SYNTHETIC OLIGORIBONUCLEOTIDES: A MESSENGER RNA AND PURIFICATION OF ISOLEUCINE TRANSFER RNA BY AFFINITY CHROMATOGRAPHY

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosphy

1975

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SYNTHETIC OLIGORIBONUCLEOTIDES

DOCTOR OF PHILOSOPHY (1975)
(Biochemistry)

McMASTER UNIVERSITY
Hamilton, Ontario.

TITLE: Synthetic Oligoribonucleotides: A Messenger RNA and Purification of Isoleucine Transfer RNA by Affinity Chromatography

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NUMBER OF PAGES: xviii, 221

ABSTRACT

Chemical synthesis of oligoribonucleotides has been applied to two problems of biochemical interest. The synthesis of a model messenger RNA coding for N-formylmethionylangiotensin and use of a synthetic tetranucleotide (ApUpCpA) bound to cellulose for affinity chromatography of isoleucine tRNA were proposed.

Synthesis of the required tri- and tetranucleotide blocks for assembly of the thirty nucleotide messenger sequence was carried out by the phosphotriester method developed by Neilson. In this method, a nucleoside destined to become the 5'-terminal residue, protected with the acid labile 2'-O-tetrahydropyranyl group, and except for uridine, an N-benzoyl group, is further protected by a 5'-triphenylmethoxyacetyl group which can later be removed selectively. This protected nucleoside is phosphorylated at the 3'-hydroxyl with 2,2,2-trichloroethylphosphate which is subsequently coupled to the 5'-hydroxyl of the second nucleoside, protected as the original one. Addition of further nucleosides is accomplished by the same two step procedure. Yields of coupled products appeared to depend upon the sequence and nucleoside being coupled, and ranged from 20-70%.

Relative rates of hydrolysis of the 5'-protecting group and N-benzoyl groups also depended upon the sequence of nucleotides used and an undesirable extent of loss of N-benzoyl groups was found particularly in sequences containing adjacent adenosine and cytidine residues or

cytidine and cytidine residues. Results obtained in these reactions were adequate to allow two block coupling reactions to be attempted, and the products of these two coupling reactions were isolated. However, on completely deprotecting these products, the yields of the expected oligonucleotides was 0%, precluding further characterization. After complete deprotection of the shorter oligonucleotides, synthesized by the stepwise addition of nucleosides, low but adequate yields were obtained which allowed confirmation of their structures.

These unexpected difficulties with the apparently unique properties of different protected oligonucleotides together with the length of the synthetic pathways involved resulted in the decision not to continue towards the objective sequence. The sterically hindered mechanism of coupling and possible binding to and degradation by Zn/Cu couple used in complete deprotection steps were discussed to account for these difficulties, especially with longer sequences. Potential solutions, based on these hypotheses were proposed.

The decision was also made to proceed with the application to affinity chromatography.

Model studies demonstrated feasibility of incorporating 5'-phosphates or 3'-phosphate-linked decane diol as possible sites for attachment of the affinity site to cellulose. Coupling of protected sequences to cellulose via phosphodiester or triester linkages were not suitable, but coupling to cellulose via phosphodiester after deprotection was demonstrated. Affinity chromatography of isoleucine labelled and leucine labelled bulk-tRNA did not indicate evidence for specific binding.

Theoretical treatment of the affinity experiment showed the validity of the idea and suggested that availability of binding sites was the critical parameter. Nuclease digestion of the oligonucleotide cellulose confirmed this hypothesis. Consideration of other matrices, spacers and coupling methods, as well as improvement of yields on deprotection were recommended in order to increase the availability of binding sites.

This thesis I wish to dedicate to the memory of my father, whose continued support and encouragement were deeply appreciated and instrumental in bringing me to this threshold.

ACKNOWLEDGEMENTS

I wish to express my deep gratitude to Dr. T. Neilson whose guidance and supervision were greatly appreciated and whose criticisms and encouragement throughout this work were invaluable. I am also indebted to Dr. K.B. Freeman, Dr. B. Ferrier, Dr. R. Bell and Dr. H.P. Ghosh for their advice and suggestions during the course of this work.

Many thanks must go to my colleagues and co-workers Dr. E. Werstiuk, Mr. E. Wastrodowski, Mr. T. England, Dr. B. Brownlee and Dr. R.B. Wallace for their stimulating discussions.

The excellent typing of Miss W. Morris and quality drawing of diagrams by Mrs. B. Bell are also greatfully acknowledged.

To my wife, Kathy, I shall be ever thankful for her unfailing moral support.

I thank the Medical Research Council of Canada and McMaster University for their financial support.

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ABBREVIATIONS

A	adenosine[a]
ala	alanine
asn	asparagine
asp	aspartic acid
arg	arginine
ATP	adenosine triphosphate
A_{260} unit	the amount of material contained in 1 ml of a solution having an absorbance of 1.0 at 260 nm.
bz	benzoyl
C	cytidine[a]
cys	cysteine
d	decyl
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EF	elongation factor
f-met	formylmethionine
FSH	follicle stimulating hormone
G	guanosine[a]
GDP	guanosine diphosphate
gln	glutamine
glu	glutamic acid
gly	glycine

gly	glycine
GTP	guanosine triphosphate
his	histidine
I	inosine
IF	initiation factor
ile	isoleucine
LH	lutenizing hormone
leu	leucine
lys	lysine
met	methionine
mRNA	messenger RNA
MSH	melanocyte stimulating hormone
n.m.r.	nuclear magnetic resonance
P	phosphate
Pan	pancreatic Ribonuclease
Pase	alkaline phosphatase
phe	phenylalanine
p.m.r.	proton magnetic resonance
p.p.m.	parts per million
pro	proline
pyroglu	pyrolidone carboxylic acid (pyroglutamic acid)
R_f	ratio of distance travelled by sample to that of the solvent front
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA

GTP	guanosine triphosphate
his	histidine
I	inosine[a]
IF	initiation factor
ile	isoleucine
LH	lutenizing hormone
leu	leucine
lys	lysine
MES	morpholino-ethane sulfonate
met	methionine
mRNA	messenger RNA
MSH	melanocyte stimulating hormone
n.m.r.	nuclear magnetic resonance
p	phosphate
Pan	pancreatic ribonuclease
Pase	alkaline phosphatase
p.c.	paper chromatography
phe	phenylalanine
p.m.r.	proton magnetic resonance
p.p.m.	parts per million
pro	proline
pyroglu	pyrolidone carboxylic acid (pyroglutamic acid)
R_f	ratio of distance travelled by sample to that of the solvent front
RNA	ribonucleic acid

rRNA	ribosomal RNA
R_{Up}	ratio of R_f of sample to that of Up
ser	serine
sRNA	soluble RNA (= tRNA)
SVD	snake venom phosphodiesterase
thr	threonine
t.l.c.	thin layer chromatography
Tm	melting temperature (midpoint of thermal denaturation curve)
TMS	tetramethylsilane
TPS	2,4,6-triisopropylbenzenesulfonylchloride
Trac	triphenylmethoxyacetyl[b]
tRNA	transfer RNA
trp	tryptophan
tyr	tyrosine
U	uridine[a]
u.v.	ultraviolet
val	valine

---

a. Unprotected mono- and oligonucleotides are abbreviated in the standard format (IUPAC-IUB Commission on Biochemical Nomenclature, 1970): eg. pA represents 5'-adenylic acid, Up represents 3'-uridylic acid and ApU represents adenylyl-(3'-5')-uridine.

b. In order to condense the complex names and formulae of protected nucleosides and oligonucleotides into a more clearly readable form, the following system of abbreviations has been used. Nucleosides and protecting groups are presented as recommended (IUPAC-IUB Commission

on Biochemical Nomenclature, 1970), Trac represents the triphenylmethoxyacetyl group, and a hyphen represents an internal or terminal 2,2,2-trichloroethyl phosphate group. 5'-substituents are placed to the left of a nucleoside symbol, 2'- and 3'-substituents are placed to the right in succession, and amino substituents as superscripts. Thus Trac A^{bz}-UtOH would represent N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenylyl-[3'-(2,2,2-trichloroethyl)-5']-2'-O-tetrahydropyranyluridine.

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1. INTRODUCTION

The primary objective of this thesis is to demonstrate how chemical synthesis of oligoribonucleotides may be used in the investigation of two ribonucleic acid molecules involved in protein biosynthesis. Synthesis of a sequence, designed to imitate the function of a natural messenger RNA, is proposed which would provide a basis for studying structural features of messenger RNA responsible for initiation or termination of protein synthesis. Utilization of the specific interaction of transfer RNA with a synthetic tetranucleotide is proposed as a highly selective method of purification for transfer RNA species.

Structural features of RNA molecules and their interactions in protein biosynthesis as they are currently understood are presented in historical context, chemical synthesis of oligonucleotides is reviewed, and affinity chromatography of nucleic acids is described. These foundational reviews provide a framework for interpretation of the results obtained and discussion of the significance of this research.

1.1. Historical Development of Some Important Concepts

Prior to the discovery that deoxyribonucleic acid was the genetic material (Avery, McLeod, and McCarty, 1944), it was believed that the wide variety of structural and functional capabilities observed in proteins made them the logical candidate for the genetic material. The reversal brought about by this discovery posed anew the question

of how genetic material is duplicated and required reinterpretation of the "one gene - one enzyme" hypothesis of Beadle and Tatum (1941; and Beadle, 1945) in the form of a second question: How is genetic information transferred from DNA to protein?

Chargaff's observations (1950, 1951a,b) of the one to one ratio of adenine to thymine and cytosine to guanine in the DNA of many species and the evidence of helical structure from X-ray diffraction by DNA (Wilkins, Stokes and Wilson, 1953; Franklin and Gosling, 1953) allowed Watson and Crick (1953a,b), on the basis of the hydrogen bonding possibilities of the keto and amino forms of the four bases, to propose a working model for DNA which offerred a clear answer to the first question but gave no clue to the second.

In this model, the 2'-deoxyribose portion of each nucleotide lies almost parallel to the helix axis and is joined by 3'-5' phosphate diesters (Brown and Todd, 1952) in two long chains wound around each other in a regular fashion. The four bases lie perpendicular to the helix axis, each attached to the C-1 of a deoxyribose, and hydrogen bonded to the complementary base on the strand opposite. The structure is diagrammed in figures 1 and 2. It is known as the B form of the helix.

The two strands are complementary and antiparallel and it is proposed that the total information content is contained in the sequence of bases of either strand (Dounce, 1952). Duplication of the information within this structure is accomplished enzymatically by unwinding the strands and forming the complementary sequences by using each half as a direct template for polymerization of the appropriate monomeric units to form the complementary halves (Meselson and Stahl, 1958).

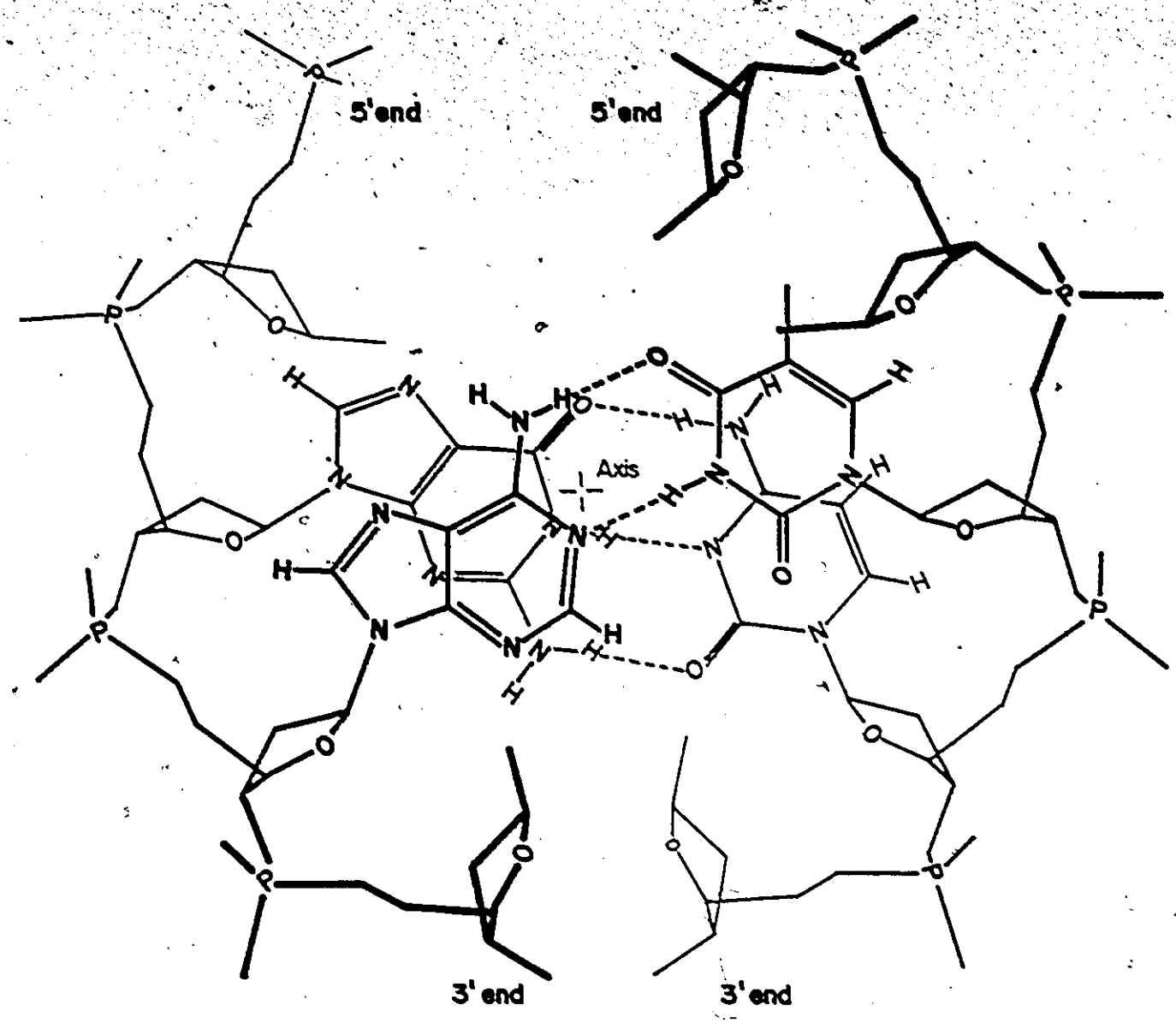

Figure 1: DNA double helix: B form, view along helix axis showing two base pairs; A-T pair above and G-C pair below.

