009 mmol) in anhydrous pyridine (0.2 ml) was added to the dark green solution and stirred for 1h. The reaction was quenched with a few drops of water, poured into water (40 ml), neutralized with 1N HCl and extracted with chloroform (4 x 30 ml). The aqueous extract was evaporated in vacuo to a small volume (< 1 ml), applied to Whatman 3MM and eluted with solvent B. A UV absorbing band (R_F 0.95) was desalted and eluted with water. The eluate (152 A_{260} units) was adjusted to pH 2 with 0.1N HCl and after 48h Avicel F tlc in solvent A indicated complete removal of tetrahydropyranyls (R_F 0.82 → 0.36). The reaction was neutralized with dilute ammonia, evaporated in vacuo to a small volume, applied to Whatman #40 and eluted in solvent A. A UV absorbing band (R_F 0.64) was desalted and eluted to yield UpU (102 A_{260} units, 61%). UpU was characterized by nuclease degradation with SVD and SPDE and comparison of its chromatographic behaviour with a sample of UpU deprotected with Zn/Cu couple.

4.5.2.2 Guanylyl(3'-5')adenosine

After the initial success in the deprotection of TracUt-UtOH a sample of TracGt$^{bz\ bz}$-AtOH (4) (10 mg) was deprotected by a similar procedure. However for removal of the N^2-benzoyl of the guanosine residue an additional step was included. After the work-up of the napthalenide reactions methanolic ammonia (5 ml) MeOH/cNH_3, 1:1) was added and stirred for 72h. The reaction was evaporated in vacuo to dryness, redissolved in a small volume of water, applied to Whatman 3MM and eluted

with solvent B. A UV absorbing band (R_F 0.85) was desalted and eluted. The eluate (132 A_{260} units) was adjusted to pH 2 with 0.1N HCl. After 48h, Avicel F tlc in solvent A indicated complete removal of tetrahydropyranyls (R_F 0.80 → 0.10). The reaction was neutralized with dilute ammonia, evaporated *in vacuo* to a small volume (< 4 ml) and chromatographed on Whatman #40 in solvent A. The appropriate UV absorbing band (R_F 0.45) was cut out, desalted and eluted to yield GpA (90 A_{260} units, 52%). GpA was characterized by nuclease degradation and by comparison of its chromatographic behaviour with a sample GpA deprotected with Zn/Cu couple.

4.5.2.3 Other Oligoribonucleotides

Protected oligoribonucleotides 7, 10, 16, 19, and $\underset{bz}{25}\ \underset{bz}{}$ were deprotected by the same procedure described above for TracGt-AtOH (4). The yields for the deprotection with the napthalene radical anion in HMPTA are summarized in Table 4.6 and compared to the yields for the other deprotection procedures. No yields were calculated for GpCpUpC and ApGpCpUpC as they were contaminated by a material that had a white fluorescence under the UV lamp. It was not possible to remove this contaminant by various paper chromatographic systems. The yields for the deprotection in THF were similar to those reported here. The only advantage of this solvent was the facile removal by evaporation *in vacuo*.

4.5.3 Methanolic Ammonia

Protected oligoribonucleotides TracUt-UtOH, 4, 7, 10, 11, 14, 16, 19, and 25 were deprotected by the same procedure. The yields for the deprotection are summarized in Table 4.6 and compared with yields for the other procedures. Only the deprotection of (7) is described in detail.

Table 4.6

Comparison of yields for deprotection procedures.

	Yields (%)		
Sequence	Zn/Cu	Napthalenide	Methanolic ammonia
UpU	63	61	52
GpA	28	52	49
GpApG	25	20	47
GpApGpC	10	22	28
GpC	22	N.D.	39
GpCpU	26	N.D.	21
GpCpUpC	18	-	28
ApG	30	29	N.D.
ApGpCpUpC	12	-	15

N.D. not done

- not calculated

4.5.3.1 Guanylyl(3'-5')adenylyl(3'-5')guanosine

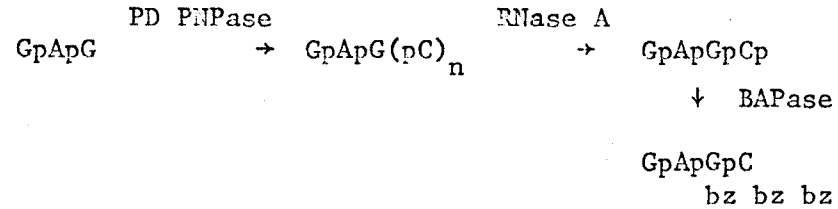
bz bz bz
TracGt-At-GtOH (7) (10 mg) was dissolved in methanolic ammonia (CH_3OH/cNH_3, 1:1) (2.5 ml) and stirred at room temperature for 72h. The solution was evaporated in vacuo to dryness, redissolved in a small volume of water (< 1 ml), applied to Whatman 3MM and eluted with solvent B. The major UV absorbing band (R_F 0.78) was desalted and eluted with water. The eluate (96 A_{260} units) was adjusted to pH 2 with 0.1N HCl and stirred at room temperature for 48h. Avicel F tlc in solvent A indicated complete removal of tetrahydropyranyls (R_F 0.73 → 0.08). The reaction was neutralized with dilute ammonia, evaporated in vacuo to a small volume, applied to Whatman #40 and eluted with solvent A. A UV absorbing band (R_F 0.20) was desalted and eluted to yield GpApG (63 A_{260} units, 47%). GpApG was characterized by SVD and SPDE degradation and by comparison of its chromatographic behaviour with a sample of GpApG deprotected with Zn/Cu couple.

4.6 Nuclease Degradation of Partially Deprotected Oligoribonucleotides

Deprotection of 4 and 19 with 1. Zn/Cu couple in DMF (2h at 50°) and 2. methanolic ammonia (72 h) and subsequent purification by paper chromatography on Whatman #40 (solvent A) gave HOGtpAtOH (R_{Gp} 3.26, 36%) and HOAtpGtOH (R_{Gp} 3.10, 24%) respectively. Nuclease degradation was monitored by the appearance of a UV absorbing compound of lower R_{Gp} (ca. 2.5) on Whatman #40 (solvent A). This compound was assumed to be the 2'-O-tetrahydropyranyl nucleotide.

4.7 Enzymatic Synthesis of Guanylyl(3'-5')adenylyl(3'-5')-guanylyl(3'-5')cytidine (GpApGpC)

The following scheme was employed for the enzymatic synthesis of GpApGpC:



GpApG was obtained by complete deprotection of TracGt-At-GtOH (7) by the modified procedure with Zn/Cu couple.

PD PNPase (25 ul, ca. 2.5 units) and RNase A (5 ul, ca. 35 units) were added to a solution containing GpApG (3.6 mM), 0.010M $Mg(OAc)_2$, 0.2M tris-Cl (pH 8.25), 0.4M NaCl and 0.04 M CDP, (75 ul) and incubated at 34° for 2h. The reaction was then placed in a boiling water bath for 5 min. and re-incubated at 34° with BAPase (10 ul, ca. 4 units) for 1h. The assay mixture was applied to Whatman #40 chromatography paper and developed in ethanol/1M ammonium acetate (pH 7.3), (60/40, v/v). The chromatogram indicated ca. 80% conversion of GpApG (R_F 0.21) to GpApGpC (R_F 0.13). The UV absorbing band corresponding to GpApGpC was desalted and eluted, final yield (3.4 A_{260} units, 64%).

GpApGpC prepared by enzymatic synthesis and GpApGpC prepared by chemical synthesis were compared by paper electrophoresis and by paper chromatography on Whatman #40 in solvent A. Both sequences had identical R_F's in these systems. GpApGpC (enzymatic synthesis) was also characterized by digestion with SPDE. The 3'-terminal nucleoside was identified as cytidine.

4.8 Formation of Duplexes Corresponding to the Double-Stranded Region of the Dihydrouridine Arm

4.8.1 Ultraviolet Absorption Studies

Absorption-temperature profiles were obtained at 240 nm (duplex I and III) and 280 nm (duplex II) in quartz cells of 1,5, or 10 mm path lengths in a Cary 118 spectrophotometer. All samples were incubated at 65° before profiles were recorded. The temperature of the cell was maintained with a circulating water bath and monitored by attaching a YSI thermisotor probe directly to the cell compartment. The solvent was 1 M NaCl, 0.01 M sodium phosphate buffer, and 10^{-4} M Na EDTA, adjusted to pH 7. The treatment of the melting data has been described (Martin et al., 1971). The enthalpy of the helix-coil transition was obtained from the slope of the plot of $1/T_m$ (in °K) and the logarithm of the total oligomer concentration (c), using equation 1 (Borer et al., 1974):

$$\Delta H^\circ = \frac{R \, d \ln c}{d \left(1/T_m\right)} \quad (\ln c/4 \text{ for non-identical strands}) \qquad \text{Eq. 1}$$

The enthalpy was also calculated from the slope at the midpoint of a plot of fraction of bases paired (f) and temperature, using equation 2 (Applequist and Damle, 1965):

$$\frac{d f}{d T_m} = \frac{N \Delta H_1}{6 R T_m^2} \qquad \text{Eq. 2}$$

Extinction coefficients in 1.0 M NaCl at 25°C at 260 nm for GpApGpC, GpCpUpC and ApGpCpUpC were calculated to be 30.1 x 10^3 M^{-1} cm^{-1}, 29.8 x 10^3 M^{-1} cm^{-1} and 36.0 x 10^{-3} M^{-1} cm^{-1}, respectively.

4.8.2 Circular Dichroism Studies

Circular dichroism spectra were recorded on a Cary 61 spectrometer equipped with a Neslab thermostated bath to maintain the temperature of the cell compartment within $\pm 0.5°$. 1 cm path-length cells were used. The oligomers were dissolved in 1.0 M NaCl, 0.01 M sodium phosphate, 10^{-4} M Na EDTA (pH 7.0) at concentrations of about 2×10^{-5} M.

4.8.3 Nuclear Magnetic Resonance Study

Proton magnetic resonance spectra were recorded on a Bruker WH 90 MHz spectrometer equipped with fourier transform (Nicolet minicomputer) to enhance the signal-to-noise ratio. Typically 200 to 300 90° pulses were required and transforms involving 8 K (real) actual data points were employed. Field/frequency stabilization was achieved by locking on the deuterium signal of the solvent. Spectra were also recorded on a Varian Associates HR 220 MHz spectrometer equipped with a computer of average transients. The position of the resonances were measured relative to the methyl peak of a t-butanol standard (ca. 10^{-2} M) and are reported in ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Solvent was prepared by lyopholyzing a solution of 1.0 M NaCl, 0.01 M sodium phosphate (pH 7.0) and redissolving in an equal volume of D_2O (ca. pD 7.2). Oligomers were dissolved in 200 to 400 microlitres at a concentration of ca. 10^{-2} M. Na EDTA (10^{-4} M) was added to improve the resolution of the spectra of the duplex.

4.9 Purification of Yeast Phenylalanyl-tRNA Ligase

Yeast phenylalanyl-tRNA ligase (EC 6.1.1.20) was purified by the method of Reid and co-workers (Schmidt et al., 1971).

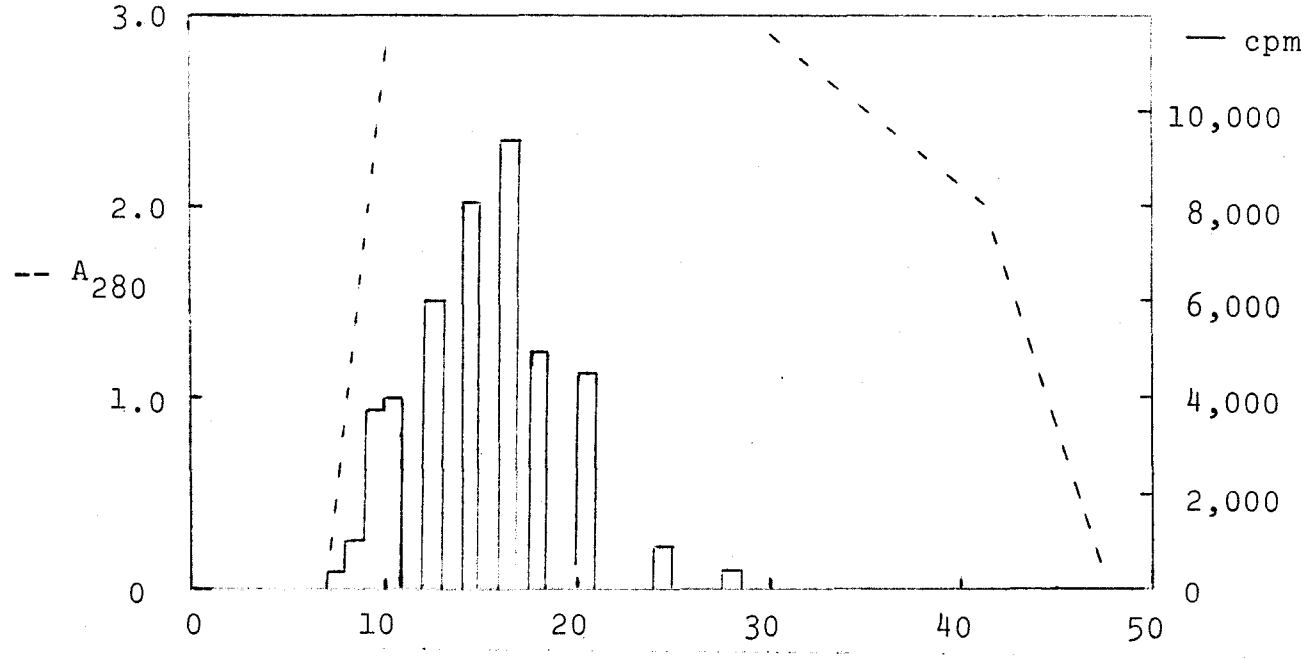
All operations were carried out at 0-4°.

A 1 lb. cake of frozen baker's yeast (Lallemand Inc.) was placed in a beaker with 225 ml of 50 mM tris-Cl (pH 7.5), 10 mM $MgCl_2$, and 10 mM mercaptoethanol and allowed to thaw overnight at 4°C. Cells were disrupted by a single passage through Aminco French Press at a pressure of 3000psi and a rate of ca. 20 ml/min. The crude extract (ca. 620 ml) was centrifuged at 14,000g for 1h in a Sorvall RC 2-B refrigerated centrifuge. The postmitochondrial supernatant (ca. 255 ml) was titrated to pH 8.0 with conc. ammonia and immediately subjected to ammonium sulfate fractionation. A small aliquot was removed at this stage for dialysis and activity assay.

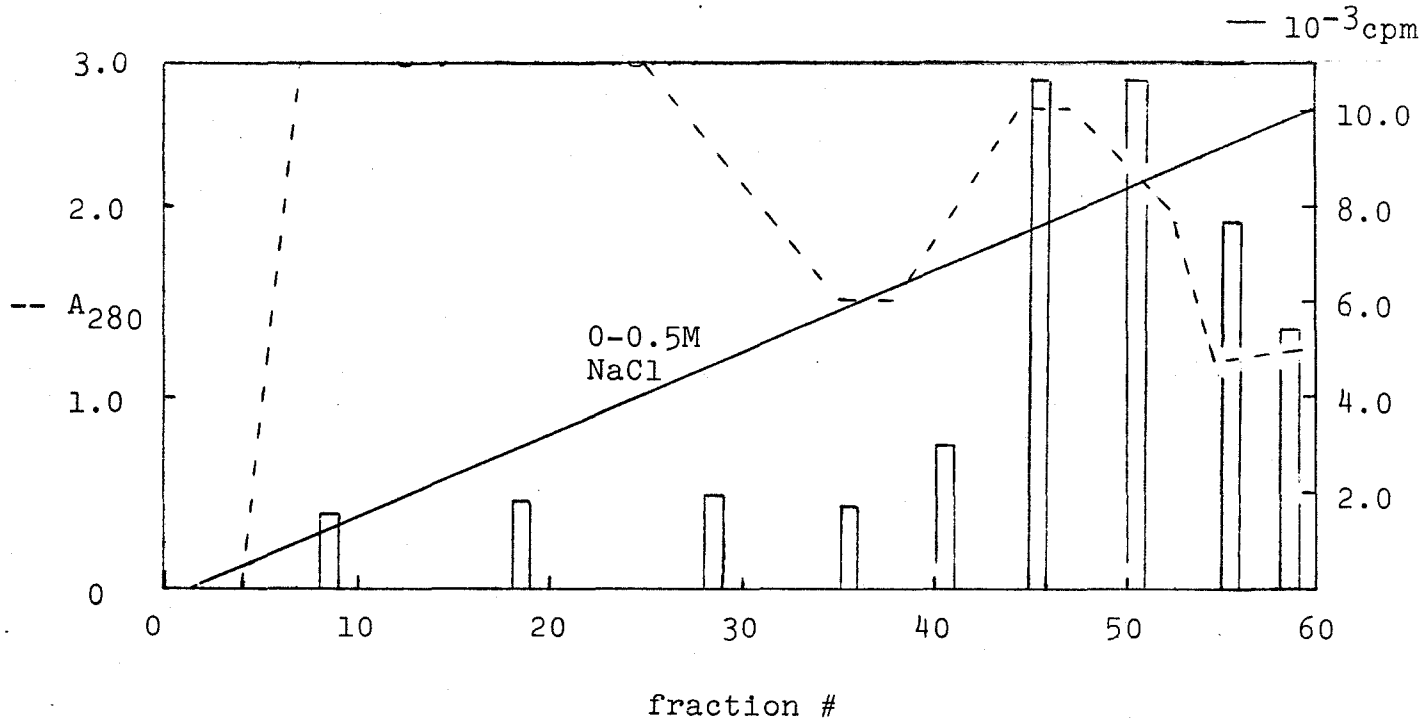
Solid ammonium sulfate (76 g) was added slowly over a period of 30 min. to the supernatant (50% saturation at 0°). After stirring for an additional 30 min. the solution was centrifuged at 14,000g for 1h. The supernatant (290 ml) was adjusted to 70% saturation by the gradual addition of further quantities of ammonium sulfate (36 g). After stirring for 30 min. the suspension was centrifuged at 14,000g for 1h. The precipitate was redissolved in 50 ml of 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 1 mM EDTA and 10% glycerol (v/v) and dialyzed against the same buffer (2 x 1 litre for 2h).

The dialyzed material was applied to a Sephadex G-200 column (6.5 cm x 14.5 cm) equilibrated with the glycerol-containing phosphate buffer above. The column was eluted with 1 litre of the same buffer at a flow rate of ca. 75 ml/h and 25 ml fractions were collected and assayed for enzyme activity. The elution of protein was followed at 280 nm with an Isco UA-2 ultraviolet analyzer. The elution profile of this column is illustrated in Figure 22a.

Figure 22. Purification of yeast phenylalanyl-tRNA ligase.



a. Gel filtration on Sephadex G-200 of ammonium sulfate fraction (5,400mg ; 11.2eu/mg).



b. Gradient elution of Sephadex G-200 fraction (3,350mg ; 24.8eu/mg) on DEAE-Sephadex.

Figure 22. cont'd.

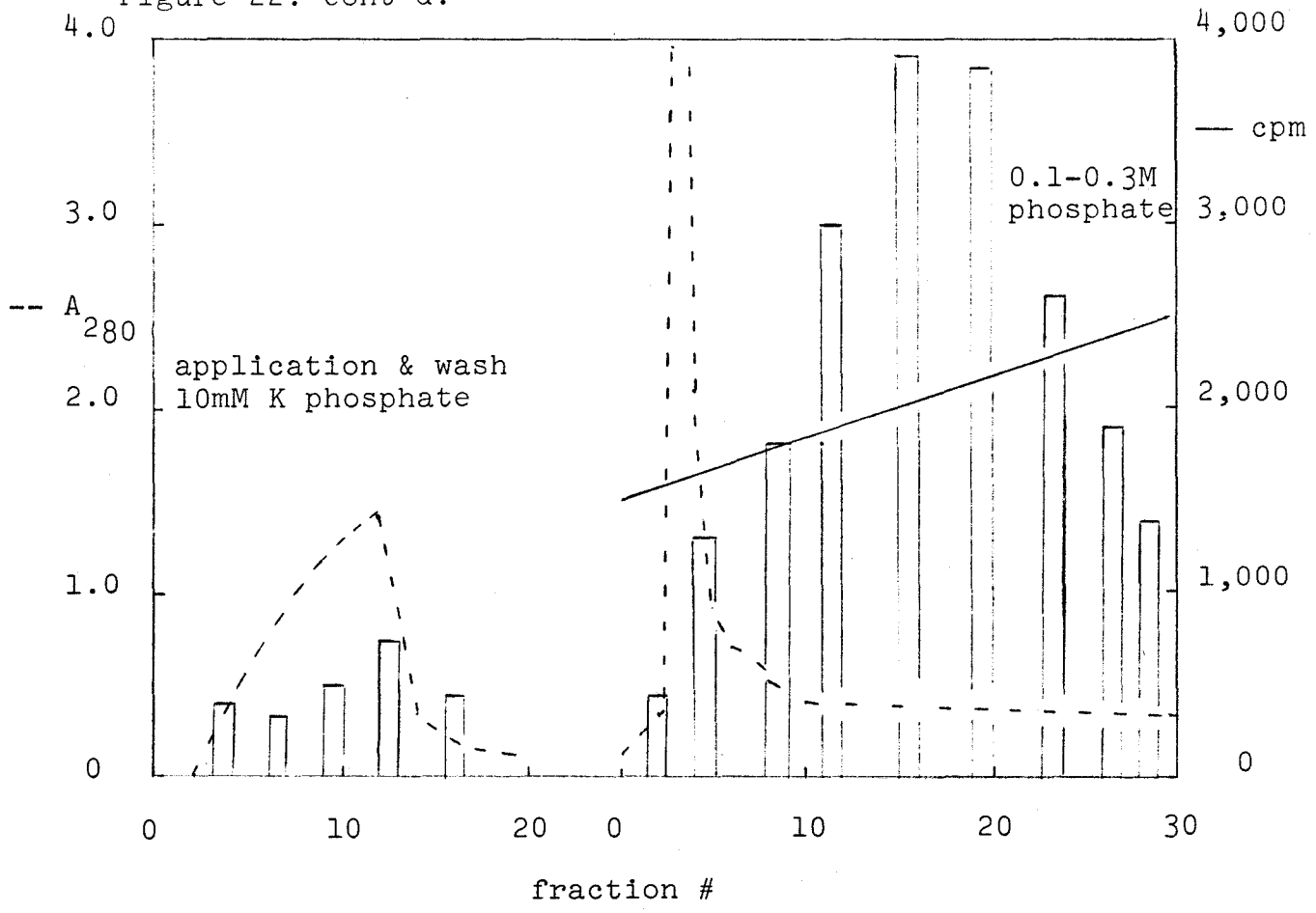


Figure 22c. Gradient elution of DEAE-Sephadex fraction (1,220mg , 44eu/mg) on a column of hydroxylapatite.

The turbid, enzyme-containing fractions from the Sephadex G-200 column (#9 → 20, 240 ml) were immediately applied to a DEAE-Sephadex A-25 column (3.2 cm x 43 cm) equilibrated with 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 2 mM EDTA and 10% glycerol (v/v). The column was eluted with a linear sodium chloride gradient (750 ml equilibration buffer and 750 ml buffer plus 0.5M NaCl) and ca. 30 ml fractions were collected and assayed for enzyme activity (Figure 22b) However it was very difficult maintaining an even flow rate and the enzyme eluted over a large volume.

The enzyme-containing fractions from the DEAE-Sephadex A-25 column (#40 → 60, 620 ml) were adjusted to 20% glycerol and applied to a column of hydroxylapatite and powdered cellulose (4:1, w/w, 4.6 cm x 2cm) equilibrated with 10 mM potassium phosphate (pH 7.2, 5 mM mercaptoethanol, 1 mM and 20% glycerol. The column was eluted with 400 ml of a linear gradient from 100 to 300 mM potassium phosphate (pH 7.2), both buffers containing 5 mM mercaptoethanol, 1 mM EDTA and 20% glycerol. The flow rate was maintained at 100 ml/h and 15 ml fractions were collected and assayed. However a large amount of enzyme pre-eluted from the column during application. The enzyme was precipitated by ammonium sulfate fractionation (85%) and centrifugation (14,000g for 1h) and applied to a second hydroxylapatite column. The elution profile of this column is illustrated in Figure 22c.

The enzyme-containing fractions from the hydroxylapatite column (#10 → 24, 240 ml) were mixed with an equal volumn of 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol and 1 mM EDTA and adjusted to 85% with ammonium sulfate (260 g). After stirring for 4h at 0°, the

suspension was centrifuged at 14,000g for 1h. The precipitate was redissolved in 10 mM potassium phosphate (pH 7.2), 5 mM mercaptoethanol, 1 mM EDTA and 10% glycerol, (30 ml) and mixed with an equal volume of glycerol and stored at -20°C.

4.9.1 Assay Procedures

Protein concentrations in the pooled fractions at each stage of purification were determined by the method of Lowry et al., (1951). Typically, aliquots (10-100 µl) were made up to 0.5 ml with water and a solution of 2% Na_2CO_3, 0.1N NaOH, 0.02% potassium tartrate and 0.01% copper sulfate (2.5 ml) was added and mixed. After standing for 10 min. at room temperature, diluted (1:1) phenol reagent (Folin-Cicoalteau) (0.25 ml) was added and mixed thoroughly. After 30 min. the absorbance at 700 nm was read and compared to the values for bovine serum albumin standards. At early stages in the purification the aliquots were dialyzed to remove low molecular compounds which interfered with the assay.

Enzyme activity was assayed in a reaction mixture (0.20 ml) which contained 0.10M tris-Cl (pH 8.0), 0.050M $MgCl_2$, 0.010M ATP, 0.001M dithiothreitol, 0.04 mg bovine serum albumin, 2 mg unfractionated tRNA (baker's yeast), 0.0001M L-phenylalanine [alanine-3-^{3}H(N)] (80 mCi/mmol) and an aliquot of enzyme - containing solution (5-10 µl, 1-2 units). The reaction was assayed at 37° and 50 µl aliquots were removed at 1, 4 and 10 minutes, applied to Whatman 3MM filter paper discs (2.4 cm) and dropped immediately into ice-cold 10% trichloroacetic acid. Filters were washed three times in ice-cold 10% trichloroacetic acid (15 min), once in ice-cold ethanol/ether (50:50, v/v) and once in ice-cold ether.

After drying, the filters were counted in 7.5 ml of a toluene based scintillation fluid (4g PPO plus 0.05g POPOP/1 toluene) at 3% efficiency to 2% error. One unit is defined as the amount of enzyme which incorporates 1 nmole of phenylalanine into tRNA in 10 min. at 37° under these conditions. (In this assay 1 nmole is equivalent to 5,340 cpm.)

4.10 Inhibition of the Aminoacylation of Yeast $tRNA^{Phe}$ by Duplexes Corresponding to the Double-Stranded Region of the Dihydrouridine Arm

The reaction mixture for the aminoacylation assay contained 50 mM sodium cacodylate (pH 5.8, titrated at 20°), 20 mM magnesium chloride, 2.5 mM ATP and 0.1 mM L-^{3}H-phenylalanine (100 mCi/mmole). Typically, oligoribonucleotides were dissolved in 150 µl of this solution at a concentration of 5×10^{-4}M, heated to 65° for 5 minutes and cooled in an ice-bath for 30 minutes. Enzyme (2.6 units, ca. 2×10^{-12}M) was added and incubated with the oligomers for 1h at 0-4°C. Aminoacylation was initiated by the addition of yeast $tRNA^{Phe}$ (0.04 mg, ca. 7×10^{-10}M) and incubation at 25°. Aliquots (25 µl) were removed at 1, 4, 7, 10, 15, and 20 minutes, applied to Whatman 3MM filter discs and washed and counted as described above. (Under the conditions of the assay 100% charging is equivalent to ca. 670 cpm.) All assays were performed in duplicate.

5. RESULTS AND DISCUSSION

5.1 Synthesis of the Amino Acid Acceptor Arm of *E. coli* $tRNA_{II}^{Tyr}$

The protected oligomer blocks, Trac $^{bz\ bz}$Gt-GtOH, Trac $^{bz\ bz}$Gt-Gt-UtOH, and Trac $^{bz\ bz}$Ct-CtOH, required for the proposed scheme (Figure 14) for the assembly of the hexanucleotide and the nonanucleotide corresponding to the amino acid acceptor arm of *E. coli* $tRNA_{II}^{Tyr}$ were successfully synthesized. Unfortunately low yields and very slow condensations were experienced for the reactions involving guanosine residues. As the coupling of oligomer blocks had been observed to give much lower yields than the addition of a single nucleoside (20 to 30% versus 60 to 80%, Neilson and Werstiuk, 1974), the block couplings between guanosine residues (eg. Trac $^{bz\ bz}$Gt-GtOH with HO$^{bz\ bz}$Gt-GtOH) would have most likely been the limiting step in this synthesis. It was therefore decided to terminate the project. However with the recent introduction of the condensing agent MST and the acid salt of mono-2,2,2-trichloroethyl phosphate to our phosphotriester method, the synthesis of an amino acid acceptor arm is now feasible.

A chemical method for the insertion of a terminal 5'-phosphate group was also developed. This procedure was demonstrated for the protected oligoribonucleotides GpG, CpU and ApUpU (Neilson *et al.*, 1975). The synthesis of pGpG was an important model as most tRNA molecules have pG at the 5' terminus.