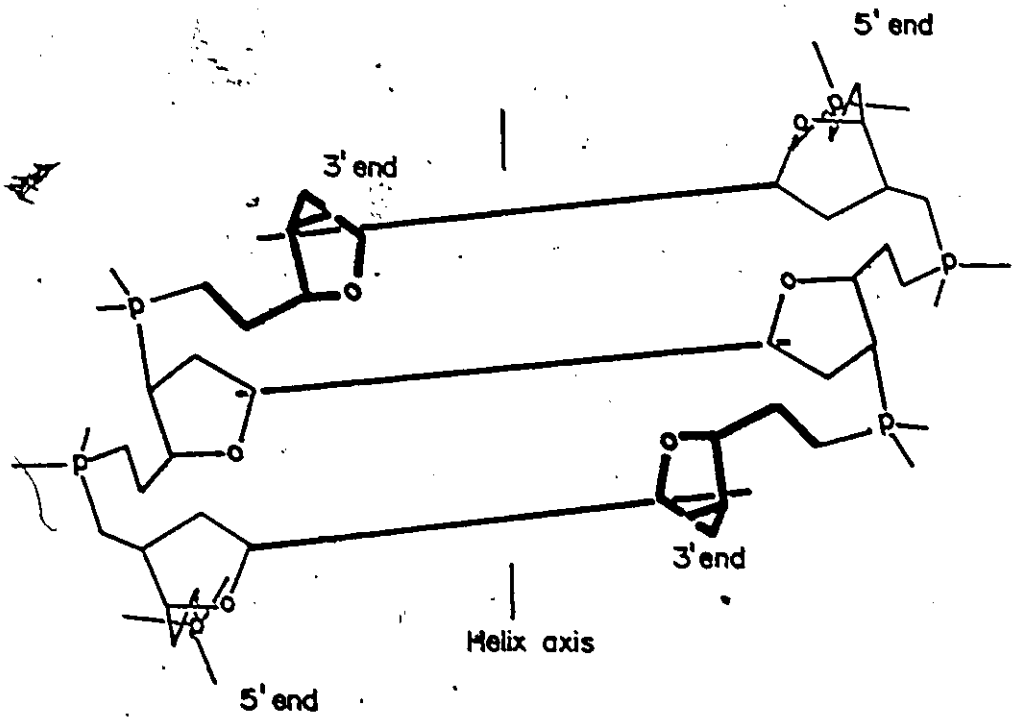

Figure 2: DNA double helix: B form, view perpendicular to helix axis.

It could not be seen how the same template could specify sequences of amino acids in proteins. Even with the growing body of evidence (see Crick, 1958; and Watson, 1963 for reviews) that RNA was involved in protein biosynthesis, the mechanism for coding amino acid sequences by nucleic acid sequences was still not apparent.

Hoagland et al. (1958) are credited with the discovery of a low molecular weight species of RNA, which came to be called transfer RNA (tRNA), to which amino acids became attached after activation and before incorporation into protein. Crick (1958) explained the requirement for an RNA template [or messenger RNA as it was subsequently named by Jacob and Monod (1961)] and a low molecular weight species of RNA to carry amino acids and align them in the correct sequence by reading or translating the genetic code, expressed as the order of nucleotides in the messenger. The adapter hypothesis, as this explanation is called, is dependent on the ability of a sequence of RNA to form a structure analogous to the DNA double helix by base pairing with a complementary sequence*. The ability of part of the sRNA molecule to form such a complementary structure with mRNA provides the mechanism by which the code is read. The sequential linkage of adjacent amino acids carried by sRNA molecules as they read the messenger is carried out in association

* The uracil base found in RNA is functionally equivalent to the thymine base found in DNA but the ribose sugar is not entirely analogous to the 2'-deoxyribose. In a helix containing ribose sugars, the conformation of the sugar is C-3 endo rather than C-3 exo, the helix is elongated, and the bases are tilted from the perpendicular to the helix axis (Arnott and Hukins, 1973) giving the A form of the helix. The base-pairing remains otherwise the same.

with ribonucleoprotein particles called ribosomes. This process will be discussed in a later section.

Crick (1958) expected the adaptor to be as small as a tri- or tetranucleotide but Hoagland's sRNA (now called transfer RNA or tRNA) with molecular weight of approximately 25,000 fulfills this role.

The RNA transcribed from DNA to serve as a template for protein synthesis was at first believed to be the ribosomal RNA but its uniform size and base composition made this belief untenable. The unstable RNA originally observed in virally infected cells and later in uninfected cells (Hershey et al., 1953; Volkin and Astrachan, 1956) met the criteria for a direct template for protein synthesis. The demonstration of synthesis of polyphenylalanine with a cell free protein synthesis system dependent on added synthetic polyuridylic acid (Nirenberg and Matthei, 1961) showed clearly the template ability of RNA and was the first step in cracking the genetic code.

Thus the flow of information in biological systems was understood: from DNA to DNA (generation to generation) and from DNA to RNA (genes to site of protein synthesis) and from RNA to protein (messenger template to final gene product). This is regarded as the Central Dogma of molecular biology. In this scheme of information transfer from gene to protein, three classes of ribonucleic acid participate: tRNA, the adaptor; mRNA, the carrier; and rRNA, a structural component of ribosomes. In order to understand fully the roles of these molecules in protein biosynthesis and its control it is necessary to learn about their structures and to learn how these structures fulfil their roles.

1.2. Messenger RNA and the Genetic Code

After slow recognition of the concept of mRNA and eventual proof of its existence, the attack was focused on the nature of the genetic code. Lack of suitable methods of purification and analysis precluded direct study of natural messengers but the successful demonstration by Nirenberg and Matthei (1961) of the use of a synthetic polyuridylic acid template for protein synthesis offered a solution to the problem.

Many investigators contributed to solving the genetic code using synthetic ribonucleotide polymers having random sequences of known composition, or perfectly alternating sequences of two, three, and four bases (Matthei et al., 1966; Nirenberg et al., 1966; Khorana et al., 1966; Nirenberg et al., 1963; Speyer et al., 1963; Thatch et al., 1966; Wahba et al., 1966; and references therein). The results of these experiments clearly indicated that the code was a nonoverlapping triplet code: each triplet of nucleotides in the messenger directed incorporation of a single amino acid into a polypeptide.

For example, an alternating polymer of uridine and cytidine when translated would give an alternating polymer of the amino acids serine and leucine, indicating that codons for these two amino acids were UCU and CUC but not indicating which codon corresponded to which amino acid. These codon assignments could be determined using random polymers of uridine and cytidine having different compositions where the frequency of occurence of each triplet would differ.

It could also be seen for most amino acids that there must be several codons which would code for a given amino acid on the basis of

codon frequency and base composition of the polymers.

The key to the final codon assignments came with the discovery of Nirenberg and Leder (1964) that binding of tRNA to ribosomes could be stimulated by tri- and larger nucleotides. In this way coding properties of each of the 64 triplets were determined.

The genetic code as it was finally established by these methods is shown in table 1. This code has been widely confirmed and found to be common to all species thus far examined.

Confirmation of the code which did not depend on synthetic polynucleotides came from examination of sequences of proteins coded by genes with frameshift and point mutations (Streisinger et al., 1966; Wittmann and Wittmann-Liebold, 1963; Yanofsky, 1963).

The frameshift mutation is so called because it results in reading of codons out of phase with the normal codons as the messenger is read in the 5'-terminal to 3'-terminal direction (Wahba et al., 1966). The new codons then each consist of two bases from one in-phase codon and one base from the adjacent in-phase codon. The resultant protein then differs drastically from the normal from that point onward in the amino terminus to carboxy terminus addition of each amino acid or until another frameshift corrects the reading frame (Biship, Leahey and Schweet, 1960; Dintzis, 1961; Goldstein and Brown, 1961). Amino acid sequence analysis of normal and mutant proteins allows a unique reconstruction of the nucleotide sequence on the basis of known codon assignments which is totally consistent with these assignments. In fact, the two possible shifts (+1 and +2 or + and -) give redundant confirmation.

Table 1

The genetic code showing the amino acids coded by each of the 64 triplets of nucleotides. The first, or 5'-proximal nucleotide is shown in the left column, the second in the top row, and the third on the right.

1st ↓ / 2nd →	U	C	A	G	3rd ↓
U	phe	ser	tyr	cys	U
	phe	ser	tyr	cys	C
	leu	ser	Ochre	Opal	A
	leu	ser	Amber	trp	G
C	leu	pro	his	arg	U
	leu	pro	his	arg	C
	leu	pro	gln	arg	A
	leu	pro	gln	arg	G
A	ile	thr	asn	ser	U
	ile	thr	asn	ser	C
	ile	thr	lys	arg	A
	met	thr	lys	arg	G
G	val	ala	asp	gly	U
	val	ala	asp	gly	C
	val	ala	glu	gly	A
	val	ala	glu	gly	G

Point mutations are changes in a single base resulting in a change of a single amino acid in the gene product. Since three bases are present in each codon, two will remain unchanged; thus limiting possible changes in the amino acid coded. The changes in amino acids observed were those expected from the presumed codon and mode of action of the mutagen applied. For example, a change of leucine to phenylalanine in a protein after treatment of cells with nitrous acid may be explained by the deamination of cytidine by nitrous acid, resulting in adenosine being incorporated at this point in the opposite strand of DNA in subsequent generations, and consequently thymidine in the position of the original cytidine. If the messenger is now transcribed from this opposite strand then uridine will be incorporated at the site where cytidine was formerly incorporated, presumably changing the codon from UCU coding for leucine to UUU coding for phenylalanine.

Some mutations resulted in production of polypeptides containing only the amino terminal portion of a known protein. The point of termination could always be attributed to codon changes from coding triplets to one of the noncoding triplets: amber, ochre, and nonsense or opal. Apparently, these three codons served as periods in the punctuation of the genetic code (Ganoza, 1966).

Studies on natural proteins (Waller, 1963) indicated that methionine was often found as the amino terminal residue. Since some initiating signal to establish the correct reading frame would seem necessary, the methionine codon AUG was regarded as a likely candidate (Thatch, 1966). Synthetic messengers beginning with a methionine codon were initiated more readily than any others and at

lower magnesium concentrations, which also increased the fidelity of translation.

The simplistic view of a messenger RNA consisting of a single strand of RNA beginning with the sequence AUG, ending with a termination triplet and containing 3 X n (where n is a small integer) nucleotides in between to code for a polypeptide is not adequate to describe those messengers which have been isolated and studied. Many partial sequences of viral genomes, which serve also as messengers, and cellular messengers, are now available (Brawerman, 1974). These data show no initiator codon at the 5'-ends nor termination codons at the 3'-ends. In multicistronic messengers such as the MS2 genome (Min-Jou et al., 1972) the termination codon of one message is separated from the initiator of the following message by a sequence whose presence and function are not understood (Jukes and Gatlin, 1971).

In most eukaryotic mRNA (with the exception of histone message (Adesnik and Darnell, 1972; Greenberg and Perry, 1972) the 3'-end of the molecule consists of polyadenylic acid (Molloy et al., 1972; Sheldon et al., 1972), the function of which is still a hotly debated issue (for review see Brawerman, 1974). This poly-A tail has allowed messenger RNA to be isolated by oligo-dT cellulose affinity chromatography (e.g. Edmonds, Vaugn and Nakazoto, 1971).