The low yields obtained for the deprotection of GpG and its derivatives cannot be fully explained. However they may have been due to

side reactions associated with the use of TPS as similar problems were experienced in later syntheses.

In retrospect it may have been fortunate that this project was abandoned as additional problems were anticipated with the formation of the duplex corresponding to the amino acid acceptor arm. Sequences like GpGpUpGpGpGpG which are rich in G residues form aggregates, even in the presence of the complementary oligomer (Podder, 1971). Also it must be noted that Ikehara's group has been unable to form an active acceptor arm with the oligomers synthesized by the phosphodiester method. Whether this reflects upon the nature of their synthetic sequences or on the original charging experiments for the tRNA fragments (Imura et al., 1969) is unknown.

5.2 Synthesis of the Double-Stranded Region of the Dihydrouridine Arm

Four combinations of condensing reagent and salt of mono-2,2,2-trichloroethyl phosphate:

1. TPS and the bis(cyclohexylammonium) salt
2. MST and the bis(cyclohexylammonium) salt (unpublished results)
3. TPS and the acid salt (unpublished results) and
4. MST and the acid salt

have been studied in the phosphorylation and coupling reactions of our general phosphotriester synthesis of oligoribonucleotides. Only the combination of mesitylenesulfonyl-1,2,4-triazole and the acid salt of mono-2,2,2-trichloroethyl phosphate provided significant improvement over the earlier endeavours with TPS and the bis(cyclohexylammonium) salt. In the syntheses reported in this thesis a greater than 50% increase in yields was obtained (Table 5.7). Of special significance was the improved efficiency in coupling purines, for example, 17 with 6 and 4 with 6.

The MST-condensed reactions proceeded smoothly to completion without additional quantities of coupling agent, a necessity with similar reactions with TPS. The reaction times were generally shorter and coloration was considerably reduced. Similar improvements for the synthesis of the nonanucleotide corresponding to the anticodon loop of E. coli $tRNA_f^{Met}$ have been reported by Werstiuk and Neilson (1976).

Sequences terminating in C residues (11 and 22) were more difficult to phosphorylate than sequences of comparable length ending in A, G, or U. An explanation for this observation was not evident as TracCtOH^{bz} itself phosphorylated readily. In later syntheses, additional equivalents of activated phosphate were sufficient to overcome this reduced reactivity (Table 4.3).

The successful synthesis of the DHU neck and the anticodon loop has firmly established MST as the coupling reagent and the acid salt of mono-2,2,2-trichloroethyl phosphate as the phosphorylating agent. Stepwise synthesis of oligomers up to five residues is now the most convenient approach. The synthesis of longer oligomers, up to a nonanucleotide, has been accomplished by block coupling. It is important to note that MST also provides improved yields for these condensations.

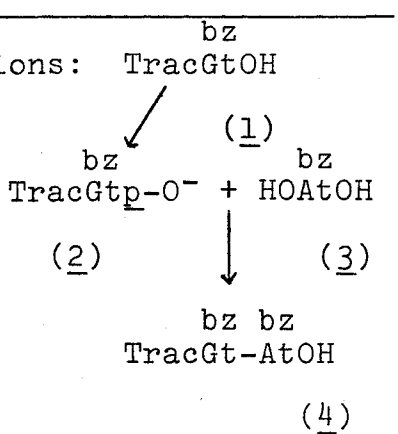
To achieve faster and more efficient condensation reactions, arylsulfonyltetrazoles (Figure 23) have also been studied as condensing agents in a phosphotriester synthesis of deoxyribooligonucleotides (Stawinski et al., 1976). The higher reactivity of these compounds relative to the corresponding triazole derivative can be attributed to tetrazole being a superior leaving group. Unfortunately, arylsulfonyltetrazoles decompose

Table 5.7

Comparison of TPS and MST as condensing agents in oligoribonucleotide synthesis

Coupling reaction	TPS % yield	MST % yield	% improvement
1 + 3 (G + A)*	35	57	63
4 + 6 (GA + G)	17	58	240
7 + 9 (GAG + C)	-	45	-
1 + 9 (G + C)	40	73	83
11 + 13 (GC + U)	50	77	54
14 + 9 (GCU + C)	25	42	68
17 + 6 (A + G)	33	73	121
19 + 9 (AG + C)	40	60	50
21 + 13 (AGC + U)	33	55	67
23 + 9 (AGCU + C)	33	45	36

* G + A represents the following reactions:



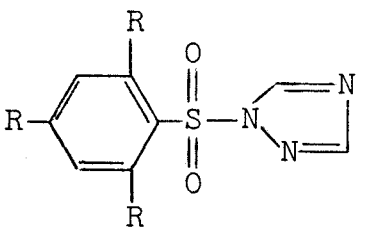
readily and must be used soon after preparation. Similar results were obtained in this laboratory in our phosphotriester synthesis of ribooligonucleotides (unpublished results).

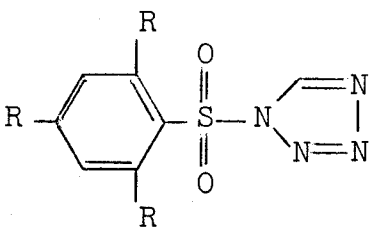
5.3 MST Activation of Sodium Triphenylmethoxyacetate

Model studies with HOUtOH indicated that MST was an acceptable condensing reagent for the 5' esterification of protected nucleosides with triphenylmethoxyacetate. The isolated yield of 41% compares favourably with the 53% yield obtained for the TPS activated reaction (Werstiuk and Neilson, 1972). The small portion of HOUt,Trac (16%) formed was separated from the desired product by silica gel column chromatography. However, in the case of $HOGt^{bz}OH$, the 5'-and 3'-triphenylmethoxyacetyl derivatives could not be separated as they co-eluted. It may have been possible to effect separation by 'back tritylation' of $HOGt^{bz},Trac$ before purification but this procedure was not attempted as reaction with p-anisylchlorodiphenylmethane reintroduces problems associated with HCl generation.

Although TPS-esterification was retained in this synthesis, these studies did provide some insight into the mechanism of esterification via mixed sulfonyl-carboxylic anhydrides. Apparently the bulky TPS-complex cannot approach a hydroxyl vicinal to a tetrahydropyranyl group as readily as the MS-complex. Perhaps triisopropylbenzenesulfonyl tetrazole (Stawinski et al., 1976) should be investigated as a condensing reagent for the esterification reaction. TPSTT would form the same active complex as TPS but would avoid any of the pitfalls associated with HCl generation and N-7 guanosine modification. (TPST would not be suitable as it is less reactive).

Figure 23. Novel condensing reagents.





Arylsulfonyl triazole

Arylsulfonyl tetrazole

I R = H

II R = CH_3

III R = $CH(CH_3)_2$

5.4 Deprotection of Protected Oligoribonucleotides

The integrity of the synthetic scheme was confirmed by deprotection of the protected oligoribonucleotides 4, 7, 10, 11, 14, 16, 19, 21, 23, and 25 (MST synthesis) and subsequent characterization of the desired products. The lack of serious contamination in these deprotections was attributed to both the purity of these protected sequences and to the modified deprotection procedure. In all cases the free oligomers were purified by simple paper chromatography techniques. Tedious DEAE-cellulose column chromatography was not required. Werstiuk and Neilson (1976) have also found that this modified deprotection procedure provides oligoribonucleotides of exceptional purity and biological activity. However, the yields for the deprotection were low (Table 4.4).

Preliminary studies on the deprotection of various dinucleotides indicated that the removal of 2,2,2-trichloroethyl groups by either reductive cleavage with the napthalene radical anion or by alkaline hydrolysis with methanolic ammonia was superior to reductive cleavage with Zn/Cu couple (Table 4.6). Unfortunately, yields dropped sharply with the increasing length of the sequence.

Reductive cleavage by napthalenide probably proceeds via the same mechanism as the Zn/Cu couple deprotection (Figure 24a). However in the case of napthalenide, a fluorescent contaminant was observed. As this method provided yields for the deprotection of longer sequences that were comparable to those obtained with Zn/Cu couple no attempt was made to separate the desired oligomer from the contaminant. Further, napthalenide did not simplify the deprotection procedure as anticipated. The methanolic ammonia treatment could not be eliminated as the N^2-benzoyl of guanosine

was not removed by the radical anion.

The removal of 2,2,2-trichloroethyl groups by alkaline hydrolysis with methanolic ammonia was a very promising procedure as:

1. The base labile groups were also removed, thus reducing the deprotection to a two-step procedure.

2. The elimination of the Zn/Cu couple step would avoid any problems that these cations may cause in biological assays. And,

3. The yields for the deprotection of longer sequences was slightly improved.

However, there is some dispute over the mechanism of the alkaline hydrolysis of phosphate protecting groups (van Boom et al., 1976). Instead of direct attack of the base on the phosphate and elimination of the protecting group (Figure 24b), recent studies suggest that the reaction proceeds through a cyclic intermediate which can yield either a 3'-5' or a 5'-5' phosphodiester linkage (Figure 24c). Although this mechanism was derived from studies on the deprotection of phenylphosphate esters by 0.1 N NaOH, a similar mechanism may apply for the deprotection of 2,2,2-trichloroethyl-phosphate esters by methanolic ammonia. No evidence for 5'-5' (or 3'-3') internucleotide linkage was observed in the nuclease degradation but if methanolic ammonia deprotection is to be used a closer examination of this possibility is warranted.

Recently, the facile removal of phosphate protecting groups by tetrabutylammonium fluoride in tetrahydrofuran was reported (Ogilvie et al., 1976). This deprotection appears to involve direct attack of the fluoride ion on the phosphate and displacement of trichloroethanol (phenol). The phosphorofluoridate intermediate is rapidly hydrolyzed under the aqueous

conditions of the work-up and purification. Although only deoxyribodinucleotides were investigated, this procedure may be suitable for the deprotection of the longer sequences of the ribo series.

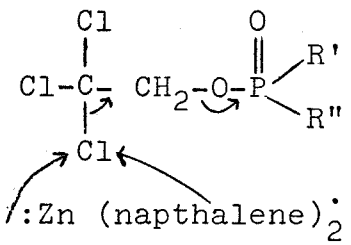
All oligoribonucleotides used in the subsequent duplex formation and aminoacylation inhibition studies were deprotected by the modified procedure with Zn/Cu couple. In some instances sequences were rechromatographed on Whatman #40 in solvent B; but at no time was purification by DEAE-cellulose column chromatography required.

5.5 Nuclease Degradation of Partially Deprotected Oligoribonucleotides

In the modified deprotection procedure, oligomers bearing only 2'-O-tetrahydropyranyl protecting groups were isolated by paper chromatography. The susceptibility of these sequences to nuclease degradation (SVD and SPDE) was checked as nuclease resistant sequences have been suggested as probes of the structure and function of nucleic acids in cellular systems (eg. Barrett et al., 1974). Studies on HOGtpAtOH and HOAtpGtOH indicated that these sequences were resistant to SPDE but were rapidly degraded by SVD. These results are consistent with the observation that naturally occuring 2'-O-methyl nucleosides do not hinder the degradation of RNA by these nucleases but that 2'-O-acetyl groups completely inhibit the action of SPDE (Laskowski, 1971 and Bernardi and Bernardi, 1971). As 2'-O-tetrahydropyranyl groups did not prevent nuclease degradation, no subsequent studies on the ability of these modified oligomers to base pair or to pass through a cell membrance were undertaken. However, it was noted that the change in hyperchromicity with SVD degradation of HOGtpAtOH (4.9% at 260 nm) was not as great as for GpA (8.3% at 260 nm). Apparently the bulky 2'-O-tetrahydropyranyl group interferes with the stacking of the bases.

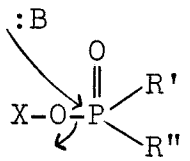
Figure 24. Deprotection of phosphate protecting groups.

a. reductive cleavage



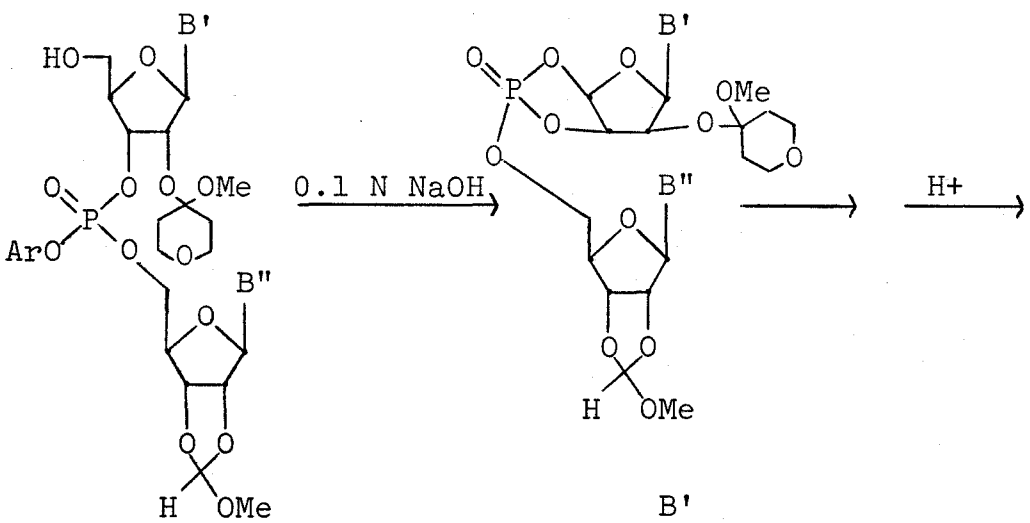
R',R" = nucleoside, or oligonucleotide

b. alkaline hydrolysis



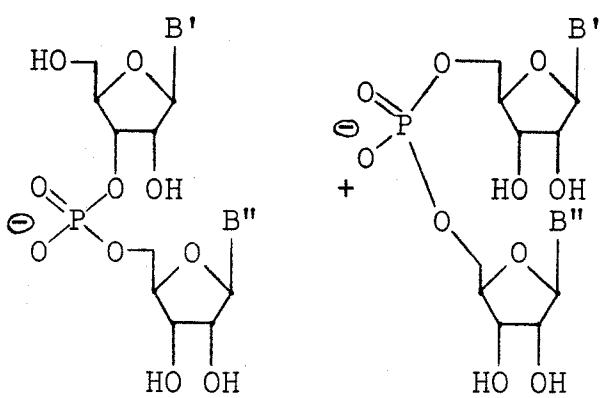
X = protecting group

c. formation of cyclic intermediates during alkaline hydrolysis.



B',B" = uracil-1-yl

Ar = C_6H_5 , 2-ClC_6H_4, 2,4-$Cl_2C_6H_3$



Decreased hypochromicity with increasing bulk of the 2'-substituent has been previously reported (Alderfer et al., 1974).