Although mRNA is single stranded, most of the observed primary sequences have numerous potentially complementary regions. One very striking feature which has appeared in several messages surrounds the initiation codon: a hairpin loop with the initiator codon centered at the end of the loop in the unpaired region, as shown in figure 3. It

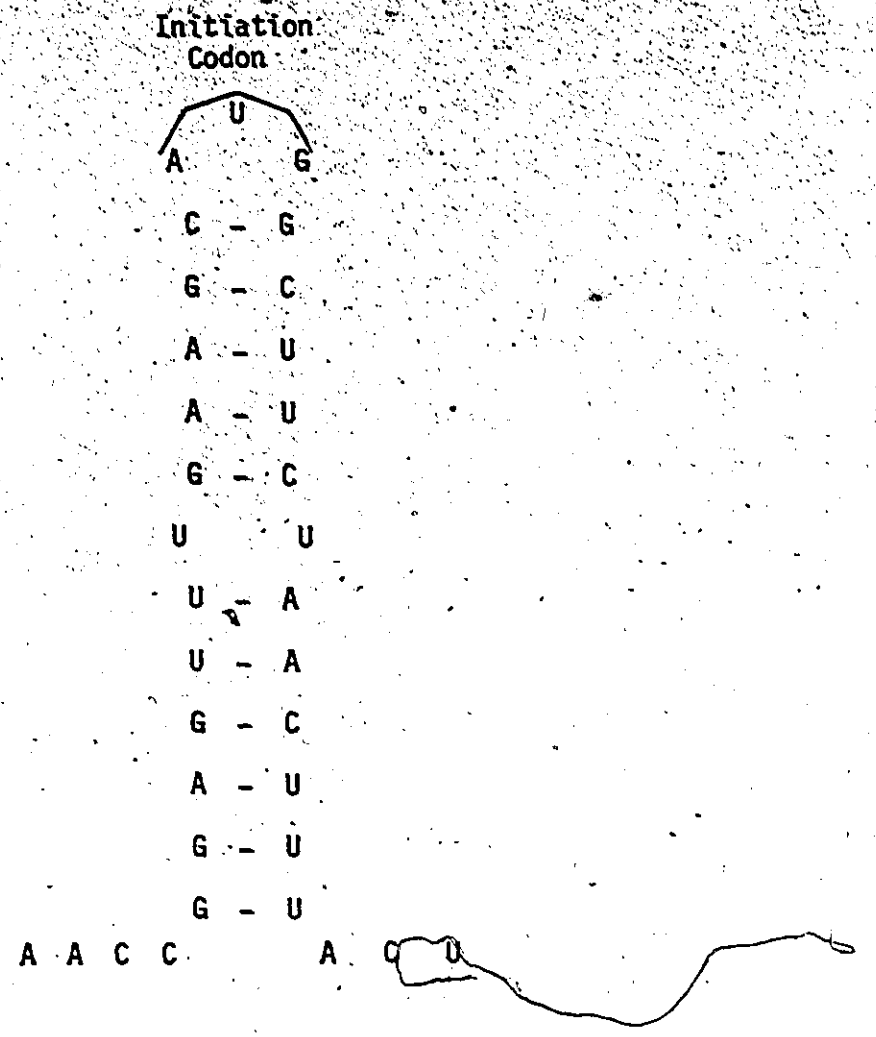

Figure 3: Initiation codon and hairpin loop of MS2 coat protein gene. (Min-Jou et al., 1972).

is obvious that since initiator codons do not appear at the beginning of the messenger, some mechanism is required to assure their recognition for the initiation process to occur. The mechanism of this recognition and features required in the messenger for recognition are not understood.

The ability of mRNA to perform its task of carrying genetic information from genes to the site of protein synthesis is due to a specific code or relationship between the sequence of nucleotides, taken by threes, and the amino acids appearing in the polypeptide. Synthetic sequences played a crucial role in establishing the genetic code and in the discovery of its punctuation: the initiation and termination triplets. Finally, there is much more to a real messenger than its coding portion but the function of these additional sequences and secondary structure is not yet understood.

1.3. Transfer RNA and Protein Biosynthesis

The role of tRNA as an adaptor dictates two structural features common to all tRNAs. The amino acid acceptor site and the decoding or anticodon site are found in a matrix of several other features, both common and unique.

Amino acids are attached to the 3' terminal nucleoside by the amino acid:tRNA ligases (E.C. 6.1.1....), often called "synthetases". Each of these enzymes is capable of recognizing the 3' terminal sequence of tRNA: CpCpA; and some as yet unknown features of tRNA to select the correct tRNA molecule. Although the enzyme binds to the anticodon

(Dube, 1973), the anticodon is not the recognition site. The amino acid is then esterified to the 2'- or 3'- hydroxyl of the terminal adenosine residue with release of pyrophosphate from ATP.

The specific base sequence of the anticodon can then pair with the codon of mRNA in association with ribosomes in order to align that amino acid for incorporation into the growing polypeptide chain. Anticodons are actually found near the midpoint of sequences of 75-90 nucleotides comprising tRNA molecules.

The sequences of all tRNAs so far determined can be folded to allow pairing of complementary sequences, giving the cloverleaf shape shown in figure 4. This folding always leaves the anticodon triplet exposed in the unpaired portion of loop II. This schematic representation is not adequate to describe the three dimensional structure as each of the double stranded regions is helical and the "arms" are apparently not extended. Figure 5 shows the three dimensional structure of phenylalanine tRNA obtained from X-ray crystallography(Rich, 1974). Even in this form, it is certain that the anticodon region is exposed and accessible.

The anticodon loop exhibits flexibility even though constrained at the stem end. Fuller and Hodgson (1967) proposed that the bases of the anticodon and two bases on the 3' side of these would be stacked in the same helix as the stem even though not base paired. Ghosh and Ghosh (1970) showed that the bases on the 5'-side and the anticodon may stack in an extension of the stem helix also but on the opposite side of the helix when the Y base adjacent to the anticodon in phenyl-

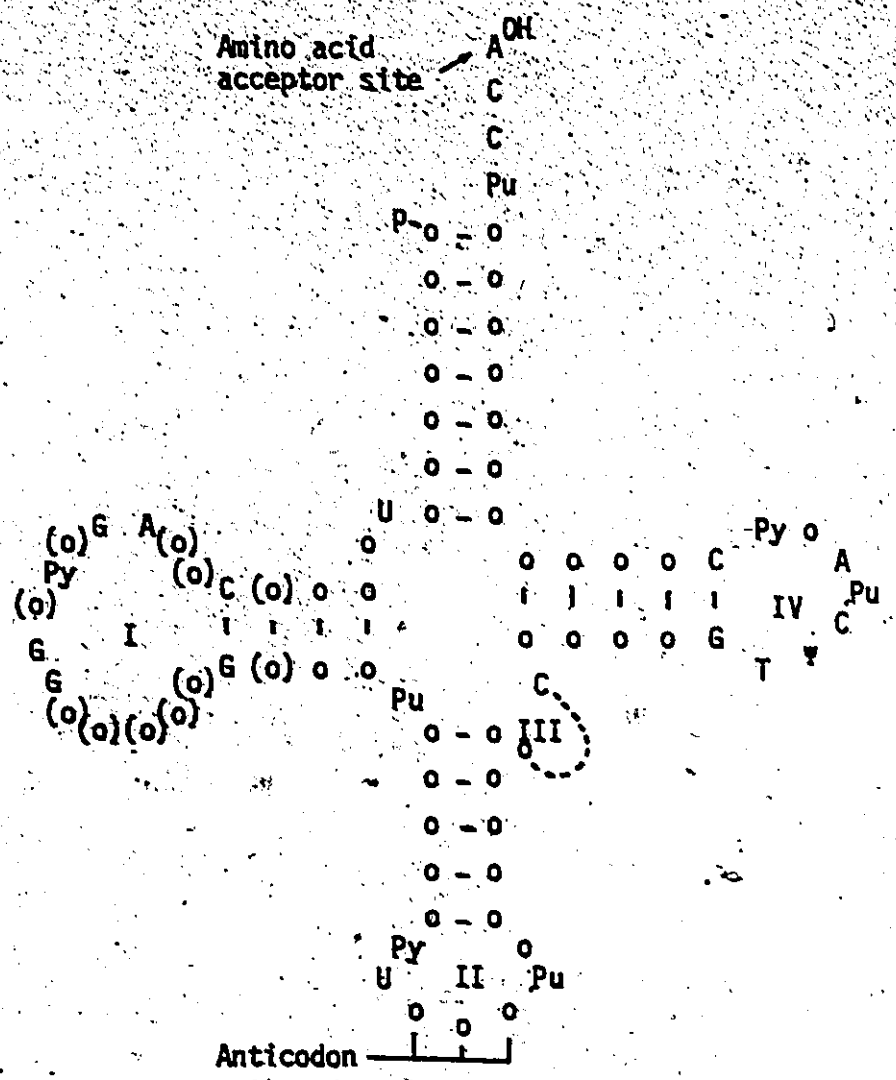

Figure 4: General secondary structure of tRNA: the cloverleaf model showing features common to most tRNA molecules. Nucleotides specified are constant (Pu = purine, Py = pyrimidine , o = variable)and (o) or ---- are optional.

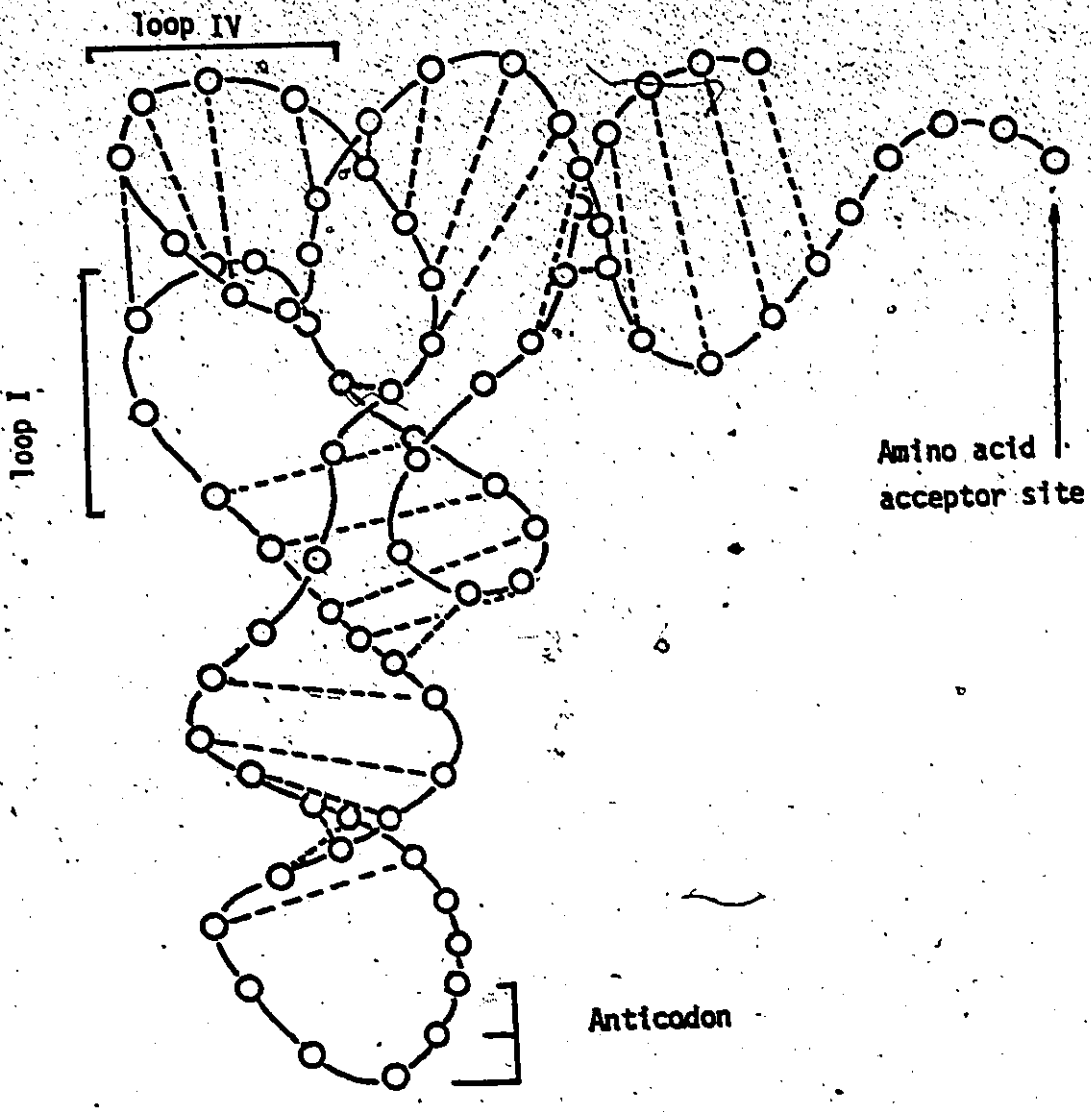

Figure 5. Phenylalanine tRNA tertiary structure.

alanine tRNA is altered. This conclusion is also supported by the experiment of Uhlenbeck et al. (1970) which showed that tetranucleotides complementary to the anticodon and 5'-adjacent base would bind specifically to tRNA.

Flexibility in the anticodon region also allows the recognition of several codons by one tRNA species. The mechanism of "wobble" pairing proposed by Crick (1966) encompasses the observed degeneracy in the third position of codons. Thus very minor alterations in the conformation of anticodons at the nucleotide opposite the third base of the anticodon allow recognition by G of both U and C, by U of A and G, and by I (inosine) of A, U and C. The efficiency of recognition of various codons may be controlled by modifications of nucleotides in the anticodon, or adjacent to it (see Sen, 1974 for review).

How the structure of the initiator tRNA allows it to perform its role is not obvious. There is usually more than one tRNA species coding for methionine, one of which initiates protein synthesis by recognising the codons AUG or GUG, while the other(s) respond only to the AUG codon at internal points in the coding portion of the messenger. The methionine amino group of the charged initiating species can be formylated enzymatically, while that of other species can not, but this modification is neither necessary nor sufficient to allow a tRNA to initiate protein synthesis.

How is protein synthesis initiated? How is it continued and terminated? The main features of these well reviewed subjects (Lengyel and Soll, 1969; Lucas-Lenard and Lipman, 1971; Haselkorn and Rothman-Denes, 1973) are summarized here.

The ribonucleoprotein particles which constitute the protein synthetic apparatus in living cells are composed of stoichiometric proportions of each of three species of RNA and several dozen different protein subunits. These subunits are organized in a specific three-dimensional arrangement and the entire molecular assembly is characterized by a sedimentation value of approximately 70S for those of prokaryotes and 80S for those of eukaryotes.

Each 70S units consists of two subunits, one of 30S and one of 50S. The smaller unit binds mRNA in the presence of a small protein, Initiation Factor 3*. This complex is enlarged by the addition of Initiation Factor 2, GTP and fmet-tRNA. Addition of Initiation Factor 1 and a second molecule of GTP is prerequisite to stable binding of the 50S subunit as it allows the release of IF-1-GDP complex formed in tRNA binding and GDP formed by hydrolysis of the second GTP. The second GTP hydrolysis apparently reflects movement of the fmet-tRNA from the "A" site on the 50S subunit to the "P" site, and concurrent realignment of the initiator codon of the messenger RNA with the P site, and the new codon with the A site. Now the ribosome is loaded and primed for elongation of the "peptide" formylmethionine.

Elongation is accomplished by binding of aminoacyl tRNA to the codon at a site with the aid of Elongation Factor T and GTP. The

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* More recent data suggested that mRNA binding may occur to the 70S complex in the presence of IF-3 (Hawley et al., 1974).

arrangement of these binding sites allows the aminoacyl ends of the two tRNAs to come close together where a protein subunit of the 50S subunit catalyzes displacement of the peptide from the tRNA in the P site by the amino group of the amino acyl-tRNA in the A site. This has the effect of transferring the peptide chain from the tRNA in the P site to the one in the A site. After removal of the discharged tRNA from the P site, GTP hydrolysis is coupled to translocation of the message and peptidyl tRNA to the P site exactly as in the last stage of initiation.

When a termination codon appears in the A site, it is recognized by a protein, Termination Factor R_1 or R_2, which activates the peptidyl transferase, resulting in hydrolysis of the peptidyl-tRNA linkage. Dissociation of the two ribosomal subunits and remaining tRNA and factors follows but its mechanism is not understood.

Protein synthesis is a remarkable, dynamic assembly of protein factors, ribosomal proteins, three rRNA species, mRNA and tRNA, each having a different function, and driven by the familiar energy provided by ATP and GTP hydrolysis. How can we understand the interaction of these many parts? Remarkable progress was made by direct examination of these parts, isolated and purified from natural sources, but therein lay the limitation of this method of investigation: progress was dependent on the ability of investigators to isolate, purify and characterize natural products.

The rich strike of synthetic oligo- and polynucleotides opened a new mine of alternatives for study of the genetic code and its implementation. Now newer methods of synthesis have opened new veins for exploitation.

1.4. Chemical Synthesis of Oligonucleotides

1.4.1. Principles and Requirements

The natural nucleic acids are linear polymers of 2' deoxyribo- or ribofuranosides of four heterocyclic bases: adenine, guanine, cytosine, and uracil (thymine in DNA), as shown in figure 6, and are often found as a double helix with a complementary nucleotide polymer. The specific 3' - 5' phosphodiester linkages in these polymers is necessary for both structural and functional requirements. The objective of forming these linkages selectively is more easily stated than accomplished though, because nucleotides may contain as many as five potentially reactive sites: 2',3', and 5' hydroxyl groups, aromatic amino group, and phosphate groups.

Enzymes, using preformed nucleoside di- or triphosphates, can catalyze the formation of 3' - 5' linkages even in the presence of these other reactive sites. In the presence of a pre-existing template, a complementary sequence is synthesized and in the absence of a template, a random sequence is produced. Chemists envy the ease with which enzymes selectively catalyze the formation of these linkages but wish to specify or create, as well as copy, nucleotides sequences.

Two problems are apparent here: the ability to select or determine the sequence of bases and the ability to form 3' - 5' phosphodiesters selectively in the presence of other potentially reactive sites. The use of blocking groups or protecting groups (the latter term will be used throughout this work to avoid confusion with the term "block" which will refer to a short sequence of nucleotides) to mask the

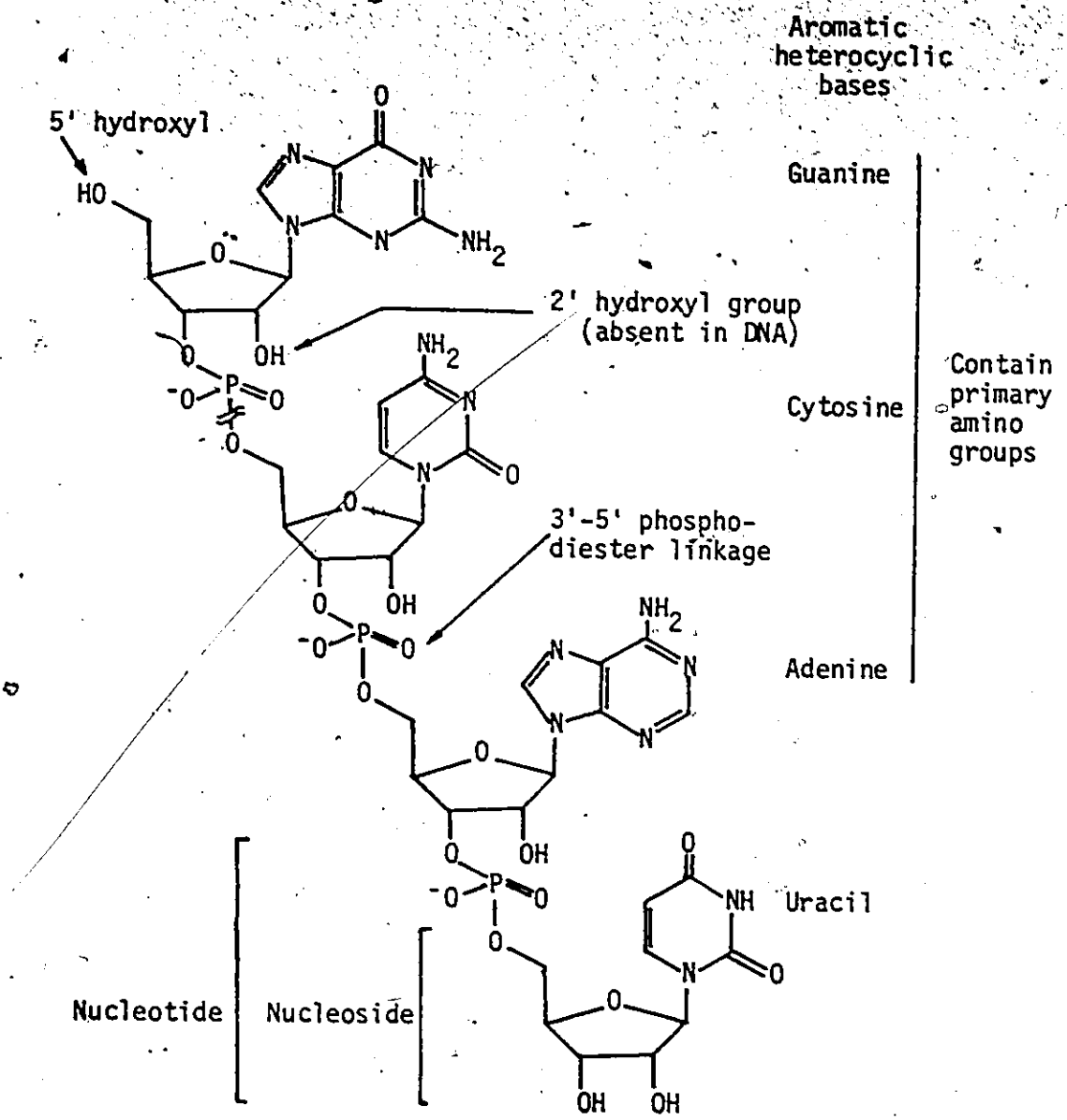

Figure 6. Features of oligoribonucleotide primary structure.

reactive sites not involved in the desired esterification of a phosphate group, is applicable to both problems.

These protecting groups must be removable under sufficiently mild conditions that the desired sequence can be generated in the "native" form. Use of protecting groups which are selectively removable in the presence of other protecting groups would permit further reactions at these selected sites; such as the 3' or 5' hydroxyl, or terminal phosphate moieties. Coupling of units with only one unprotected reactive site followed by selective deprotection at a new site; or use of a two step coupling procedure allow creation of unique sequences by repetition of these steps with appropriate monomeric or oligomeric units. Selection of a suitable strategy for assembly of a sequence will determine the tactics required for obtaining suitably protected monomeric or oligomeric units.

Since quantitative chemical reactions are rare, and even small deviations from this ideal will result in appreciable quantities of side products after several steps, suitable methods of purification of the desired products are required. The characteristics of protecting groups and the type of side reactions (and therefore side products) occurring will determine which methods can be used and which will be effective.

Oligonucleotide synthesis by chemical methods is dependent on strategic selection of a synthetic route and selection of protecting groups whose incorporation and removal are compatible with the chemistry of coupling nucleosides by phosphate esters. Purification methods must be selected by considering properties of the protecting groups used and the side products expected. In the following sections, some of the

chemistry and characteristics of protecting groups which have been used in synthesis of oligonucleotides, methods and reagents for formation of phosphate esters, strategies for assembly of protected nucleotides, and finally, purification procedures for protected and deprotected sequences will be discussed. Particular emphasis is placed on discussion of methods which were used in this work.

1.4.2. Protecting Groups for Oligonucleotide Synthesis

Many protecting groups have been used to protect two or more of the five* reactive sites in various synthetic schemes. For this reason protecting groups will be discussed with respect to three functional categories: the phosphate, protected throughout synthesis in the triester method or transiently during addition and selective deprotection of one structural unit by either the di- or triester methods; the primary amino function, protected throughout synthesis; and finally, the sugar hydroxyl functions. This last category, although it provides no explicit distinction between different hydroxyl groups, will be discussed by indicating the sites at which each protecting groups has been used.

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* The additional reactive sites of heterocyclic bases such as N-1 of adenine and N-7 of guanine have not been found to require protection although some small proportion of side reactions may occur at these sites.

1.4.2.1. Phosphate Protecting Groups

Four principle classes of phosphate protecting groups are represented in the alphabetical listing in table 2. These are the phosphoramidates and phosphorothioates, convertible to phosphates by treatment with isoamyl nitrite or aqueous iodine respectively; substituted aromatic alcohols, removed by basic hydrolysis (except for 18); substituted ethanols, removed by β-elimination mechanisms; and a heterogeneous class including the benzyl, benzhydryl, t-butyl, and cyclic protecting/activating groups not listed in the table.

Ikehara has established the phosphoramidates (2 and 3) (Ohtsuka et al., 1970) as a flexible and promising tool in nucleotide synthesis as a terminal phosphate protecting group removable under mild neutral conditions, as a suitable linkage for polymer-bound synthesis and as a potential activation method for synthesis of phosphodiesters. The S-ethyl phosphorothioate (11) of Nussbaum and Tiberi (1965) is structurally and functionally very similar. It has been shown to be a similarly flexible protecting group (Cook et al., 1969a,b) stable under basic conditions, removable under mild neutral conditions and capable of being activated by mild oxidation with iodine for condensation with other reagents to give esters and pyrophosphates.

The uses of various substituted phenyl protecting groups, removable under a range of alkaline conditions, have been demonstrated by Reese and co-workers, Narang and co-workers and Ikehara and co-workers. (For example, see Cusack, Reese and van Boom, 1973). Some have been used simply as the triester protecting group preventing pyrophosphate formation, chain cleavage, or esterification at the internucleoside phosphate sites while others have also been used to aid

Table 2.