5.6 Enzymatic Synthesis of Guanylyl (3'-5') adenylyl (3'-5') guanylyl (3'-5') cytidine

The scheme for the enzymatic synthesis of GpApGpC was designed to give a high yield of product and to prevent randomization of the nucleotide sequence by the degradative action of PD PNPase. In the initial reaction an oligo C tail is added to the 3' end of the primer, GpApG. RNase A in the assay mixture then cleaves the oligomer to yield GpApGpCp. The 3'-terminal phosphate of this tetranucleotide inhibits PD PNPase. Finally BAPase gives the desired sequence, GpApGpC. Even if randomization does occur in the initial reaction, the only possible product, GpApC, could be easily separated in the purification step.

The synthesis of GpApGpC by this enzymatic approach demonstrates that sequences prepared by chemical synthesis are biologically active as they function as enzyme substrates. The enzymatic synthesis also confirms the structure of GpApGpC obtained by chemical synthesis.

5.7 Formation of Duplexes Corresponding to the Double-Stranded Region of the Dihydrouridine Arm

5.7.1 Ultraviolet Absorption Studies

Duplex formation was indicated by cooperative absorption-temperature profiles and the concentration dependence of the melting temperature.

Figure 25 illustrates a typical melting transition for duplex III and the determination of the melting temperature. This data is replotted in Figure 26 (fraction of bases paired (f) and temperature) and from the slope at the midpoint an enthalpy for the helix-coil transition was calculated (-39.1 Kcal/mole, Eq. 2). Although this value was not in good

agreement with the enthalpy value (-26.5 Kcal/mole) predicted by the method of Borer et al., (1974), Gralla and Crothers (1973) have suggested that the uncertainty in the temperature dependence of the absorbance makes unreliable the enthalpy determined from the shape of the melting curve.

In Figure 27 the expected inverse-linear relationship for a plot of $^1/T_m$ (in °K) and the logarithm of the total oligomer concentration (c) is demonstrated. The enthalpy of the helix-coil transition calculated from the slope of this plot (-24.4 Kcal/mole, Eq. 1) was in much better agreement with the predicted value. However it is unusual that the enthalpy value calculated from a plot of $^1/T_m$ and ln c is lower than that obtained from a plot of (1-f) and T; normally the opposite is observed.

The T_m and enthalpy values calculated for duplex III are consistent with the experimental data reported for other short duplexes (Table 5.8). The T_m for duplex III is lower than the T_m's for the self-complementation of CpCpGpG (Arter et al., 1974) and the hybridization of GpGpGpC and GpCpCpC (Podder et al., 1971). This reflects the destabilization of the internal A=U base pair relative to a G≡C base pair.

The melting transition profiles for duplex I were similar to those observed for duplex III. At comparable oligomer concentrations, duplex I had T_m's that were within experimental error ($\pm$ 1°) of those observed for duplex III. This result was not anticipated as from an analysis of oligomer-polymer interactions Borer et al., (1974) have concluded that the unpaired bases in the polymer at either end of the complementary oligomer would markedly stabilize the complex. Thus the unpaired adenosine in duplex I was expected to increase the stability of this duplex relative to duplex III. As no enhanced stabilization was observed, it would appear

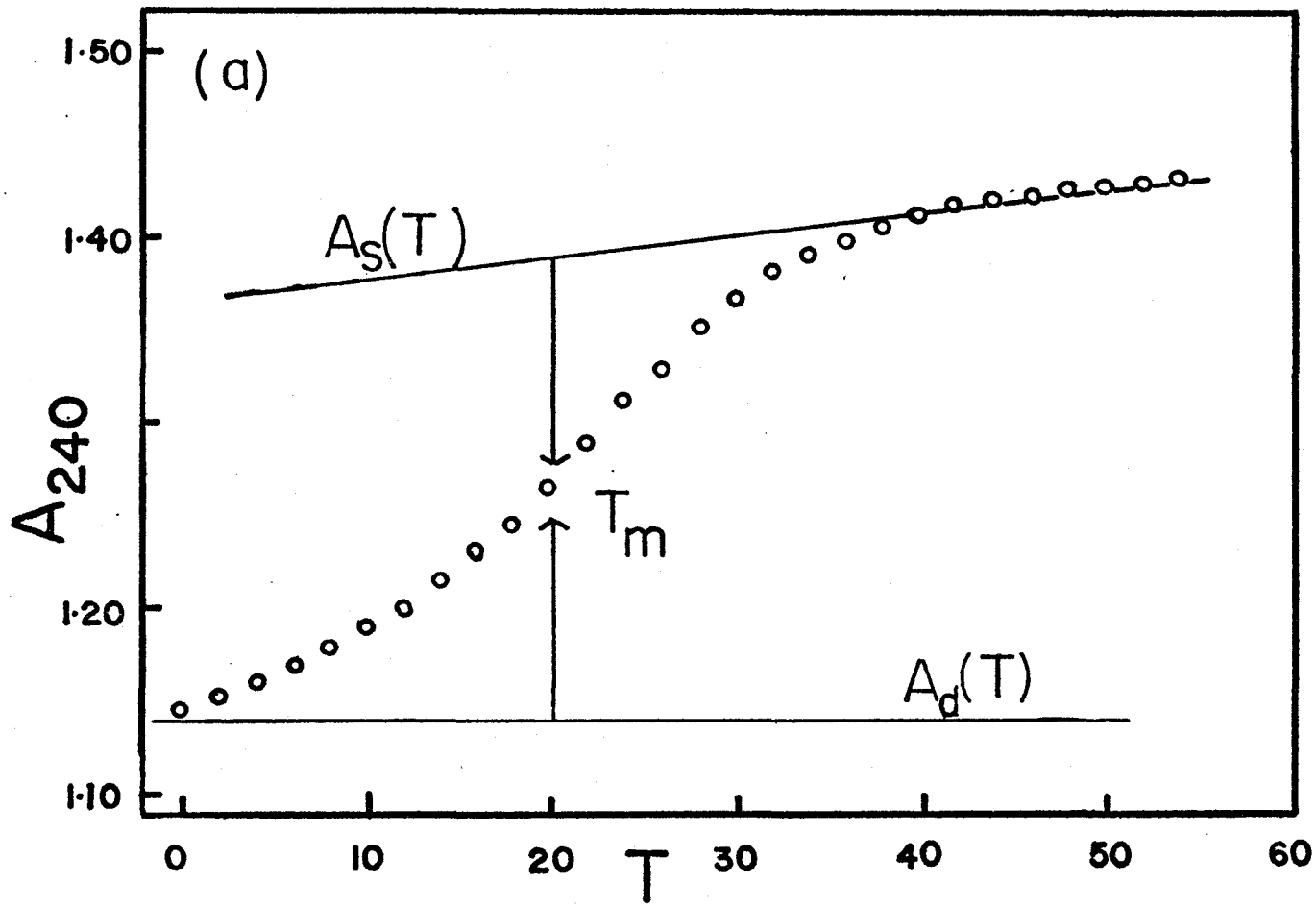


Figure 25. Melting transition of duplex III , ($c=6.2\times10^{-4}$, 1mm cuvet) , A_sT and A_dT single- and double-strand limiting curves.

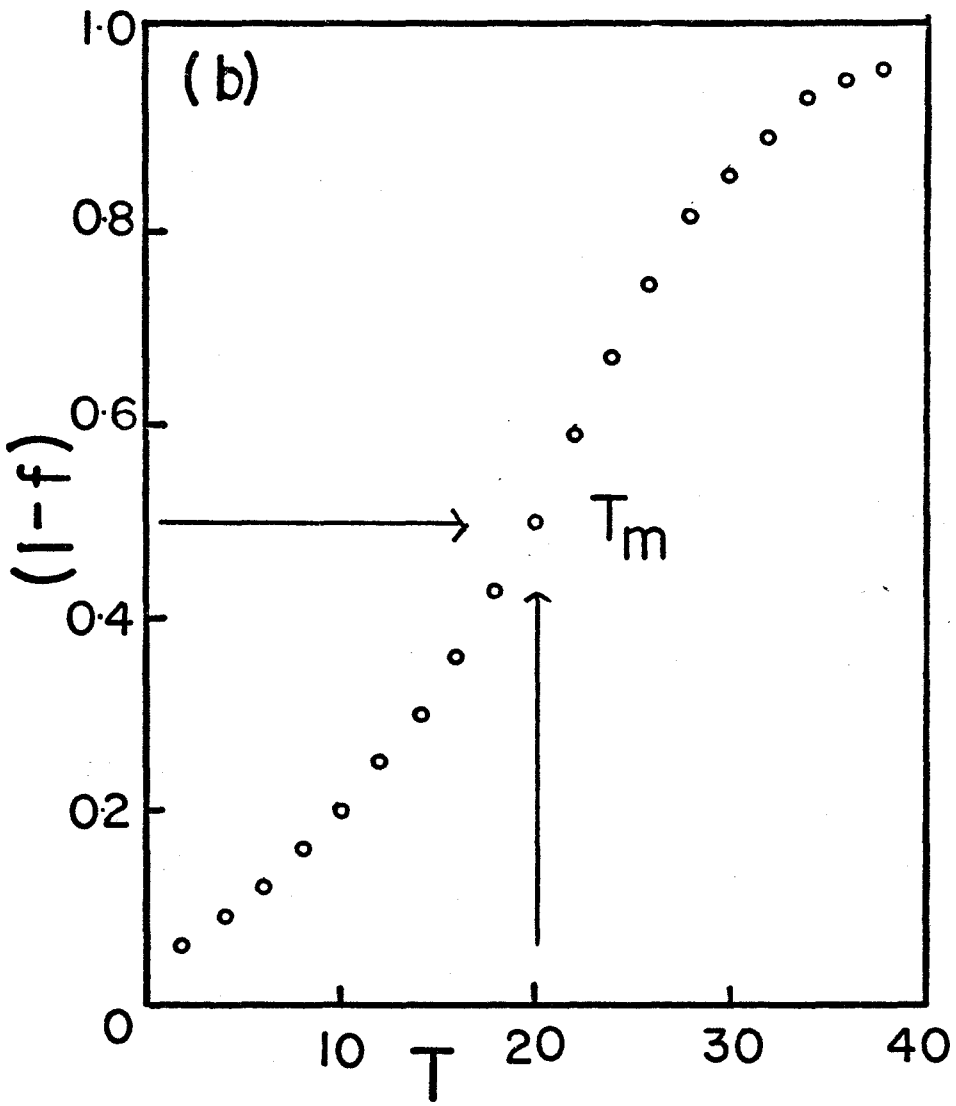


Figure 26. Plot of fraction of bases unpaired (1-f) calculated from data in figure 25

where $f(T) = \frac{A_s(T) - A(T)}{A_s(T) - A_d(T)}$.

that an oligomer-polymer interaction is a poor model for predicting the effect of a single, 'dangling' base on the stability of a short helix. However an additional factor must be considered in the study of duplex I formation - the self-complementation of ApGpCpUpC (duplex II).

Borer et al's method predicts that duplexes I, II and III would have the same enthalpy and T_m for the helix-coil transition (if the effect of 'dangling' bases is ignored). Unfortunately this model does not consider the difference in terminating a short helix with A=U versus G≡C base pairs. As a duplex of only four base pairs probably exists in an 'all or none' state (Podder, 1971 and Arter et al., 1974), the extent of 'fraying' (Patel and Hilbers, 1975) should be an important contribution to the stability of these duplexes. Terminal A=U base pairs are more susceptible to 'fraying' than G≡C base pairs (Pohl, 1974); consequently the T_m of duplex II which has two terminal A=U base pairs would be expected to be lower than the T_m's for I and III, both of which have two terminal G≡C base pairs. Duplex II was in fact found to have a T_m that was significantly lower than the T_m's for I and III (Table 5.8). This would also indicate that the nature of the terminal base pairs has a larger effect on duplex stability than the contribution of 'dangling' bases.

Ravetch et al., (1974) have reported a $T_m < 0°$ for ApGpCpU at 1.8×10^{-4} M. Perhaps an extension of the present study in which the stability of the duplexes formed by ApGpCpUpC and ApGpCpU were compared would provide important information on the effect of 'dangling' bases. At any rate, duplexes I and III are poor models for this type of study as I could be complicated by the presence of II. Although duplex I can be expected to dominate from the difference in T_m's of II and III, the extent to which duplex II may be present

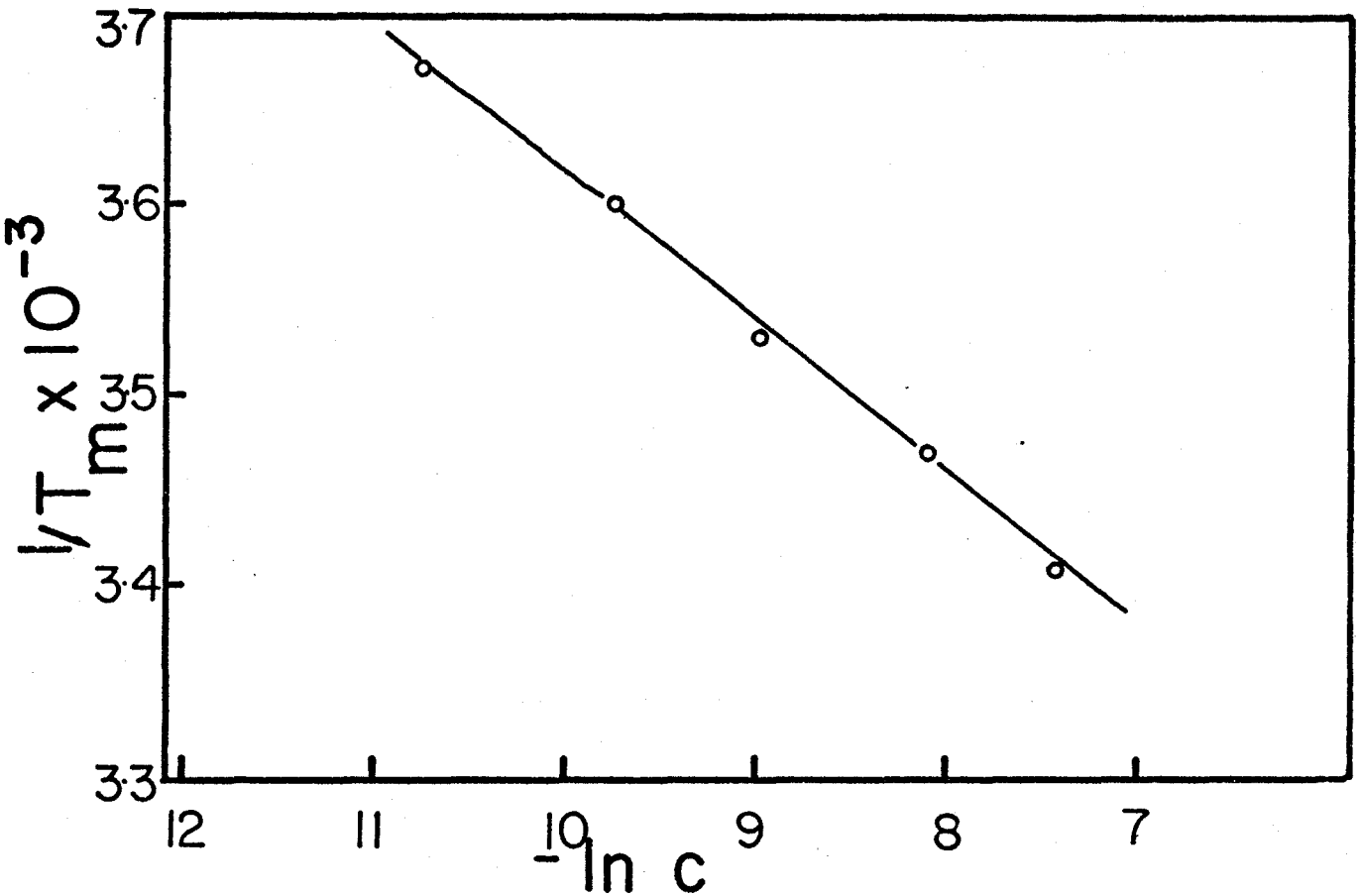


Figure 27. Duplex III , plot of $1/T_m$ (^oK) and logarithm of total oligomer concentration.

is unknown.

As a study of the interaction of these oligomers and duplexes with yeast phenylalanyl-tRNA ligase was intended, the stability of duplex III under conditions optimal for both aminoacylation and tRNA binding was explored. In 50 mM tris buffer (pH 7.5) and 10 mM Mg^{+2}, a common buffer in aminoacylation assays (eg. Dudock et al., 1971), no significant difference in the T_m was observed. As low pH increases the affinity of the ligase for the native tRNA (Befort et al., 1970), the stability of duplex III at pH 6.0 (0.010 M sodium phosphate buffer, 1.0 M NaCl) was also checked. Again no change in T_m was detected.

5.7.2 Circular Dichroism Study

As circular dichroism is extremely sensitive to the relative geometry of the base chromophores (Borer et al., 1973), a cd study was initiated to gain further information on duplex formation.

In Figure 28 the cd spectrum of GpApGpC plus GpCpUpC is compared with the spectra of the separate oligomers. Two features of this spectra stand out, the position of the λ max (cf. 270 nm for GpApGpC plus GpCpUpC with 272.5 nm for the separate oligomers) and the large negative ellipticity at 210 nm. Obviously the cd spectra for GpApGpC plus GpCpUpC is not simply a summation of the spectra of the single strands. On the basis of these differences it was concluded that GpApGpC was hybridizing with GpCpUpC to form duplex III.

With elevated temperature three significant changes were observed in the cd spectra of duplex III (Figure 29):

1. a shift in λ max from 270 nm to 272.5 nm,
2. a rapid decrease in the negative ellipticity

and 3. a more gradual and less dramatic decrease in the positive ellipticity.

When ellipticity (210 nm and 270 nm) was plotted with temperature, cooperative melting transitions were obtained. As similar temperature-dependent changes in the cd spectra of the single strands were not observed, these changes were probably due to the helix-coil transition. T_m's of 8° and 17° (210 nm and 270 nm, respectively) were calculated from these ellipticity-temperature profiles. It would appear that the negative ellipticity reflects duplex formation more accurately than the positive ellipticity as the T_m of the same sample in the uv study was 5.5°. Perhaps the positive ellipticity reflects some other feature of the oligomers' conformation.

The cd spectra of ApGpCpUpC plus GpApGpC and ApGpCpUpC were also studied and the formation of duplexes I and II, respectively, were established by the shift in λ max (I, 272 → 274 nm; II 272.5 → 275 nm) and the cooperative ellipticity-temperature profiles. Again the T_m's calculated from the decrease in the negative ellipticity (210 nm) with temperature compared more favourably with the T_m's of the uv studies.

It would have been informative to have studied the concentration dependence of the T_m as calculated from the cd spectra of the duplexes and compared the results with the uv study. However the large volume of the cd cell (ca. 2.7 ml) necessitated relatively large quantities of oligomers to obtain concentrations suitable for duplex formation. As other studies were planned it was decided to conserve these sequences.

Table 5.8

Melting properties of various duplexes

Duplex	T_m ($c=10^{-4}M$)+	$-\Delta H^o$ (kcal/mole) obs. (Eq.1)	$-\Delta H^o$ (kcal/mole) predicted (Borer et al.,1974)	$-N\Delta H_1$ (kcal/mole) obs. (Eq.2)
GAGC/GCUC (III)	17.7	24.4	26.5	39.1
GAGC/AGCUC (I)	16.0	24.4	26.5+?	30.7
AGCUC (II)	6.0	29.3	26.5+?	25.9
A_4U_4 (Martin et al.,1971)	11.5	51	55.7	46
CCGG (Arter et al.,1974)	18.5	33	40.4	-
GGGC/GCCC (Podder,1971)	>35	21.6	42.1	-
A_2GCU_2 (Borer et al.,1974)	28.3	46	42.9	-

+ For non-complementary helices $c=4\times10^{-4}M$.

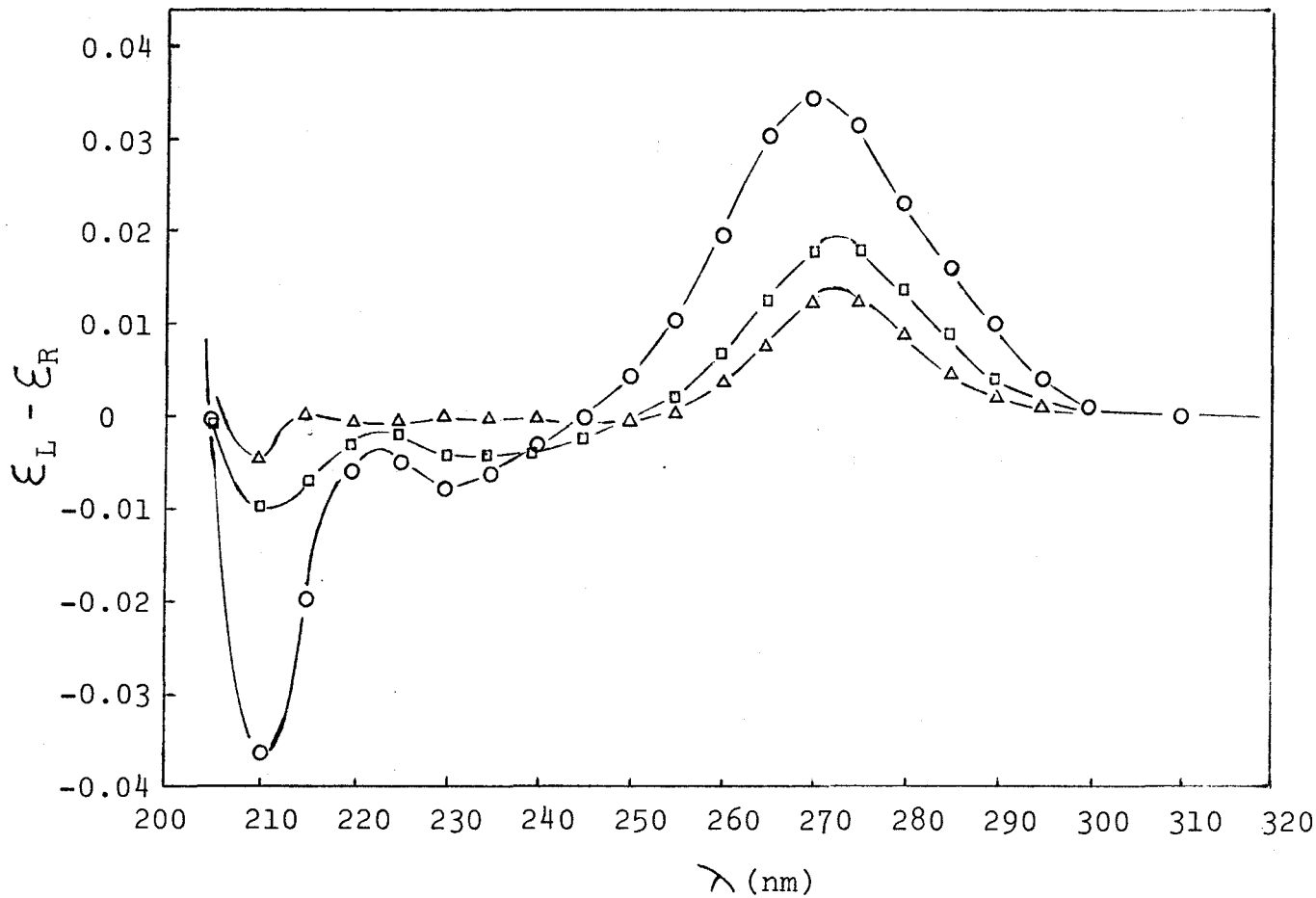


Figure 28. Circular dichroism spectra, △ - GpApGpC (1.9×10^{-5}M), □ - GpCpUpC (1.8×10^{-5}M) and ○ - GpApGpC (2.1×10^{-5}M) + GpCpUpC (2.1×10^{-5}M), 0^{o}.

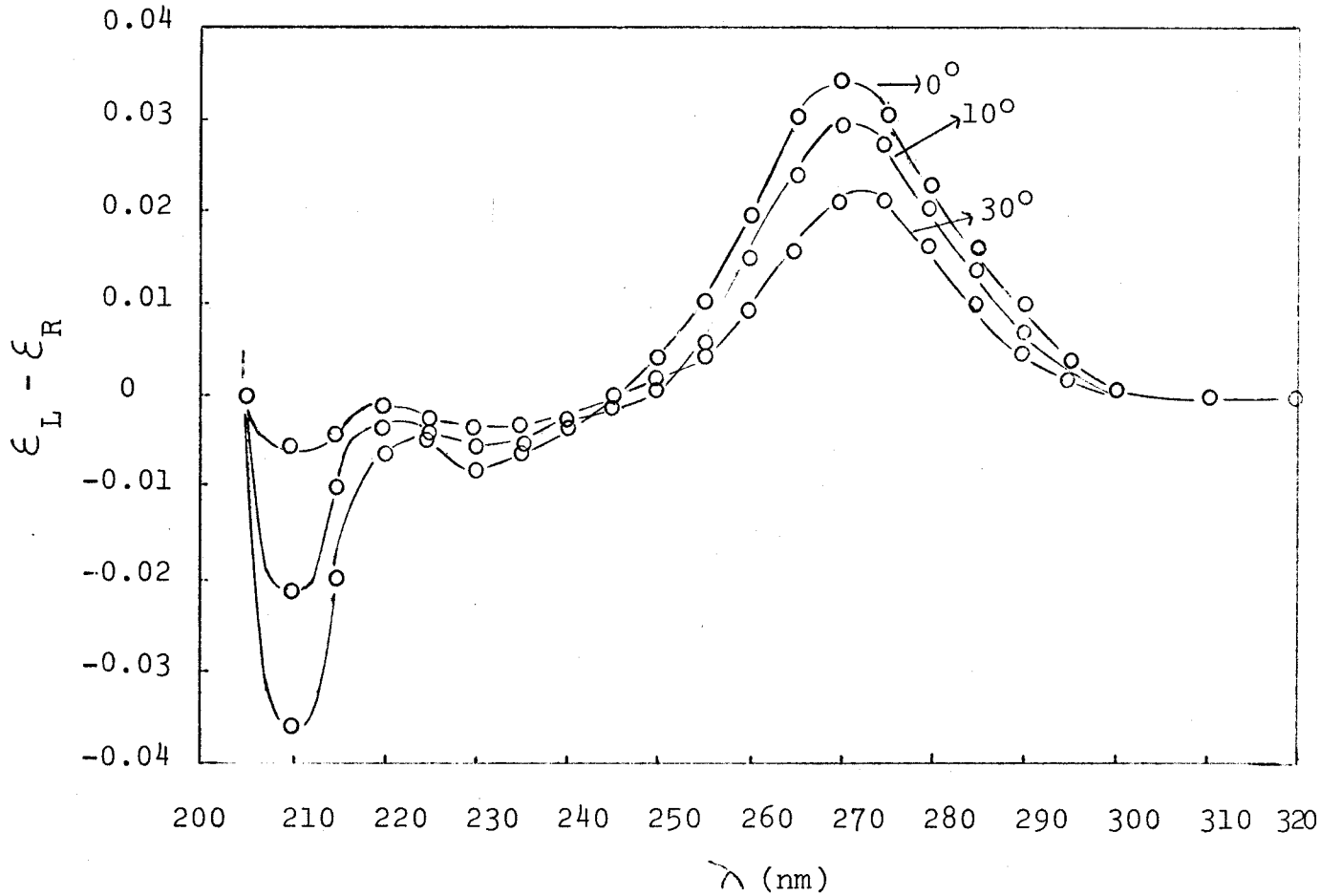


Figure 29. Temperature-dependent changes in circular dichroism spectra of duplex III ($c = 4.2 \times 10^{-5}$M).

5.7.3 Proton Magnetic Resonance Studies

Only the pmr spectra of duplex III was examined in this study as it was anticipated that the interpretation of the spectra of duplex I would be complicated by the presence of II.

In the original high-resolution pmr studies, the formation of a double-stranded helix in tRNA was demonstrated by the resonances for the hydrogen-bonded ring NH protons (G_1H and U_3H) of Watson-Crick base pairs (for review see Kearns and Shulman, 1974). The formation of short helices by self-complementary oligonucleotides has also been demonstrated in a similar manner (Patel and Hilbers, 1975; Arter et al., 1974; and Kan, Borer and Ts'o, 1975). Although the resonances of the exchangeable protons can be assigned to specific base pairs in a helix of defined sequence (Shulman et al., 1973 and refs. therein), these resonances provide little information on helix stability as they broaden and disappear at temperatures well below the melting temperature.

More recent studies have also examined the temperature dependence of the chemical shifts for the resonances of the nonexchangeable aromatic and anomeric protons (Arter et al., 1974; Borer, Kan, and Ts'o, 1975). When the temperature is lowered there are dramatic, predominantly upfield, changes in the chemical shifts of these resonances. This is mainly due to the diamagnetic shielding of aromatic ring currents in neighboring bases. When the chemical shifts are plotted against temperature, cooperative melting curves are obtained. Thus, a T_m for the helix-coil transition can be calculated from the midpoint of these curves.

5.7.3.1 Assignment of the Resonances of the Nonexchangeable Protons

Partial assignment of the resonances of the 21 aromatic and anomeric protons of duplex III (Table 5.9) was accomplished by comparison of the spectra of duplex III with the spectra of the isolated tetranucleotides.