Posphate protecting groups for oligonucleotide synthesis

	Group	Reagent or Activator	Removal Conditions	Reference
1.	2-acetyl-2-methyl-ethyl	DCC	7 N NH_4OH, 30 min, r.t.	Soll and Khorana, 1965
2.	Anilidate	DCC/aniline	Aq. isoamyl nitrite	Ohtsuka *et al.*, 1970
3.	Anisidate	DCC/anisidine	Aq. isoamyl nitrite	Ohtsuka *et al.*, 1970
4.	Benzaldoxime	H^+	2 N NaOH, 8 h, r.t.	Narang *et al.*, 1970a
5.	Benzhydryl	Diphenyldiazomethane	80% Acetic acid, 15 h, r.t.	Cramer and Scheit 1962
6.	Benzyl (diester)	Dibenzylchloro-phosphate	Pd/H_2	Atherton *et al.*, 1945
	(triester)	Phenyldiazomethane	NaI/acetonitrile, 80°	Scheit, 1967
7.	t-Butyl	Trichloroacetonitrile	20% Acetic acid, 45 min, 60°	Cramer *et al.*, 1962b
8.	benzylhydracrylamide	DCC	2 N NaOH, 6 h, r.t.	Narang *et al.*, 1970a
9.	O-Chlorophenyl	TPS/chlorophenyl-phosphate	0.1 N NaOH, 3h, r.t.	Reese and Saffhill, 1968
10.	4-Chloro-2-nitrophenyl	DCC	2 N NaOH, 15 min, 100°	Narang *et al.*, 1970b
11.	Cyanoethyl (diester)	DCC/cyanoethylphos-phate or cyanoethanol	7 N NH_4OH, 18 h, r.t. or 0.1 N NaOH, 5 min, 50°	Tener, 1961
	(triester)			Letsinger and Ogilvie, 1969

Table 2 (continued)

Group	Reagent or Activator	Removal Conditions	Reference
12. S-ethylphosphorothioate	DCC/S-ethylphosphorothioate	Aq. I_2, 4 h, r.t.	Nussbaum and Tiberi, 1965
13. 1-methyl-2-acetyl-ethyl	DCC	7 N NH_4OH, 30 min, r.t.	Soll and Khorana, 1965
14. Phenyl	Phenylphosphorodichloridate	0.1 N NaOH, 5 min, r.t.	Reese and Saffhill, 1968
15. 2-Phenylmercaptoethyl	DCC	Na metapriodate, 2 N NaOH	Narang *et al.*, 1972
16. 2-(α-pyridyl)ethyl	DCC	2 M NaOMe, 48 h, 0°	Freist *et al.*, 1970
17. Trichloroethyl	DCC, or TPS/trichloroethanol or trichloroethylphosphate	Zn-Cu in DMF, 2h, 50°	Eckstein, 1967a
18. Tritylaminophenyl	DCC	Aq. I_2	Ohtsuka *et al.*, 1973

purification by providing an aromatic, non-polar binding site at a terminal phosphate for column chromatography or solvent extraction. The removal of tritylaminophenyl groups by aqueous iodine treatment is unique in this class.

The most extensively used phosphate protecting groups have been the cyanoethyl group of Tener (1961) and the trichloroethyl group of Eckstein (1967a,b). The cyanoethyl group can be removed by mild alkali treatment and has been used successsfully in many applications to di- and triester methods of nucleotide synthesis.

The sensitivity of this protecting group to alkali has enabled its use as a terminal phosphate protecting group during addition of one mononucleotide unit. Subsequent removal of the cyanoethyl group can then be accomplished selectively to allow activation of the phosphate for addition of the next protected mononucleotide. The group is readily introduced by reaction of cyanoethanol with a suitably activated phosphate at the orthophosphate, monoester, or diester level. Addition to a phosphodiester yields a triester which no longer carries a charge. This principle feature of the triester method greatly increases the solubility of oligonucleotides in non-polar solvents aiding both reaction and purification as attested in later discussion of the phosphotriester method itself.

The trichloroethyl group is similar: its ability to be removed selectively allows its application to both di- and triester methods of synthesis. Its introduction can be accomplished at suitably activated phosphate groups bearing zero, one or two esters also. Greater base

stability is the major feature distinguishing it from the cyanoethyl group. While this group can be removed under alkaline conditions, the preferred and selective method of cleavage is reductive β-elimination under one of several conditions: active zinc-copper couple in DMF, or zinc dust in 80% acetic acid or pyridine-acetic acid. (Eckstein 1967a). The two latter conditions were considered too drastic; the zinc in acetic acid procedure was found to cause modification of N-benzoyl cytosine (Cook et al., 1969a). However, neutral conditions with zinc-copper couple have been used successfully for deprotection of oligonucleotides synthesized by the triester method by several authors. (e.g. Neilson, 1969; Catlin and Cramer, 1973). The simplicity and success of application of the trichloroethyl group to both deoxyribo- and ribonucleotide synthesis and the experience available in this laboratory made this group the one of choice for the proposed synthesis. The remaining phosphate protecting groups in table 2 which have not been discussed and the cyclic activation/protecting groups proposed by Ramirez (see Ramirez and Ugi, 1974) have not been widely used or have shown unacceptable disadvantages in the procedures used to remove them.

1.4.2.2. Amino Protecting Groups

Protection of the primary amino function of the heterocyclic bases is a much simpler story. All but one of the amino protecting groups (4) listed in table 3 are fairly common carboxylates which can be introduced by full acylation of a free or partially protected nucleo-

Table 3.

Amino protecting groups for oligonucleotide synthesis.

	Group	Reagent	Removal	Reference
1.	Acetyl	Chloride, anhydride	OH^-	Gilham and Khorana, 1959
2.	p-Anisoyl	Chloride, anhydride	OH^-	Khorana *et al.*, 1961
3.	Benzoyl	Chloride, anhydride	OH^-	Khorana *et al.*, 1961
4.	Dimethylamino-methylene	DMF-dimethylacetal/H^+	H^+,OH^-	Zemlicka *et al.*, 1966
5.	Isobutyloxy carbonyl	Isobutylchloroformate	NH_4OH	Ogilvie and Letsinger, 1967
6.	Isobutyroyl	Chloride	OH^-	Kumar and Khorana, 1969
7.	Naphthoyl	Chloride	OH^-	Ralph *et al.*, 1963

side or nucleotide using the acid chloride or anhydride, followed by selective de-O-acylation and purification.

Since the lability of amide linkages under alkaline conditions are different for each of the heterocyclic bases (Ralph and Khorana, 1961; Werstiuk and Neilson, 1973) for any given protected nucleoside, defined conditions for removal of protecting groups are not listed in the table. Early work on oligonucleotide synthesis employed the acetyl group but its use was soon displaced by use of the more stable benzoyl and p-anisoyl groups. These are currently in widespread use.

Application of still larger aromatic groups such as the naphthoyl or dimethoxytrityl groups to protection of the N-2 position of guanine in order to increase the solubility of its protected derivatives was not successful as the bulk of these groups apparently interfered with reaction at the 5'-hydroxyl (Ralph et al., 1963). Introduction of the isobutyroyl group for protection of the guanine amino group (Kumar and Khorana, 1969) was probably for the same reason: this group offered less steric hindrance compared to the benzoyl group. With the greater stability of the guanine amides, greater lability of the acyl protecting groups was not a problem.

Treatment of nucleosides or oligonucleotides containing O-benzoylpropionyl groups with hydrazine in buffered pyridine (Letsinger, et al. 1967) was also found to remove the N-aroyl protecting groups. This advantage or disadvantage will be discussed with the O-protecting groups.

One further feature of the N-aroyl protecting groups is important. They impart a greater acid lability to the N-glycosidic linkage of purines. This detail must be included in the consideration of selectively re-

movable groups for other sites and in the logistics of complete deprotection.

Difficulties with acid and base lability were not anticipated as the benzoyl group has been chosen for work in this laboratory for reasons discussed in the next section, and the appropriately protected adenosine and cytidine residues were available (Neilson and Werstiuk, 1971a). These proved satisfactory in oligonucleotide synthesis (Neilson and Werstiuk, 1971b) as did the analogously protected guanosine residue later (Wastrodowski, 1972).

1.4.2.3. Hydroxyl Protecting Groups

The wide variety of hydroxyl protecting groups which have been tried is indicative of the vigor of the search for suitably labile and stable protecting groups which can be used compatibly with various synthetic strategies. The site at which a particular group will be useful is determined by two principle considerations. Selective insertion at one site would be an obvious advantage, but few reagents are sufficiently selective without additional help, such as the presence of other protecting groups, or suitable methods of isolating the desired position isomers. The second consideration is the set of conditions under which the group can be or will be removed. This consideration divides the field into four classes of protecting groups which are discussed: acid labile, base labile, catalytically removable, and stable. Since this is clearly inter-related with the whole scheme of synthesis to be planned, selection of protecting groups for particular hydroxyl groups

Table 4.

Hydroxyl protecting groups in oligonucleotide synthesis

Group	Site*	Reagent or Activator	Removal Conditions	Reference
1. Acetyl		Acid Chloride	OH^-	Gilham and Khorana, 1958
2. p-Anisoyl		Acid Chloride	OH^-	Khorana *et al.*, 1961
3. Benzoyl		Acid Chloride	OH^-	Khorana *et al.*, 1961
4. Benzoylformyl		Acid Chloride	Aq. pyridine/r.t. or OH^-	Letsinger and Ogilvie, 1969 Chladek and Smrt, 1964
5. β-Benzoylpropionyl	3'	DCC	Hydrazine/pyridine/Acetic Acid	Ogilvie, 1967
6. Benzyl	2'	Benzyl Chloride/KOH	Pd/H_2	Griffin *et al.*, 1966
7. Benzylidene	2'-3'	Benzaldehyde/ZnCl	Pd/H_2/20% Acetic Acid	Cramer *et al.*, 1964
8. t-Butyldimethylsilyl		Chloride/Imidazole	Tetrabutylammonium fluoride	Ogilvie, 1973
9. Chloroacetyl		Anhydride	2-mercaptoethylamine	Cook and Maichuk, 1970
10. Chlorophenoxyacetyl		Acid Chloride	OH^-	van Boom *et al.*, 1971

Table 4 (continued)

Group	Site*	Reagent or Activator	Removal Conditions	Reference
11. Cyclic carbonate	2'+3'	Ethyl Chloroformate	OH^-	Michelson, 1959
12. Cyclic Phosphate	2'+3'	Mononucleotide/DCC	H^+,OH^-	Gilham and Khorana, 1959
13. Dihydrocinnamoyl		Acid Chloride	Chymotrypsin	Sachdev and Starkovsky, 1969
14. 4-Dimethylamino-benzylidene	2'+3'	Aldehyde/H^+	OH^-,H^+	Cramer *et al.*, 1964
15. 2,4-dimethoxy-benzylidene	2'+3'	Aldehyde/H^+	OH^-,H^+	Cramer *et al.*, 1964
16. Dinitrophenyl-sulfenyl	3',5'	Acid Chloride	Thiophenol, Raney Nickel	Letsinger *et al.*, 1964
17. 1-Ethoxyethyl	2'	Ethylvinyl ether/H^+	H^+	Chladek and Smrt, 1964
18. Formyl		Trimethylorthoformate	OH^-	Griffin *et al.*, 1967
19. Methoxymethyl-idene	2'+3'	Trimethylorthofor-mate H^+	H^++ Formate; OH^-	Griffin *et al.*, 1967
20. Methoxyethyli-idene	2'+3'	Trimethylorthoace-tate H^+	H^++ Acetate; OH^-	Fromageot *et al.*, 1967
21. Methoxyacetyl	3'	Orthomethoxyacetate/ H^+	OH^-	Reese and Stewart, 1968

Table 4 (continued)

	Group	Site*	Reagent or Activator	Removal Conditions	Reference
22.	Methoxytetrahydropyranyl	2'	Methoxydihydropyran/H^+	H^+	Reese et al., 1970
23.	Methoxytetrahydrothiopyranyl	2',5'	Methoxydihydrothiopyran/H^+	H^+	van Boom et al., 1972
24.	di-Methoxytrityl	5'	Chloride	H^+	Smith et al., 1962
25.	p-Methoxytrityl	5'	Chloride	H^+	Smith et al., 1962
26.	Napthylisocynate	5',3'	Chloride	Stable	Agarwal et al., 1972
27.	p-Nitrophenylchloroformate	5',2',3'	Chloride	OH^-	Letsinger and Ogilvie, 1967
28.	Phenylisocynate	5',3'	Chloride	Stable	Agarwal et al., 1972
29.	Phenoxyacetyl	5'	Acid Chloride	OH^-	van Boom et al., 1971
30.	Pivaloyl		Acid Chloride	OH^-	Weimann and Khorana, 1962a
31.	Tetrahydropyranyl	2',5'	Dihydropyran/H^+	H^+	Smith and Khorana, 1959
32.	Tetrahydrathiophenyl	2',3'	Dihydrothiophene	$AgNO_3$	Cohen and Steele, 1966

Table 4 (continued)

Group	Site*	Reagent or Activator	Removal Condition	Reference
33. Trityl	5'	Chloride	H^+	Gilham and Khorana, 1959
34. Trityloxyacetyl	5'	TPS	$NH_4OH/MeOH$	Werstiuk and Neilson, 1972

* The site specified is the site at which the group has found utility. If no entry is made, then the group is presumed to have been used at any of the hydroxyl sites. No distinction has been made with respect to use in the ribo- or deoxyribonucleosides except where the group is bifunctional and used for both 2' and 3'. "+" is and, "," is or, ";" is followed by, and "/" is with.

will be discussed in this context also.

Acid labile protecting groups have been used at 2'-, 3'- and 5'-hydroxyl sites. Much of the early work on deoxyribonucleotide synthesis done by Khorana and his co-workers utilized the trityl group for protection of the 5'-hydroxyl. This was a natural extension of the use of this group, selective for primary hydroxyl groups, from its established place in carbohydrate chemistry. Conditions for removal of this group (80% acetic acid, 100°) allowed depurination of significant extent, especially with N-substituted nucleosides. This drawback was virtually absent in the more labile methoxytrityl and dimethoxytrityl groups subsequently introduced (Smith et al., 1962). These latter groups are still used extensively in deoxyribonucleotide work but to a lesser extent in work with ribonucleotides, as some migration of the phosphate from the 3' hydroxyl to the 2' under the acidic conditions of removal have been reported. (Rammler and Khorana, 1962).

The tetrahydropyranyl group (Smith and Khorana, 1959) is still more labile but lacks selectivity for any particular site. It has subsequently found extensive use as a 2' protecting group in ribonucleotide synthesis, as phosphate migration under its removal conditions (pH 2) is negligible (Griffin et al., 1968). How selective insertion of this group at the 2' site is accomplished will be discussed later.

The presence of a chiral center in the tetrahydropyranyl acetal prompted Reese and co-workers to seek other alternatives. The 4-methoxytetrahydropyran-4-yl (Reese et al., 1970), 4-methoxytetrahydrothiopyran-4-yl and 4-methoxytetrahydrosulfonopyran-4-yl (van Boom et al., 1972) groups offer a range of acid lability in symmetrical (non-chiral) ethers.

Use of ethyl vinyl ether in the same capacity was of less value because still greater acid lability of this group resulted in loss of the group under conditions of coupling.

The other principle subclass of acid labile groups are the cyclic 2',3' -ylidenes. These rings are opened under mildly acidic conditions to give one free hydroxyl and the corresponding esterified hydroxyl. The lability of some of these compounds has prevented their general use in the coupling steps of oligonucleotide syntheses. While there is some selectivity for formation of the 3' ester and 2' hydroxyl, this procedure alone is not sufficiently selective. The discovery by Michelson et al. (1955) that only the 3',5' diacetate of adenosine could be crystalized from a transacetylation melt of 2',3',5' triacetyladenosine allowed Reese and co-workers to isolate 3',5'-di-O-acetyl nucleosides formed by mild acid treatment of the 5'-acetyl, 2',3'-methoxyethylidene nucleosides (Fromageot et al., 1967). Similarly, choice of the benzoyl group as the N protecting group conveniently permitted the selective crystalization of the 3'-acetyl, N, 5'-dibenzoyl derivatives of guanosine and cytidine (Wastradowski, 1972; Neilson and Werstiuk, 1971a).

Reaction of these 3',5' diacetylated nucleosides with dihydropyran with an acidic catalyst followed by basic hydrolysis concluded this selective insertion of the 2' acid labile protecting group. The same approach is used in forming the 2'-O-methoxytetrahydropyranyl derivatives.

While the methoxytetrahydropyranyl group has the advantage of symmetry (no chiral center is formed with this reagent) and the slightly greater acid lability compared to the tetrahydropyranyl group, when this work was begun, the symmetrical group had not been extensively tried and

the 4-methoxydihydropyran reagent was not as readily available. The tetrahydropyranyl group was chosen for the 2'-hydroxyl in this work, and synthesis of the N,2'-O-protected nucleosides could be accomplished as described (Griffin et al., 1968; Neilson and Werstiuk, 1971a; Neilson, Wastrodowski, and Werstiuk, 1973).

Most of the base labile protecting groups except for the cyclic phosphate groups are carboxylic acid esters. Some of these (acetyl, benzoyl, p-anisoyl) are identical to and sometimes used concurrently as N-protecting groups. While most groups have been used in the 3' or 5' positions, Khorana and co-workers have found acetyl and benzoyl groups sufficiently labile in the 2' position to allow their removal without phosphoryl migration, (pH <10) e.g. Lohrmann et al., (1966), but this decision was also strongly influenced by the preferred use of dimethoxytrityl groups in the 5' position whose removal required sufficiently strong acidic conditions to allow 2' deprotection if an acid labile 2' protecting group were used. As an alternative to this scheme, the series of groups of increased alkali sensitivity: methoxyacetyl, triphenylmethoxyacetyl, and phenoxyacetyl; have been variously used by several authors.

One advantage in using the triphenylmethoxyacetyl group is its facile and characteristic detection on silica gel t.l.c. plates with the use of a ceric sulfate-sulfuric acid spray followed by strong heating. This procedure develops a strong yellow coloration in the presence of a trityl containing compound and a more orange coloration with methoxytrityl containing compounds, due to the formation of triphenyl methylene ions. As these groups have been used

exclusively for the 5'-position this method permits a simple identification of the 5'-terminal starting materials and products. Because of the bulk of the trityloxyacetyl group it can be inserted at the 5'-hydroxyl selectively in ribonucleosides having a 2'-O-tetrahydropyranyl group. These reasons, and the lability of this group under mild basic conditions made it the protecting group of choice for this work.

The β-benzoylpropionyl group, although base labile, can be removed selectively by treatment with hydrazine hydrate in acetic acid buffered pyridine. While these conditions are mild and do not cleave other commonly used hydroxyl protecting groups, N-benzoyl groups of cytosine and adenosine are removed.

Since the final step of deprotection is usually the removal of the 2'-protecting groups, concurrent removal of the N protecting groups is acceptable and/or desirable.

A suitable representative of the subclass of silyl protecting groups developed by Ogilvie (1973) is the t-butyldimethylsilyl group. It is acid labile to different extents at primary and secondary sites and this property has allowed selective preparation of 2'- or 3'-monosubstituted nucleosides by selective cleavage of the 2'(3'), 5'-disubstituted nucleoside with 80% acetic acid at 100° (Ogilvie et al., 1974). These conditions are suitable for preparing protected nucleosides but less so for deprotection of a synthetic oligonucleotide. Removal of this group can be accomplished with tetrabutyl ammonium fluoride which does not affect other commonly used protecting groups, but which has recently been shown to allow acyl migration from a cis hydroxyl group (Dodd et al.

1975). Rapidity and selectivity in preparation of these derivatives and the high yields afforded make this group a promising addition, with reservations, to the arsenal of protecting groups available.

The stable isocyanate derivatives were developed for the purpose of quantitatively and irreversibly sealing terminal 3'- or 5'-hydroxy groups remaining unreacted after addition of one protected unit against any further reaction which would lead to formation of sequence isomers during synthesis either in solution or on a polymer support.

The highly aromatic naphthylisocyanate provides a terminal group with a high affinity for trityl cellulose, allowing the error sequences, terminated by this protecting group, to be removed rapidly and effectively from the desired product, which lacks highly aromatic groups, after deprotection (Agarwal *et al.*, 1972).

1.4.2.4. Manipulation of Protecting Groups

Discussion of the features of protecting groups for phosphate, amino and hydroxyl functions has been limited to those which have been extensively used and found to be practical and those which are promising but have not yet had time to be used extensively. In each section, the choice of protecting group for use in this work was made and explained briefly. Now the whole picture of the protection of the mononucleosides as used in this work can be seen.

Insertion of the tetrahydropyranyl protecting group selectively depends on the selective isolation of 3', 5'-diesterified nucleosides which are found to crystalize preferentially from mixtures of the 3',5'-

and 2',5'-isomers. These are formed by reaction of the free nucleoside (except for guanosine, where the N^2-benzoyl nucleoside is used because of its greater solubility) with trimethylorthoacetate with acid catalysis to give the 2',3'-methoxyethylidene derivative, acetylation (or benzoylation in the case of cytidine and guanosine) of the 5' hydroxyl and opening of the ethylidine ring with mild acid treatment.

After treatment with dihydropyran and mild acid catalysis, removal of the 3',5'-acyl groups allows isolation of the two diastereoisomers of the 2'-O-tetrahydropyranyl derivatives. Guanosine and cytidine have their N-benzoyl protecting groups at this point but adenosine requires perbenzoylation and selective de-O-benzoylation.

The bulk of tetrahydropyranyl groups adjacent to 3'-hydroxyl sites prevents formation of 3'-3' internucleotide bonds, eliminating the need for a 3'-protecting group (Neilson, 1969). Similarly this bulk allows selective acylation of the 5'-hydroxyl group with triphenylmethoxyacetate using TPS as the activating reagent. Phosphorylation of the only remaining unprotected site, the 3'-hydroxyl, with 2,2,2-trichloroethylphosphate is the first of two steps in the coupling procedure. The second is activation of the 3'-terminal inserted phosphodiester and reaction with the 5'-hydroxyl of the new nucleoside (again, the steric hindrance provided by the tetrahydropyranyl groups prevents 3'-3' coupling). Complete deprotection is afforded by treatment of the protected sequence with zinc/copper couple in DMF, methanolic ammonium hydroxide and finally, 0.01 N HCl; to remove the phosphate protecting groups, 5'-and N-protecting groups and 2'-protecting groups in that order. High yields and selectivity of reaction in these procedures compared to those discussed

above provide justification for these choices.

1.4.3. Phosphorylating and Condensing Reagents

In order to form mono, di, or tri-esters of phosphoric acid, it is necessary to activate the phosphate for nucleophilic attack by the weakly nucleophilic hydroxyl group of a nucleoside.

One of the most well known agents used in oligonucleotide synthesis is dicyclohexylcarbodiimide (DCC). This dehydrating agent has been used principly to activate phosphomonoesters (nucleotides or phosphates esterified with a protecting group). The activation and coupling reactions are comparatively rapid with minimal side reactions. This reagent does not react with free alcohols, allowing activation of a phosphate in the presence of the hydroxylic component. Some side reactions with unprotected amino groups have been observed but no problem was encountered with protected amino groups. This data was gleaned from Smith et al., (1958); Weimann and Khorana, (1962a,b) and others of the extensive publications of Khorana and co-workers. DCC will not form diesters of monoesterified phosphoramidates, allowing them to be used as terminal protecting groups (Ikehara, 1974).

The use of arenesulfonyl chlorides have also been investigated (Jacob and Khorana, 1964; Lohrmann and Khorana, 1966). These reagents appeared to give a more rapid reaction but the sterically hindered mesitylene sulfonyl chloride (MS) or triisopropylbenzenesulfonyl chloride (TPS) were found to give less reaction at the 5' hydroxyl groups of nucleosides compared to p-toluenesulfonyl chloride. These reagents are

preferable to DCC for formation of phosphotriesters and throughout this work, TPS has been used in phosphorylation and coupling reactions.

Both DCC and arenesulfonyl chlorides have been used in joining adjacent nucleoside units by formation of phosphodiester and triester linkages and in phosphorylation of nucleosides with a monoester protected phosphate.

Many phosphorohalides of various types have been used to phosphorylate partially protected nucleosides with moderate success, but these methods of phosphorylation have generally been found less satisfactory because of side reactions with the heterocyclic bases and formation of pyrophosphate linkages during the workup steps (see Khorana, 1961; and Narang and Wightman, 1973 for review and references). Phosphorus oxychloride is capable of phosphorylation and subsequent coupling of a nucleoside to another nucleoside without re-activation of the phosphorylated nucleoside (Mungall et al., 1974). While these methods appear rapid and convenient, the yields have been moderate.

Trichloroacetonitrile (Cramer et al., 1962a,b) has been used successfully in the preparation of phosphomonoesters such as 2,2,2-trichloroethyl phosphate (Eckstein, 1967).

Activated phosphates have been found using arylsulfonyl imidazolides (Berlin et al., 1973), triazolides (Katagiri et al., 1974), picryl chloride, isoxazolium salts, and carboxylic acid chlorides (see Narang and Wightman, 1973, for review). Activation of a phosphate by reduction of phosphoramidates and phosphorothioates have been mentioned above. Phosphate intermediates in oligonucleotide synthesis have been found to be too labile under both acidic and basic conditions to be useful

but Letsinger (1975) has reported the rapid synthesis of phosphotriesters using p-chlorophenyl phosphorodichloridite and 2,2,2-trichloroethyl phosphorodichloridite at very low temperatures followed by oxidation to the phosphotriester. Details of this procedure are to be published shortly.

While there are a great variety of effective phosphorylating and activating agents, the particular requirements found in the synthesis of oligonucleotides may limit the field greatly. Triisopropylbenzene sulfonyl chloride has been used in this laboratory successfully and is the reagent used throughout this work.

1.4.4. Approaches to Oligonucleotide Synthesis

Armed with the knowledge of protecting groups, and phosphorylation and coupling methods available, strategies of synthesis of a nucleotide sequence are considered under two basic philosophies.

Coupling of a nucleotide unit to a growing chain offers the advantage of requiring formation of only one phosphate ester bond per nucleotide coupled. This approach requires that both the nucleotide unit and the growing chain be protected at every site but the desired site of reaction and that the coupling reaction be followed by selective removal of one protecting group to complete the cycle.

The second approach, coupling of a nucleoside unit, protected at all but one or two sites, requires phosphorylation of the growing chain. Thus the growing chain must be protected at all but the site of phosphorylation. Coupling of nucleoside units with one unprotected site must be

followed by selective deprotection as above while coupling of nucleosides with two unprotected sites requires selective coupling to the desired site. The latter has the advantage of eliminating one cycle of deprotection. Other procedures involving the presence of both a phosphate and hydroxyl group on the units to be added have been used for polymerization of nucleotides.

Weimann and Khorana (1962b) studied the efficiency of linking protected deoxynucleoside 3'-phosphates with deoxynucleoside 5'-hydroxyls compared to the efficiency of linking 5'-phosphates with 3'-hydroxyls and found that the latter was preferable. The former approach was found preferable for ribonucleotide synthesis (Lohrmann et al., 1966).