The resonances of GpApGpC were identified by the procedure of 'incremental assignment' (Figure 30a) (Borer et al., 1975). First, the pmr spectra of GpA, GpApG and GpApGpC were obtained at high temperature (ca. 70°) to avoid inter- and intra-strand interactions. Comparison of the spectra of GpApG and GpA lead to unambiguous assignment of the resonances of $G(3)H_8$ and $G(3)H_{1'}$. Similarly, comparison of GpApGpC and GpApG lead to the assignment of $C(4)H_6$, $C(4)H_5$ and $C(4)H_{1'}$. $C(4)H_6$ and $C(4)H_5$ were identified on the following basis:

1. The chemical shift. Deshielding by the phosphodiester bond shifts the H_5 resonance of pyrimidines about 1.8 ppm upfield into the region of the anomerics.

2. The coupling constant. The H_6 and H_5 doublets have temperature-invariant coupling constants ($J_{5,6}$ = 7.6 Hz , obs. 7.5 Hz). (N.B. the $H_{1'}$ resonances have temperature-sensitive coupling constants ($J_{1'-2'}$ < 6 Hz)).

And 3. A decoupling experiment. Irradiation of $C(4)H_5$ caused the $C(4)H_6$ doublet to collapse to a sharp singlet.

Finally, the assignment of the resonances of GpA was based on the data of Ts'o and co-workers (1969).

Only the low field resonances of the aromatic protons of GpCpUpC (Figure 30b) could be assigned in this study. The resolution at 90 MHz

was not sufficient to distinguish the resonances of the four anomeric and three H_5 protons. In the region of 7.5 to 8.0 ppm in the pmr spectra of GpCpUpC a singlet and two doublets were observed. The singlet was easily identified as the G(4)H_8 resonance. As the intensities of the two doublets were in the ratio of 2 to 1, it was assumed that they represented the C(1)H_6 plus C(3)H_6 and the U(2)H_6 resonances, respectively. This assumption was confirmed by measurement of the coupling constants ($U\ J_{5,6}$ = 8.1 Hz, obs. 7.9 - 8.3 Hz and $C\ J_{5,6}$ = 7.5 Hz, obs. 7.2 - 7.9 Hz).

Finally comparison of the pmr spectra of duplex III with the spectra of GpApGpC and GpCpUpC allowed direct assignment of all the low field resonances (Figure 31). As the spectra of duplex III and the isolated strands were obtained at 70°, there was no change in the chemical shifts of these resonances from inter-strand interactions. Confirmation of the assignment of the H_8 resonances of G(1), G(3), G(4) and A(2) was derived from an isotope exchange experiment. After two weeks at -20°C the intensities of the H_8 resonances were observed to have decreased. Exchange of these protons with solvent has been previously reported in oligonucleotides labelling experiments (eg. Miller et al., 1974). As the resonances of the anomeric and H_5 protons of GpCpUpC were not assigned, it was not possible to identify the resonances in the high field region of the pmr spectra of duplex III.

Table 5.9

Assignment of resonances of the nonexchangeable protons.

$$\overrightarrow{\text{GpApGpC}}$$
1 2 3 4
$$\text{CpUpCpG}\overleftarrow{\phantom{\text{CpUpCpG}}}$$

Proton	Chemical shift (ppm)	Proton	Chemical shift (ppm)
$G(1)H_8$[a]	7.87	$C(1)H_6$	7.78
$G(1)H_{1'}$[b]	5.72	$C(1)H_5$	-
		$C(1)H_{1'}$	-
$A(2)H_2$	8.15	$U(2)H_6$	7.83
$A(2)H_8$	8.26	$U(2)H_5$	-
$A(2)H_{1'}$	5.98	$U(2)H_{1'}$	-
$G(3)H_8$	7.90	$C(3)H_6$	7.78
$G(3)H_{1'}$	5.78	$C(3)H_5$	-
		$C(3)H_{1'}$	-
$C(4)H_6$	7.74	$G(4)H_8$	7.97
$C(4)H_5$	5.85	$G(4)H_{1'}$	-
$C(4)H_{1'}$	5.87		

[a] $G(1)H_8$: G(1) refers to the guanosine in base pair number 1 of duplex III.
H_8 refers to the aromatic proton in position 8 of the purine ring.

[b] $G(1)H_{1'}$: refers to the anomeric proton of G(1).

Figure 30a. 'Incremental assignment' of resonances of the pmr spectra of GpApGpC (70°C, GpApGpC = 6.1×10^{-3}M).

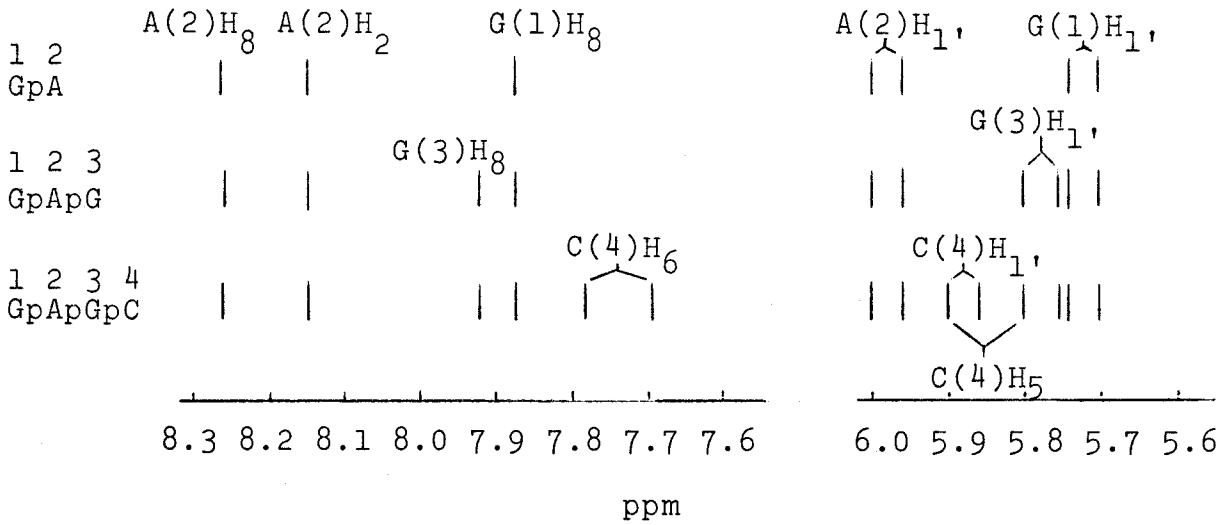
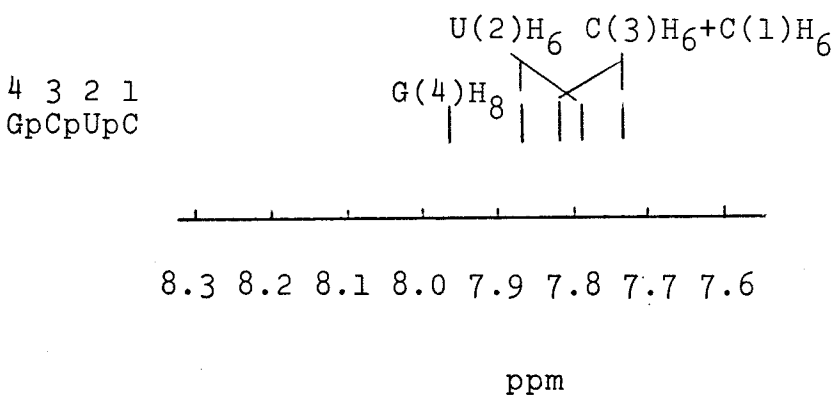


Figure 30b. Assignment of the lowfield resonances of the aromatic protons of GpCpUpC (70°C, GpCpUpC = 7.3×10^{-3}M).



5.7.3.2 Temperature Dependence of Chemical Shifts

Before mixing GpApGpC with GpCpUpC, the temperature dependence of the chemical shifts for the low field resonances of the isolated tetranucleotides was studied (Figure 32a and b, respectively). These resonances experienced only small changes in their chemical shifts over the 65 to 15 degree temperature range (N.B. The H_8 resonances of G(1) and G(3) are not plotted as they were poorly resolved at lower temperatures.). As these changes were essentially linear, it was considered that a random process such as base stacking was responsible. This study also demonstrated that the C doublet of GpCpUpC did in fact contain the resonances of two protons.

With decreasing temperature the resonances of duplex III were observed to broaden rapidly. This broadening reflects the reduced segmental motion of the duplex relative to the unstacked single strands. It was also observed that at room temperature fine white filaments were formed in the pmr sample. Borer et al., (1975) have suggested that short helices can aggregate by end-to-end stacking to form an extended helix. At any rate, as a result of the reduced tumbling of the molecule it was necessary to increase the number of pulses from about 250 at 70°C to 2000 at 25°C.

Figure 33 illustrates the temperature dependence of the chemical shifts of the low field resonances of the aromatic protons of duplex III. Dramatic and predominantly upfield shifts were observed. Comparison of the biphasic transitions of duplex III with the linear transitions of the isolated strands strongly suggests duplex formation. It is important to note that these temperature dependent changes in the pmr spectra of duplex III were completely reversible. The spectra at any temperature could be reproduced. Unfortunately, only the $A(2)H_2$ resonance could be followed

... accurately at the low temperatures. It was originally planned that the G(3)H_β resonance would be followed ....

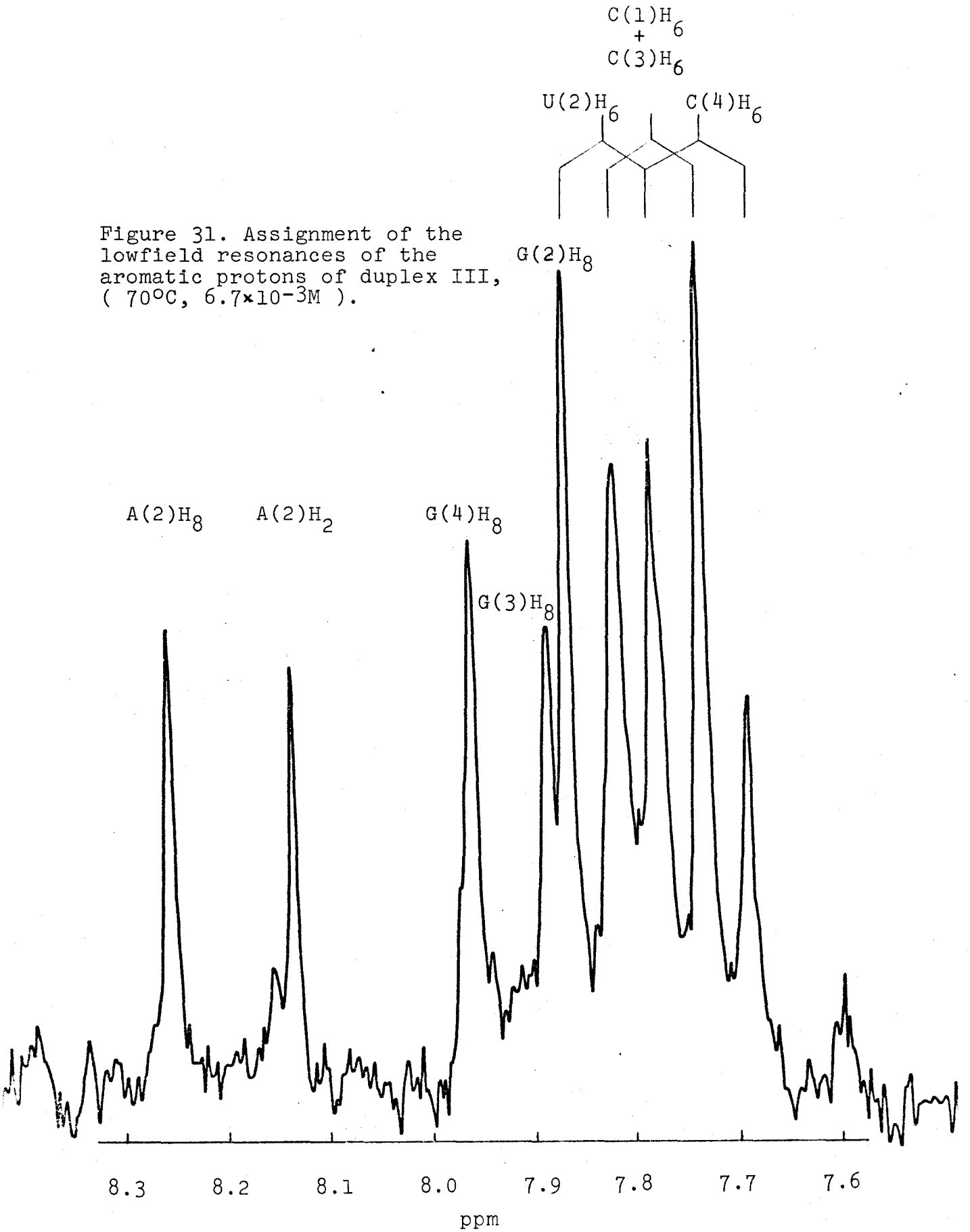


Figure 31. Assignment of the lowfield resonances of the aromatic protons of duplex III, (70°C, 6.7×10^{-3}M).