Both methods used by Khorana and co-workers have applied the first philosophy. N-protected deoxy-5'-nucleotides with 3'-acetate groups were successively coupled to deoxy 5'-dimethoxytrityl, N-protected nucleoside and the 3'-acetates are removed by mild alkaline hydrolysis to make way for the next addition. In the ribonucleotide series, presence of the 2'-hydroxyl group protected by an ester group forbade alkaline deprotection of the new oligonucleotide. Instead, synthesis proceeded in the 3'- to 5'-direction by addition of 5'-dimethoxytrityl, N-protected, 3'-nucleotides, and the 5' group was removed by acid hydrolysis after each addition.

Narang and co-workers and Cramer and co-workers have used a different route: addition of monodeoxynucleotides carrying a 3'-protected phosphate followed by selective removal of the phosphate protecting group. The trichloroethyl group is admirably suited to this job as it can be removed reductively.

Letsinger has applied the second philosophy in triester synthesis of oligodeoxynucleotides (Letsinger and Ogilvie, 1969). Phosphorylation of a free 3'-hydroxyl with cyanoethyl phosphate was followed by coupling with a nucleoside bearing a 3'-benzoylpropionyl group. Use of the methoxytrityl group as the 5'-protecting group allowed either terminal to be deprotected for extension of the chain.

Neilson and co-workers have similarly applied the triester method to the synthesis of oligoribonucleotides. Coupling is afforded after phosphorylation of the free 3'-hydroxyl with trichloroethyl phosphate. The bulk of 2'-O-tetrahydropyranyl groups prevents coupling of 3' sites to each other as explained previously. The 5'-hydroxyl is protected with a triphenylmethoxyacetyl group which can be selectively removed under mild basic conditions to enable extension of the sequence by block coupling.

While stepwise, or single unit, addition is suitable for synthesis of short sequences, the difficulty encountered in purifying longer sequences synthesized in this way make it prohibitive for the synthesis for longer sequences. As a result, methods have been developed for selective deprotection of blocks of oligonucleotides for incorporation into longer sequences by specific coupling or by polymerization of blocks.

Some of the advantages of the triester method of synthesis were discussed with the protecting groups used. Two major advantages over the diester approach must be emphasized. The first is non-reactivity of the triester linkage compared to the diester. Khorana and co-workers have found that addition of an activated mononucleotide unit to a chain containing diester linkages requires a greater excess of the mono-

nucleotide unit with increasing chain length. Yields, too, decrease with chain length due to internal chain cleavages induced by activation of the diester linkages, and formation of triesters subject to alkaline cleavage.

The second advantage is the decreased polarity of the triester containing sequences and the resultant increased solubility in the solvents of the reactions and in non-polar solvents enabling rapid and large scale purification using alumina or silica gel chromatography.

Attempts to circumvent the tediousness of stepwise and block synthesis due to the required purification after each coupling by applying a polymer bound or solid phase approach, which worked well in peptide synthesis, have not met with unequivocal success.

Letsinger and Mahadevan (1965, 1966) have used a polystyrene polymer with the nucleotide sequence coupled through an amide linkage to a cytidine residue, protected at the 5'-end with a trityl group. Cleavage from the insoluble resin could be accomplished with sodium hydroxide in dioxane-ethanol.

Hayatsu and Khorana (1967) have used a soluble atactic polystyrene support containing methoxytrityl groups as the 5'-hydroxyl attachment site for synthesis, extending the work of Cramer et al., (1966). The major difficulty encountered with this system was the impossibility of using the established aqueous deprotection conditions and the resulting requirement for non-aqueous conditions, which were less efficient. The heterocyclic bases required deprotection before release of the synthetic sequence under acidic conditions but the benzylamine treatment used was found to give side products with N-anisoylcytidine containing sequences.

Melby and Strobach (1967,1969a,b) were able to apply nearly the same methods as Khorana had used except that use of a crosslinked insoluble polymer allowed more suitable deprotection conditions to be used. They were able to synthesize a variety of di- and trinucleotide sequences, including all four bases, in a satisfactory manner.

Letsinger et al., (1967) have also used a popcorn polystyrene with a carboxylic ester linkage to the 3'-hydroxyl group. The methoxytrityl group is also used as the 5'-protecting group, but its removal is necessary at each step for chain extension.

Phosphoramidate linkages at the 5'-end to an insoluble polymer were initially presented by Blackburn et al., (1967) and have lately been extended to use in the ribonucleotide series by Ohtsuka et al., (1972).

Koster (1972a,b; Koster and Heyns, 1972) has presented an extended study of solid phase supports for oligonucleotide synthesis in the deoxy series using a ribonucleotide with an unprotected 2'-site adjacent to the 3'-site where the deoxy sequence is attached. Glass, Sephadex LH-20, polyethylene glycol are among the supports tried by this author.

Yip and Tsou (1971) developed the first reported polymer bound oligoribonucleotide synthesis on a succinylated isotactic polystyrene. In this scheme, 5'-nucleotides, protected at the 2'-hydroxyl with a benzoyl propionyl group analogous to the 5'-attachment of a nucleoside to the resin bound succinyl group, and at the 3'-hydroxyl with a methoxyacetyl group, are coupled to the 3'-hydroxyl freed by mild alkaline hydrolysis after each addition. Cleavage from the resin is accomplished

with hydrazine as for removal of the 2'-groups.

Although the advantages of a polymer bound approach are enticing, there appear to be some fundamental difficulties with the efficiency of coupling reactions on the polymer supports, removal of protecting groups, and removal from the resin. Many of these difficulties appear to stem from problems with solubility of the resin or with reactions in a two phase system. Since yields are not quantitative, the purification of the desired product sequence after its cleavage from the resin is also a major problem.

1.4.5. Purification Methods

A variety of minor side reactions occur during coupling reactions either on a polymer support or in solution and as a result, an isolation of the major products must be carried out from a variety of impurities which are similar in nature. Purification of protected sequences synthesized by the triester method can be accomplished rapidly and on large scales by silica gel chromatography in non-polar solvents. In the present work, the eluant was methylene chloride containing concentrations of methanol increased in a stepwise manner, which eluted components in order of increasing polarity. With the stepwise mode of synthesis proposed for this current work, purification after each operation is facilitated by the great difference in behaviour between the phosphorylated and coupled products; the former carrying a full negative charge, the latter, no charge.

Purification of protected sequences synthesized by the diester method has been accomplished by DEAE cellulose chromatography, elution being carried out with a volatile buffer such as triethylammonium bicarbonate. Unfortunately the capacity of this method is low and a single column may require several days as materials are eluted in order of increasing number of negative charges so that the product is the last to be eluted.

Several attempts to simplify the purification of diester protected sequences has resulted in a solvent extraction method for purification of dinucleotides (later used in block coupling) and the development of several highly aromatic terminal protecting groups designed to increase the binding to trityl cellulose of the compounds carrying these groups. The groups concerned have already been discussed.

Sephadex LH-20 and Biobeads-S have been used by several authors for the separation of protected sequences by gel filtration in non-aqueous media. Unfortunately, the capacity and resolution of these methods are not high and they appear to be suitable only for shorter sequences.

Purification of deprotected oligonucleotides has been accomplished mainly by paper chromatography in a variety of solvent systems or by DEAE cellulose chromatography in 7M urea by the method of Tomlinson and Tener, (1963). This latter procedure can be used at neutral pH, separating species on the basis of total negative charge (which corelates with chain length) or at acidic pH, (~3.5), separating species on the basis of base composition (as the total negative charge is now decreased by protonation of some of the heterocyclic bases). Reversed phase chromatography, long a standard method in tRNA research, has also been

considered for preparative purification of synthetic sequences.

While affinity chromatographic separations of homo-oligonucleotides have been successfull, no fully sequence specific method of purification is yet practical for these unique sequences.

1.4.6. Summary

Application of the method of Neilson for the synthesis of a messenger RNA would require the synthesis of nucleosides protected at the 2'-hydroxyl with acid labile tetrahydropyranyl groups, at the amino function of the bases adenine, guanine and cytosine with benzoyl groups and for nucleosides used at the 5'-end of the synthetic sequences, protedted at the 5'-hydroxyl with triphenylmethoxyacetyl group.

Synthesis then proceeds by a two step coupling method. First, phosphorylation of the unprotected 3'-hydroxyl with trichloroethyl phosphate and TPS is carried out. Second, after isolation of the phosphorylated intermediate, a nucleoside with a free 5'-hydroxyl is coupled, again using TPS. No 3'-protecting group is reqired because the 2'-O-tetrahydropyranyl groups effectively prevent formation of 3'-3' coupled products. Coupling of blocks of nucleotides is feasible with the potential for selective removal of the 5'-protecting group under mild alkaline conditions. Purification of protected sequences is readily accomplished by silica gel chromatography.

Complete deprotection requires three steps: removal of the trichloroethyl groups by mild reduction with Zn/Cu couple in DMF at 20-60°, hydrolysis of the 5'-triphenylmethoxyacetate group and N-benzoyl groups under alkaline conditions and finally, removal of the tetrahydropyranyl groups with mildly acidic conditions, preventing phosphoryl migration.

Purification of the deprotected product may be accomplished by paper chromatographic methods currently in use or by DEAE cellulose chromatography. Characterization of the synthetic product can then be pursued by standard enzymatic digestion procedures.

1.5. Affinity Chromatography

1.5.1. Principles and Requirements

Cuatrecasas and Anfinsen (1971) have written:

> "Affinity Chromatography figuratively describes procedures used to purify one of the components of a system consisting of two or more species whose reversible interactions reflect affinity with a high degree of biological specificity."

This principle should be ideally suited for purification of one member of a pair of wholly or partly complementary nucleic acids. However, literature dealing with affinity chromatography of natural and synthetic nucleic acids is scant compared with that dealing with proteins (see Cuatrecases and Anfinsen, 1971, for review).

To simplify perception of the requirements and principles to be discussed, an operational definition of affinity chromatography may be

helpful. Affinity chromatography is performed by immobilizing one component of the interacting system on an insoluble porous support and passing a mixture containing the other component over the support in a bathing medium conducive to selective interaction (binding) between bound and free components. The remaining parts of the mixture not adsorbed are eluted, and the adsorbed component can then be eluted separately.

Adsorption of the free component of a system to the affinity site used must have an association constant high enough (or dissociation constant low enough, these inversely related terms are variously used) to retard the passage of that component very distinctly, to allow separation to occur. For example, the K_i of p-aminophenylβ-D-thiogalactoside, a competitive inhibitor of β-galactosidase, was found to be 5 mM (K_i, or the Michaelis constant for the inhibitor, is an approximation of the dissociation constant) and this ligand was used successfully to purify the enzyme (Steers, Cuatrecasas and Pollard, 1971). This value may well be considered a maximum practical value for the dissociation constant of a complex to be used for affinity chromatography as the binding observed in this example was not stable, but only caused sufficient retardation of the enzyme to allow its selective enrichment.

The method of attachment of an affinity site to a chromatographic matrix will depend on the functional groups present and their susceptibility to modification. This simplistic view is complicated by the possibility that coupling the ligand to an insoluble matrix may alter the association constant quite drastically. Indeed it has been found necessary in many examples of affinity chromatography of proteins to couple the ligand to the matrix through a long bifunctional "spacer"

group. The minimum length required depends on the nature of the free component or target molecule and the specific ligand used, but generally a length of 1-2 nm long spacer group has been found satisfactory (for example see Steers, Cuatrecasas and Pollard, 1971).

After adsorption of the target molecule and elution of unbound materials, conditions may have to be changed in order to elute the adsorbed material. With enzymes adsorbed to a competitive inhibitor, adding a high concentration of substrate to compete for binding to the enzyme may permit its elution. Any condition which lowers the association constant can be used if it is not detrimental to the target molecule. Higher tempeature, higher or lower ionic strength, changed pH or the presence of denaturants such as urea or sodium dodecyl sulfate have been used for this purpose.

In cases where the affinity site is not uniquely selective and may bind several components from the mixture, a gradient of the elution conditions may be used to release these components serially in order of increasing stability to the conditions used.

While affinity chromatography is more rapid and selective than many conventional methods, it requires a great deal of preparative work. Selection of a suitable ligand or affinity group and its coupling to a suitable chromatographic matrix may have to be made on the basis of empirical testing of several systems under a variety of adsorption and elution conditions.

1.5.2. Affinity Chromatography of Polynucleotides

The principles discussed above have been applied in several different variations of affinity chromatography of natural and synthetic polynucleotides. While several of these investigations appeared promising most have not subsequently been pursued.

Bautz and Hall (1962) coupled T4 phage DNA to acetylated phosphocellulose using DCC in pyridine. As T4 DNA is known to contain glycosylated thymidine residues, this was believed to be the point of attachment. Hybridization of a crude RNA preparation to this matrix-bound DNA was accomplished by stopping the flow through the column regularly for long enough intervals to permit association of the complementary viral RNA. Re-application of the bound fraction of RNA from this first column to a second column containing DNA from a deletion mutant of T4 lacking the rII region allowed these authors to obtain a greatly enriched fraction of RNA, corresponding to the rII region, in the flow through fraction.