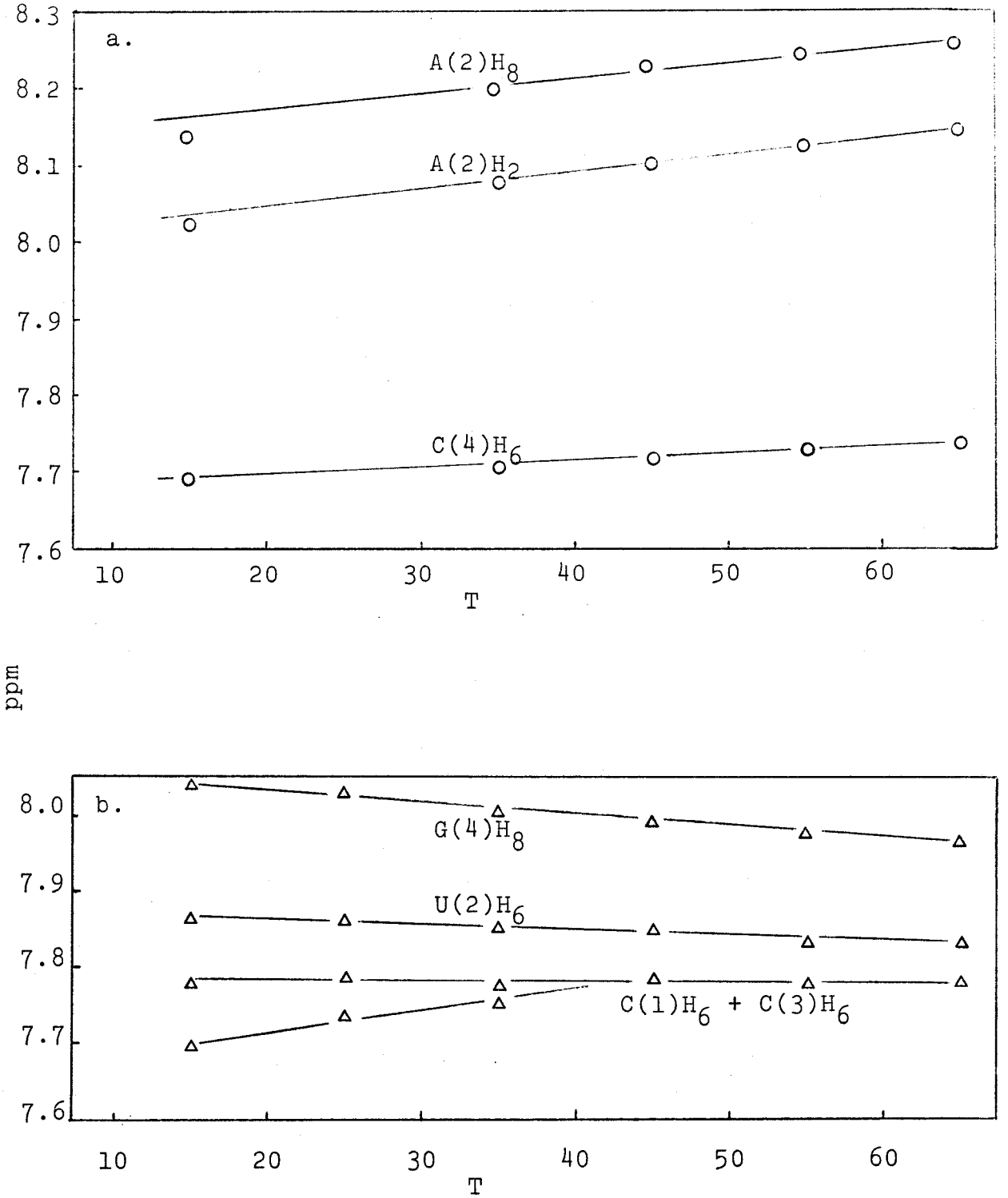
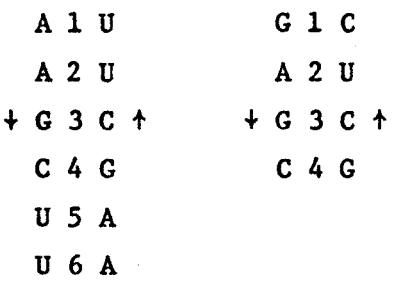


Figure 32. Temperature dependence of chemical shifts of lowfield resonances of the aromatic protons of a. GpApGpC (6.1×10^{-3}M) and b. GpCpUpC (7.3×10^{-3}M).

as this nucleotide has the same nearest neighbours as G(3) in the duplex formed by ApApGpCpUpU (Borer et al., 1975).



Therefore this resonance was expected to experience the same upfield shift in the helix-coil transition.

From the cooperative melting curve for the temperature dependence of chemical shift of A(2)H_2 (Figure 34), a T_m of 42 ± 1°C was calculated for the A = U base pair. As it was assumed that a short helix of four base pairs would melt in an 'all-or none' process, this melting temperature also represents the T_m of the helix-coil transition of duplex III. This value compares very favourably with the melting temperature (43.5°C) predicted by extrapolation of the data for the concentration dependence of T_m (U.V. absorption studies, Figure 27). The agreement of these values is especially noteworthy as it indicates that the same helix-coil transition was studied over the 10^3-fold concentration range.

The spectra of the exchangeable, N1 protons of G(1), G(3) and G(4) and the N3 proton of U(2) was not obtained in this study. Comparison of duplex III with the duplex formed by the self-complementary sequence CpCpGpG (Arter et al., 1974) suggested that these resonances would only exist at temperatures less than 10°C. Due to the problems associated with obtaining spectra at low temperature in H_2O it was decided that this information did not warrant the additional effort to obtain these spectra.

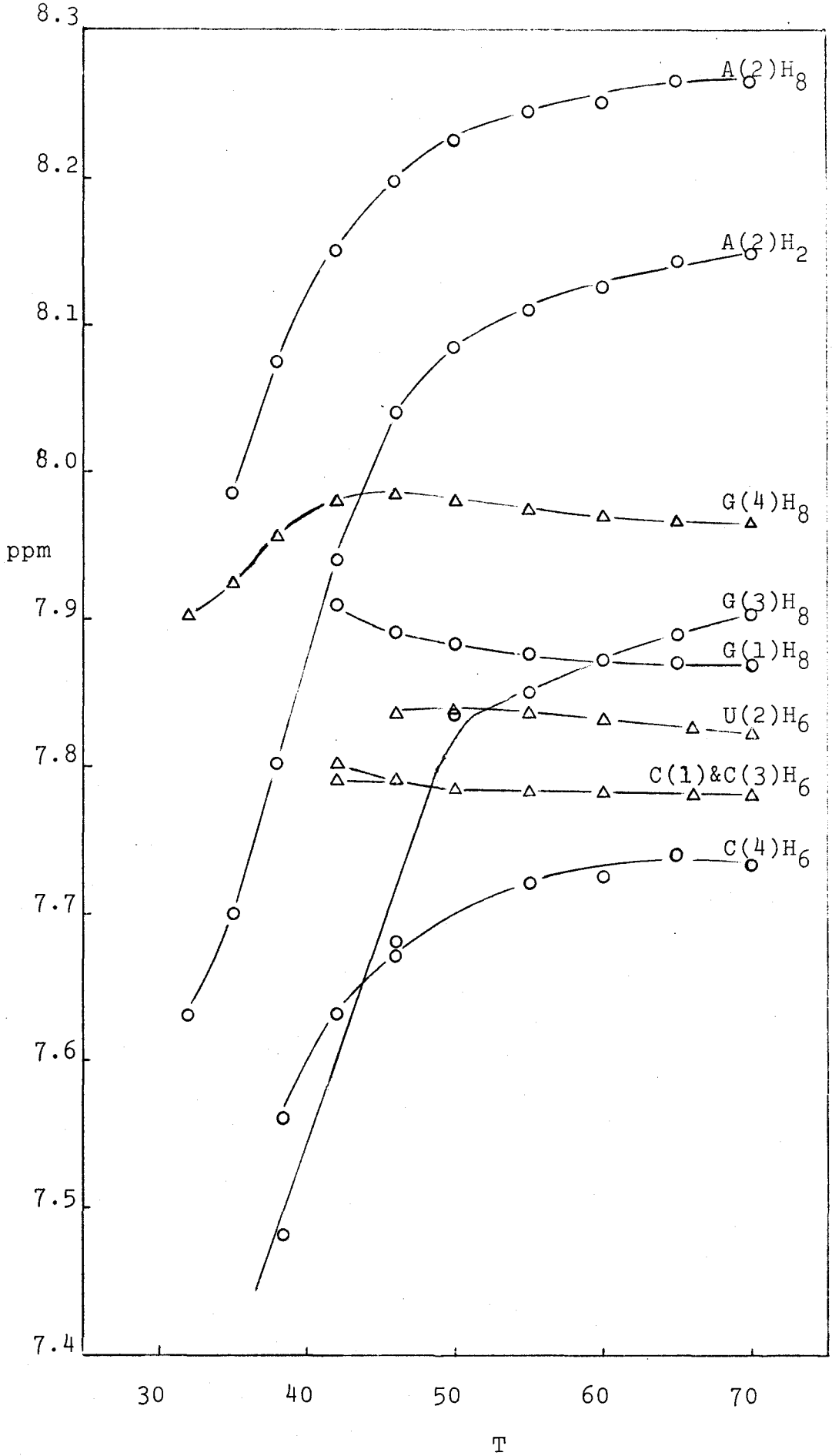
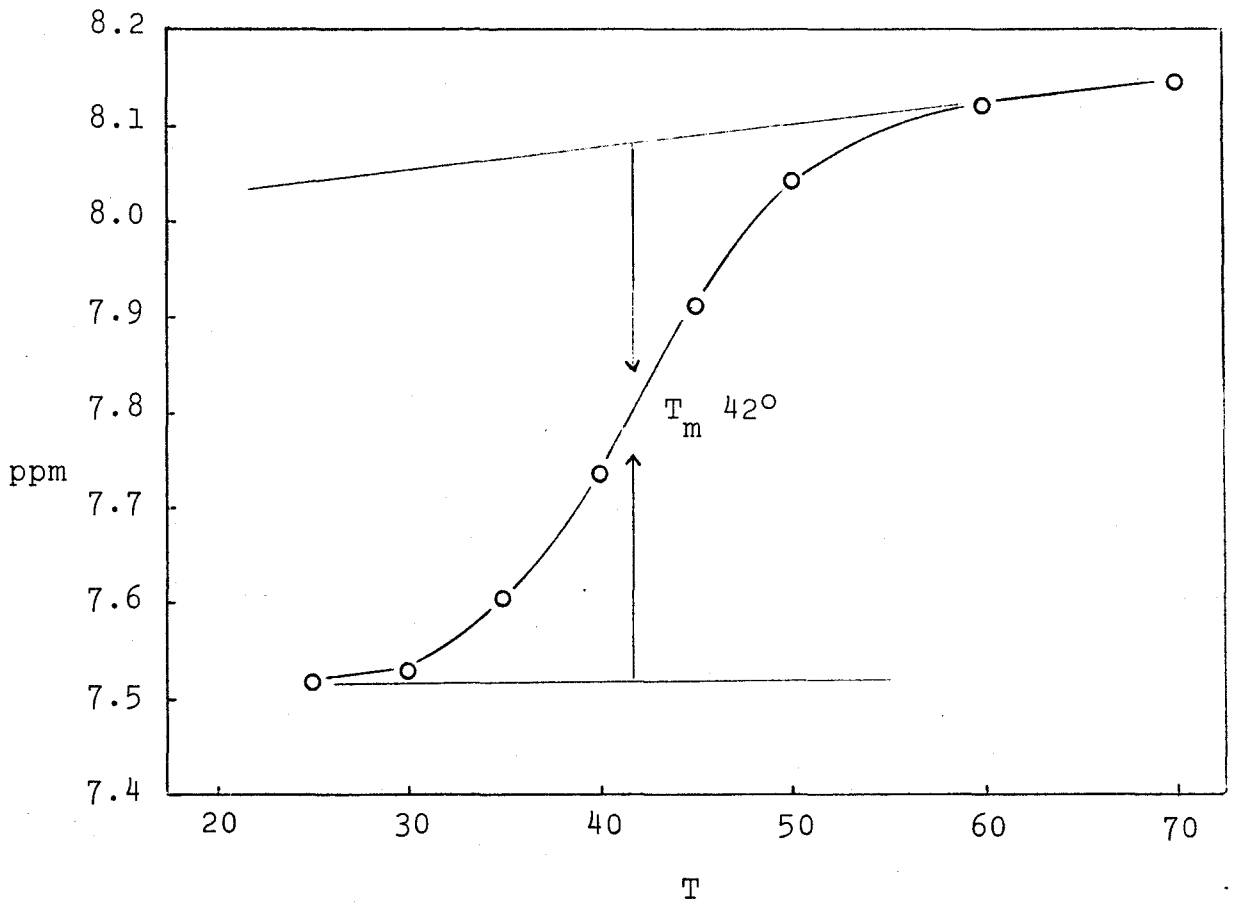


Figure 33. Temperature dependence of chemical shifts of lowfield resonances of duplex III ($c = 6.7 \times 10^{-3}$).

Figure 34. Temperature dependence of chemical shift of $A(2)H_2$ resonance (duplex III , $c = 12 \times 10^{-3} M$).



Although the pmr study of duplex III complements the other studies on short, double-stranded RNA helices (Arter et al., 1974 and Borer et al., 1975), this study is unique. This is the first pmr study of duplex formation by sequences which are not self-complementary. This factor complicates the interpretation of the spectra as the duplexes formed by self-complementary sequences have fewer resonances (compare 21 aromatic and anomeric protons of duplex III with 10 protons of $(CpCpGpC)_2$) and also have two-fold symmetry which further reduces the number of resonances by half (eg. the two terminal and two internal base pairs of $(CpCpGpG)_2$ are identical).

Two final points about the pmr spectra of these oligoribonucleotides must be made. First, they confirm the structural identity of the sequences and secondly, as no unaccountable resonances were observed, they also indicate the purity of the sequences deprotected by the modified deprotection procedure with Zn/Cu couple.

5.7.4 Summary

The formation of short, double-stranded RNA helices by the complementary oligoribonucleotides, GpApGpC, GpCpUpC and ApGpCpUpC was established by three different techniques -- ultraviolet absorption, circular dichroism and proton magnetic resonance. These studies are valuable as they provide additional thermodynamic data on duplex formation. The formation of duplexes I and III is especially significant as these duplexes --

1. correspond to a natural sequence, common to several tRNA molecules.

2. correspond to a proposed, partial recognition site for yeast phenylalanyl-tRNA ligase. And

3. represent the first demonstration of a duplex with four base pairs, one of which is an A=U pair.

5.8 Purification of Yeast Phenylalanyl-tRNA Ligase

The purification of yeast phenylalanyl-tRNA ligase is summarized in Table 5.10. Although the enzyme was not purified to homogeneity, a 100-fold purification was achieved. The specific activity of this preparation (1040 eu/mg) compares favourably with that obtained by Fasiolo and Ebel (1974) with a similar procedure (3200 eu/mg).

The apparent increase in enzyme activity after the Sephadex G-200 column may be attributed to the preceding ammonium sulfate fractionation. Although both Reid *et al.*, (1971) and Fasiolo and Ebel (1974) have reported loss of activity upon fractionation, activity has also been observed to increase on storage (Fasiolo *et al.*, 1970). Alternately, some substance which interferes with the aminoacylation assay may have been removed upon gel filtration.

The problems associated with the purification procedure were a direct result of the poor separation by DEAE-Sephadex column chromatography. Fasiolo and Ebel (1974) have experienced similar problems at this stage and have replaced this column with a DEAE-cellulose DE-11 column. They suggest that the high content of nucleic acids (*ca.* 15%) in the eluent of the Sephadex G-200 column affects the separation. Due to the large amount of protein eluted with the enzyme on the DEAE-Sephadex column, the subsequent hydroxylapatite column was seriously overloaded and most of the enzyme pre-eluted during application. Precipitation and re-application of

the enzyme to a second hydroxylapatite column gave acceptable purification.

It was decided that this partially purified enzyme preparation would be suitable for the preliminary studies on the inhibition of aminoacylation by the duplexes. However for the other studies proposed a homogenous ligase would be required. Recently a novel procedure for the purification of yeast phenylalanyl-tRNA ligase was described (Hossain, 1975). In order to avoid proteolytic fragmentation the enzyme was purified from toluene lysates of baker's yeast in the presence of the protease inhibitor, phenylmethylsulfonyl fluoride. This enzyme had an α_4 subunit structure with subunits of molecular weight 75,000 which is in contrast to earlier reports of an $\alpha_2\beta_2$ subunit structure with subunits of molecular weight 75,000 and 63,000 (Schmidt et al., 1971). Hossain suggests that proteolytic fragmentation can account not only for the differences reported in subunit structure (cp. Schmidt et al., 1971 and Failso et al., 1970), but also for the differences reported in specific activity (cp. Schmidt et al., 1971 and Fasiolo et al., 1970) and the stoichiometry for ligand binding (cp. Fasiolo et al., 1974 and Horz and Zachau, 1973). As the procedure of Hossain provides an enzyme of exceptional purity and activity, it would be the method of choice for the preparation of yeast phenylalanyl-tRNA ligase for meaningful studies on the interactions with the duplexes.

Table 5.10 Purification of yeast phenylalanyl-tRNA ligase.

Fraction	Protein(mg)	Sp.Act.(eu/mg)	Total Act. (eu)	Purificn.	Recov.(%)
I. postmitochondrial supernatant	9,950	10.3	102,000	-	-
II. ammonium sulfate fraction (50-70%)	5,400	11.2	60,300	1.1	59
III. Sephadex G-200 filtrate	3,350	24.8	83,200	2.4	81
IV. DEAE-Sephadex fraction	1,220	44	53,600	4.4	53
V. hydroxylapatite fraction	18	1,040	20,250	100	20

5.9 Inhibition of Aminoacylation of Yeast $tRNA^{Phe}$ by Duplexes Corresponding to the Double-Stranded Region of the Dihydrouridine Arm

The conditions for the inhibition assay were selected to favour both duplex formation and the interaction of duplexes with yeast phenylalanyl-tRNA ligase.

pH - As the pH optimum for the interaction of yeast $tRNA^{Phe}$ and the cognate ligase is pH 5.5, a buffer of similar pH was selected to enhance the interactions of the isolated duplexes with the enzyme. 50 mM sodium cacodylate (pH 5.8) with 20 mM magnesium chloride was employed as (1) Dudock et al., (1971) had used this buffer in the heterologous charging assays and (2) duplex formation at pH 6.0 and in the presence of 10 mM magnesium chloride had been demonstrated.

Temperature - Control assays were performed at 4, 20, 25, 30 and 37 degrees (Figure 35). Only at temperatures above 25° was significant aminoacylation observed; at 4° no incorporation above background was observed, even at times up to 1 hour.

Ligand Concentration - As the assays were performed at 25°, high concentrations of oligomers ($> 5 \times 10^{-4}$ M) were required to maintain duplex formation. For example, at $c = 6.2 \times 10^{-4}$ M only 30 to 40% of oligomers are base paired in duplex III (Figure 26). The high concentrations of oligomers relative to yeast $tRNA^{Phe}$ (7×10^{-10} M) should also ensure measurable levels of inhibition if the duplexes and the ligase interact.

Pre-incubation - The ligase plus the duplexes were incubated at 0 - 4°, in the absence of tRNA, as under these conditions duplex formation is nearly 100%.

In addition to duplexes I and III (Table 5.11), the oligomers ApGpCpU, ApGpCpUpC, GpCpUpC and GpApGpC (Table 5.12) were also studied for possible inhibition of the aminoacylation reaction. However no significant difference between assays and controls were observed at any of the time points. As it could be argued that the total number of cpm were too low to observe small differences, the assays were modified and repeated. Assays with higher specific activity [^{3}H] - phenylalanine (150 m Ci/mole), larger aliquots (50 µl) and increased amounts of tRNA^{Phe} (14×10^{-10} M) failed to demonstrate any inhibition. Assays were also performed at 35^o and aliquots were taken at shorter time intervals (1, 3, 5, 7 and 10 minutes). Again no inhibition was observed.

This failure to observe inhibition of the aminoacylation of yeast tRNA^{Phe} does not rule out the possibility of interaction between duplex I or III with yeast phenylalanyl-tRNA ligase. For example, the 5' half of yeast tRNA^{Phe} does not inhibit aminoacylation (Thiebe et al., 1973) although nuclease protection studies have demonstrated an interaction of this fragment with the ligase (Horz and Zachau, 1973). Under the conditions of this assay significant levels of inhibition (>10%) would be observed if the K_i of the duplex was smaller than about 8×10^{-4} M.

Control Aminoacylation

10 min. 150cpm - 50cpm (blk) = 100cpm or $\underline{1.5 \times 10^{-11} \text{ mole}}$

- assuming sp. act. ^{3}H-L-phenylalanine 100m Ci/mmol and 3% counting efficiency.

$$v = \frac{Vmax\ [S]}{Km + [S]}$$

$[S] = [tRNA^{Phe}] = 7 \times 10^{-10}$ M/1.

Km = 0.89×10^{-7} M/1. (Rae et al., 1973).

$$1.5 \times 10^{-11} \text{ mole/10 min./2.6 units} = \frac{Vmax\ (7 \times 10^{-10} \text{ M/1})}{.89 \times 10^{-7} \text{ M/1} + 7 \times 10^{-10} \text{ M/1}}$$

$$\underline{Vmax = 19.2 \times 10^{-10} \text{ moles/10 min./2.6 units}}$$

10% Inhibition

10 min. (cntrl - 10%) - 50cpm (blk) = 85cpm or $\underline{1.28 \times 10^{-11} \text{ mole}}$

- assume inhibition competitive, at 25°C duplex c. = 1.3×10^{-4} M (ie. 25%)

$$\frac{1}{v} = \frac{Km}{Vmax}\left(1 + {}^{[I]}/_{K_i}\right)\frac{1}{[S]} + \frac{1}{Vmax}$$

$$\frac{1}{1.28 \times 10^{-11} \text{ mole/10 min./2.6 units}} = \frac{0.89 \times 10^{-7} \text{ M/1}}{19.2 \times 10^{-10} \text{ M/1}}\left(1 + \frac{1.3 \times 10^{-4}}{K_i} \text{ M/1}\right) \cdot \frac{1}{7 \times 10^{-10} \text{ M/1}} + \frac{1}{19.2 \times 10^{-10} \text{ m/1}}$$

$$\underline{K_i = 7.65 \times 10^{-4} \text{ M/1}}$$

It would thus appear that the isolated duplex does interact strongly with yeast phenylalanine tRNA ligase and that the dihydrouridine neck does not represent a major binding site.

In full agreement with Schimmel's suggestion (1973), the interaction of weakly binding tRNA fragments with the ligase must be studied directly

rather than by merely examining the activity of the fragments in an aminoacylation assay. Perhaps one tactic for studying the interaction of ligases with small tRNA fragments is isotope exchange. Gamble and Schimmel (1974) have observed that the rate of exchange with solvent (^3H_2O) of the H_8 protons of purine residues in tRNA molecules is dependent on secondary and tertiary structure. Perhaps in the presence of cognate ligase it may be possible to demonstrate that specific bases of native tRNA or tRNA fragments have altered exchange reactivity. Schimmel's group has recently shown that the oligomer UpApG (positions 8 - 10) binds well to isoleucyl-tRNA ligases and that the U has an increased ^3H exchange reactivity.*

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*O.C. Uhlenbeck, personal communication.

Table 5.11 Inhibition of aminoacylation by duplexes I and III.

time (min.)	cpm (^{3}H) duplex I (c=5.2x10^{-4}M)	duplex III (c=5.2x10^{-4}M)	control (-oligomer)	blank (-tRNA)
1	66±1	62±2	59±5	51
5	101±2	98±2	94±6	48
10	144±6	150±3	150±5	54
15	205±4	207±5	207±4	56
20	254±5	267±6	265±5	46
30	342±4	353±14	350±7	58

Table 5.12 Inhibition of aminoacylation by oligoribonucleotides.

time (min.)	cpm (^{3}H) ApGpCpU (4.6x10^{-4}M)	ApGpCpUpC (5.1x10^{-4}M)	GpCpUpC (5.1x10^{-4}M)	GpApGpC (5.4x10^{-4}M)	cntrl	blk
1	105±4	109±3	94±6	95±3	104±7	83
5	137±6	139±5	127±8	125±5	130±2	88
10	210±6	212±15	202	194±13	193±2	82
15	248±7	262±13	255±10	258±5	246±5	85
20	345±8	335±8	323±15	323±9	328±11	94
25	417±8	404±14	409±2	416±13	400±8	89

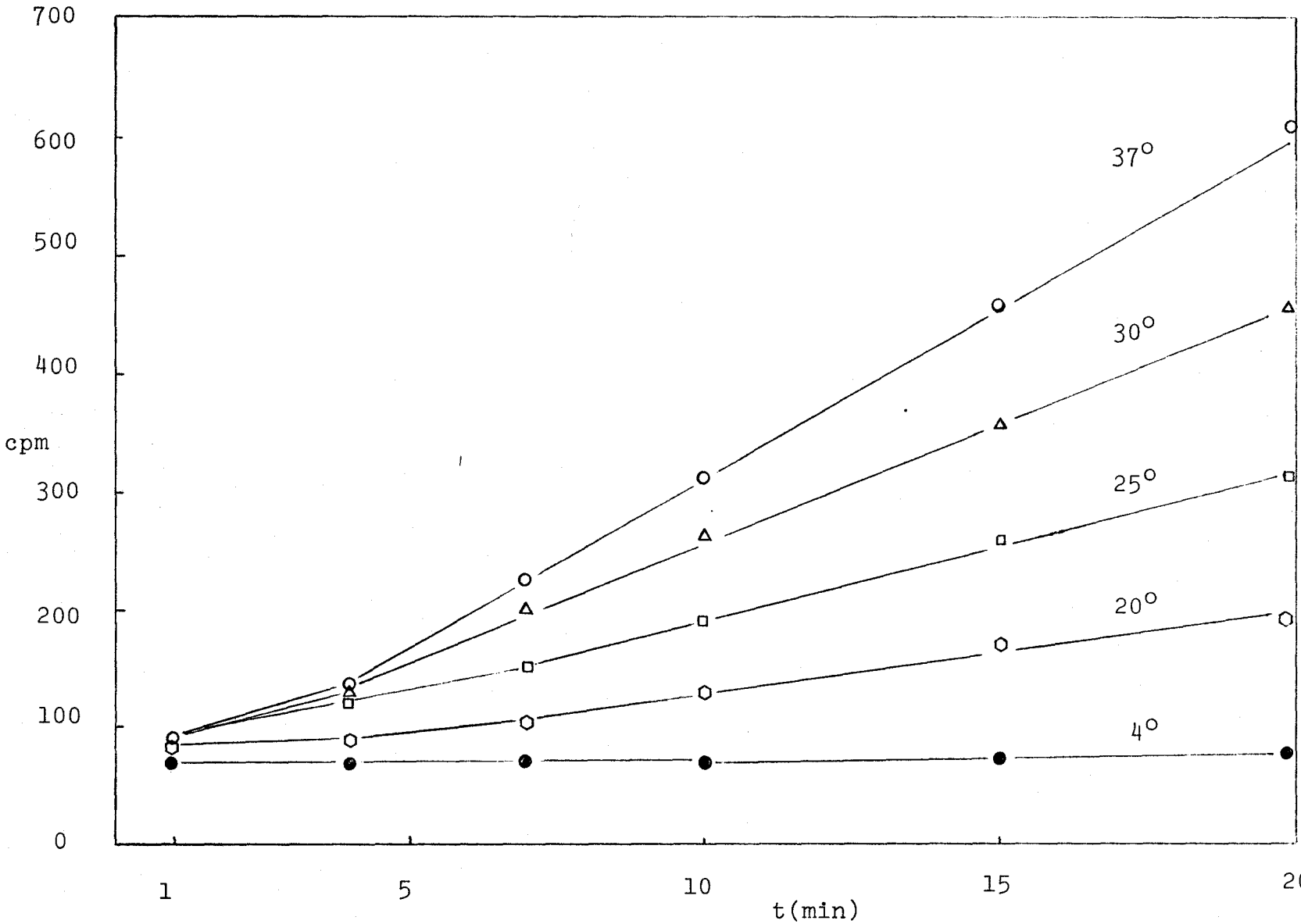

Figure 35. Temperature dependence of aminoacylation of yeast $tRNA^{Phe}$.

6. CONCLUSIONS

To obtain the desired oligoribonucleotides GpApGpC, GpCpUpC and ApGpCpUpC, it was necessary to modify both the phosphotriester synthesis and the three-step deprotection procedure.

The general phosphotriester method of Neilson and co-workers was modified to improve the yields of protected oligoribonucleotides and to avoid possible side-reactions associated with 2,4,6-triisopropylbenzenesulfonyl chloride activation. The replacement of TPS with a new condensing reagent, mesitylenesulfonyl 1,2,4-triazole and the replacement of the bis(cyclohexylammonium) salt of mono-2,2,2-trichloroethyl phosphate with its acid salt accomplished both of these goals. Of special significance was the improved yields for the coupling of purine residues. The stepwise synthesis of protected oligomers, up to a pentanucleotide, by the modified procedure is now firmly established.

The following modifications were introduced to the three-step procedure for the deprotection of oligoribonucleotides to simplify the purification and to improve the yields:

1. Shortened treatment with smaller amounts of Zn/Cu couple,
2. Increased treatment with methanolic ammonia,
3. Replacement of Dowex 50W-X8 with Chelex 100 resin, and
4. Isolation of partially deprotected sequences by paper chromatography

Although improved yields, especially for longer oligomers, were not observed, sequences of exceptional purity and biological activity were

obtained.

The complementary oligoribonucleotides prepared by the modified method were then used to study the formation of short, double-stranded RNA helices. The formation of duplexes I ($\overset{\text{ApGpCpUpC}}{\underset{\text{CpGpApG}}{\cdot\cdot\cdot\cdot\cdot}}$), II ($\overset{\text{ApGpCpUpC}}{\underset{\text{CpUpCpGpA}}{\cdot\cdot\cdot\cdot\cdot}}$) and III ($\overset{\text{GpCpUpC}}{\underset{\text{CpGpApG}}{\cdot\cdot\cdot\cdot\cdot}}$) was established by ultraviolet absorption and circular dichroism studies. The thermodynamic data for the helix-coil transitions of the duplexes was consistent with that of other short, RNA helices. However, this study was novel as it was the first demonstration of the formation of a stable duplex with only four base pairs -- one of which has an A=U pair.

The formation of duplex III was also confirmed by a proton magnetic resonance study. The agreement of the melting temperature observed in this study with the value predicted from extrapolation of the UV data is indeed noteworthy. This indicates that the same structure was examined over a 10^3-fold concentration range. Pmr studies hold particular promise as the structure of nucleic acids in solution can be examined at atomic resolution. The pmr study was unique as this was the first investigation of duplex formation by oligomers that were not self-complementary.

Finally, the formation of duplexes I and III was of signifiance as they correspond to a region common to several tRNA molecules and to a proposed, partial recognition site for an aminoacyl-tRNA ligase. Thus, they are also excellent probes for studies on the basis of specificity in protein-nucleic acid interactions. Although duplex-ligase interaction was not demonstrated by the aminoacylation inhibition assay, interaction cannot be ruled out. These investigations merely confirm the necessity of

employing more sensitive techniques in the study of ligase-tRNA fragment interactions.

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