Adler and Rich (1962) applied the same method for attaching synthetic polyribonucleotides to acetylated phosphocellulose. In this case the attachment was less efficient as the point of attachment was presumed to be the 2'-hydroxyl groups. Selective retardation of the complementary polynucleotides was demonstrated.

Gilham (1962) introduced another method: the preparation of oligo-dT cellulose by polymerization of pT with DCC in pyridine in the presence of cellulose. The column prepared in this way was found to bind poly-dA and about 6% of sRNA from *E. coli*. The amino acid acceptor activity of this material was not reported. This method was extended to

the preparation of cellulose containing other homooligonucleotides (Gilham, 1964) and for separation of oligoadenylate tracts prepared by pancreatic RNase and T1 RNase digests of Brome Grass Mosaic Virus (Gilham and Robinson, 1964).

Boulton and McCarthy (1962) trapped high molecular weight DNA in agar for DNA hybridization studies and extended the method for preparation of complementary RNA by affinity chromatography using the finely divided agar (McCarthy and Boulton, 1963).

More recently, DNA occluded in polyacrylamide has been used to purify S1 nuclease (Slor, 1975).

Nygaard and Hall (1963) also used a noncovalent attachment of DNA for RNA and DNA hybridization. They found that denatured DNA was strongly adsorbed to nitrocellulose and was still accessible for complementary base pairing either for hybridization assays or preparative affinity chromatography. The same method has been used by Bautz and Reilly (1966) for purification of RNA corresponding to the rII region of the T4 genome.

Another unusual noncovalent method of attachment to the chromatography matrix has been the adsorption on DEAE cellulose of oligonucleotides covalently linked to polyvinyl alcohol (Schott, 1974a,b).

Erhan, Northrup and Leach (1965), using essentially the methods of Gilham for preparation of homooligonucleotides bound to cellulose, showed that phenylalanine tRNA was specifically retarded on oligo-dT cellulose and lysine tRNA was specifically retarded on oligo-dA cellulose. They proposed this method for the identification of codewords of protein biosynthesis. However, as the elegant technique of Nirenberg and Leder (1964) had been successfully used for the same purpose, this paper appears

to have been ignored.

Hung (1965) has also tried affinity chromatography of tRNA on poly-U bound to polyvinylchloride by ultraviolet induced coupling through the uracil bases. The fact that serine tRNA was bound on this column suggests that photohydration of some of the uracil bases has changed the coding properties of this polynucleotide.

Sander et al., (1966) did not extended Gilham's procedures significantly but presented additional data on elution of complementary oligonucleotides from oligonucleotide-cellulose matrices.

About this time it was realized that matrix bound polynucleotides could also be used for affinity chromatography of proteins. Cozarrelli et al., (1967) prepared oligo-dT cellulose by the method of Gilham, extended the sequence with oligo-dC and hybridized poly-dI to these sites. In the presence of soluble oligo-dC, complementary to the extended, non-bonded poly-dI, DNA ligase could be bound and assayed. Jovin and Kornberg (1968) used similar methods with DNA polymerase.

Alberts has used DNA bound to cellulose for the isolation of DNA binding proteins (Alberts et al., 1968; Alberts and Herrick, 1971). The method used was simply to dry a solution containing DNA on cellulose. On resuspension of the cellulose in aqueous media, the DNA remains bound and is available for binding.

Nelidova and Kiselev (1968) have attempted purification of aminoacyl tRNA synthetases with tRNA bound to polyacrylhydrazide agar by its 3' terminal ribose after periodate oxidation.

Cyanogen bromide activated sepharose or agarose have been more recently used for binding of tRNA for isolation of antibodies (Bonnavida, Fuchs and Sela, 1970), binding of poly-C:poly-I in studies on interferon production (Wagner, Bugianesi and Shen, 1971) and for single stranded nucleic acids for hybridization affinity chromatography (Poonian, Schlabach and Weissbach, 1971). This matrix has also been used to bind an ε-aminocaproyl spacer group which has been activated via the hydrazide for binding of periodate oxidized 16 S RNA for isolation of the complementary DNA (Robberson and Davidson, 1972).

Saxinger, Ponnamperuma and Gillespie (1972) have used carbonyldiimidazole activated phosphocellulose for coupling a variety of natural and synthetic polynucleotides. Shih and Martin (1974) in studies on hybridization of SV40 DNA and RNA utilized both this method and the "Water-soluble DCC" (CMC) method developed by Gilham (1968) to couple nucleic acids to a cellulose support.

An elegant but specialized application of affinity chromatography was the demonstration of selective retardation of glutamic acid tRNA on a polyacrylamide marix containing phenylalanine tRNA bound by hydrazide coupling of its 3'-terminal, periodate oxidised ribose (Grosjean, Takada and Petre, 1973).

The use of poly-dT cellulose or poly-U Sepharose in isolating poly-A containing messenger RNA has already been mentioned and is currently in widespread use.

The only study which has demonstrated sustained effort in the development and application of oligonucleotide chromatography is the work

of Smith. In 1971, Astell and Smith reported separation of dA oligomers on columns of cellulose containing oligomers of dT bound (by the water-soluble DCC method of Gilham). Thermal elution gave excellent resolution of dA oligomers of increasing chain length (n = 6 - 12). Other methods of elution such as reversed salt gradients, formamide, dimethylformamide (DMF) or $NaClO_4$ were effective also but not as good as the increasing temperature gradient (Astell and Smith, 1972). Data on the stability of these complexes (including some heterogeneous sequences, Astell et al., 1973) and the expressed intention to use a synthetic oligonucleotide complementary to part of the messenger RNA for lysozyme from phage T4 (Doel and Smith, 1973) indicate the promise of these procuedures.

Recent studies (Gillam, Waterman and Smith, 1975) have indicated that some retardation is observed for sequences containing mispaired bases. This conclusion is different than that drawn by Schott (1974) of the all or nothing nature of binding of sequences. It appears likely that these conclusions depend upon the conditions, sequences used, and their lengths. Smith's conclusions are still consistent with the high selectivity of nucleic acid hybridization, even with shorter sequences.

In many of these examples, the affinity sites bound are of high molecular weight, and result in high selectivity for exactly complementary sequences. This dictates a low rate of association and a high association constant for the interaction. Thus these forms of affinity chromatography are designed to be performed as an all or none operation: the sample is applied, allowed time to hybridize and the unbound material eluted. Often

repetition of these steps within one column run are required to give good binding and separation.

When shorter oligonucleotides are used, as in the methods of Gilham and Smith, elution conditions may be made continuous in order to separate several species having different degrees of binding. In those studies on elution of tRNA from affinity columns, the actual site of complementarity may be only three or four nucleotides in length as we shall see.

In all but two of the foregoing studies, (those of Schott and that of Robberson and Davidson) no spacer molecule has been used to attach the affinity site to the matrix. In studies using long polynucleotides as the affinity sites, the fraction of the total length of affinity site sequence which is too close to the support to allow binding is very small. Astell and Smith (1972) indicated the deoxyoligonucleotide sequences bound to cellulose by its 5'-terminal phosphate were accessible for binding up to within one half of a nucleotide unit of the point of attachment.

While several different matrices have been used successfully for affinity site supports, cellulose and its derivatives have been most widely used. The reasons for this are probably both functional and historical.

Individual details of elution methods have not been extensively discussed here because these are usually unique and derived empirically for each application from the principles discussed previously.

From this brief review of affinity chromatography using nucleic acids, it can be appreciated that this field is still in its infancy despite its age of thirteen years.

1.5.3. Affinity Chromatography of Transfer RNA

Two approaches to affinity chromatography of tRNA have been introduced. The first (Erhan, Northrup and Leach, 1965) utilized homodeoxyoligonucleotides bound to cellulose. This method suffers from two disadvantages: the stability of interaction of complementary ribo- and deoxyribonucleotides is less stable than the corresponding homologous (ribo-ribo or deoxy-deoxy) interactions; and the homopolymeric sequences have no specific length, a factor which may destabilize the binding as sequences with exactly matching lengths have optimal stability. The general applicability of this method to other tRNA species is also limited by the nature of the sequences which can be incorporated into an insoluble matrix by this method.

The second study (Grosjean, Takada and Petre, 1973) utilized a pure transfer RNA species as the affinity site to bind another species of tRNA having a complementary anticodon. The principle drawback to this method is that it requires one pure tRNA species to bind another.

The methods available in this laboratory for the synthesis of short oligoribonucleotides of defined sequence and length should permit the design of an affinity chromatographic system for tRNA purification. The use of such a specifically synthesized affinity site would embody the advantages of both former methods, but which would lack their disadvantages. A careful consideration of this proposal in light of the properties of complementary oligonucleotides and of tRNA indicated the feasibility of such a project.

What are the factors that influence the stability of complementary oligonucleotide complexes? What are the relationships between these factors and this stability?

The nature of the sugars in the two components is important where, generally, the order of stability of complexes is RNA:RNA > DNA:DNA > RNA:DNA, and with respect to base composition of the duplex, high proportions of G:C base pairs is reflected in greater stability of the duplex than a high proportion of A:U base pairs (see Felsenfeld and Miles, 1967, for review).

The effect of high salt concentrations is increased stability of the duplex. Walker (1969) has expressed the relationship

$$Tm \propto \log [Na^+]$$

where $[Na^+] < 0.1$ M and levelling off above 1.0 M, (where stability is defined in terms of Tm, the melting temperature, or midpoint of a temperature vs. association curve) and indicated that a divalent ion such as magnesium was about 100 fold more effective in stabilizing complementary structures.

Several studies have dealt with the stability of short complementary sequences (Michelson and Monny, 1967; Lipsett, 1964; and Lipsett, Heppel and Bradley, 1961) but have not been able to agree exactly on the relationship between Tm and chain length. The observations were consistent with a decrease in stability with decreasing chain length and a decrease in stability with difference in chain length between the two components of the duplex. This last observation has also been known in hybridization studies on higher molecular weight materials. Generally,

mono-, di-, and trinucleotides were not capable of forming stable duplexes. This data is consistent with that of Astell and Smith (1971, 1972) except that Tm values tended to be higher for oligonucleotide complexes bound to an insoluble support than for the same complex in solution. Nevertheless, the stability of oligonucleotide complexes shorter than six to seven nucleotides in length was not sufficient to make affinity chromatography of these sequences practical.

The term stability has been used very loosely and in an intuitive manner thus far and its relationship to the empirical measure Tm was described. Tinoco and co-workers have investigated the stability of complementary and partially complementary nucleic acid complexes with respect to base sequence (Tinoco, Uhlenbeck and Levine, 1971; Tinoco et al., 1973). They have described stability in terms of the free energy of interaction calculated for the contribution from each possible adjacent pair of nucleotides and appropriate corrections for internal loops, end effects, etc. Free energy is related to the extent of binding by the relationship.

$$\Delta G = -RT\ln K$$

where K represents the equilibrium constant for the association reaction.

Before attempting to reinterpret the previously reviewed data in the quantitative terms of Tinoco and co-workers, it must be recalled that we are not dealing with association of short oligonucleotides per se. Presumably, it is an association of a short oligonucleotide, bound to an insoluble matrix, with a tRNA molecule having an already highly ordered structure at the site of association: the anticodon loop, which is at issue. This issue has already been considered empirically.

Hogenaur (1967) demonstrated the association of several radioactively labelled trinucleotides with unfractionated tRNA by Sephadex G-50 chromatography. In a later study, the dissociation constant for the interaction of ApUpG and methionine tRNA was measured by equilibrium dialysis and gave a value of 150 X 10^{-6} M (Hogenaur, 1970). Both studies were conducted at 0 - 5° in the presence of 10 mM Mg^{++}. This value is less than the value of 5 mM suggested as a maximum for workable affinity chromatography.

This principle has been extended in studies on the binding of many tri- and tetranucleotide sequences to methionine tRNA (Uhlenbeck, Baller and Doty, 1970) and tyrosine tRNA (Uhlenbeck, 1972), isoleucine tRNA (Schimmel et al., 1972) and 5S RNA (Lewis and Doty, 1970).

These studies indicated amply the specific nature of binding of tri and tetranucleotides to unpaired regions of these RNA molecules. The association constants (the inverse of dissociation constants) found for complementary trinucleotides were about $10^4 M^{-1}$ while those for tetranucleotides were greater than $10^4 M^{-1}$ under conditions of 1 M NaCl and 10 mM Mg^{++} at 0 - 5°. Tetranucleotides were found to bind most favorably to the anticodon and nucleotide adjacent to the anticodon on the 5' side. This association is clearly more stable than the interaction of complementary tri and tetranucleotides.

The affinity chromatography of tRNA using a tRNA binding site (Grosjean, Takada and Petre, 1973) was based on previous studies of specific interaction of tRNAs having complementary anticodons (Eisinger, 1971a,b; Eisinger, Feurer, and Yamane, 1970,1971). This interaction appears to be based on the interaction only of the three nucleotides of

the anticodon, as the anticodons of phenylanine tRNA bound to the matrix and the glutamic acid tRNA adsorbed were complementary, the binding was specific, and no similar tetranucleotidic interaction between these tRNAs could be envisaged.

Association constants for tRNA associations under conditions similar to those discussed above were about $10^5 M^{-1}$.

The evidence presented in this introduction confirms the feasibility of the proposal that tRNA should be selectively bound to an affinity column containing a tetranucleotide complementary to the anticodon and one adjacent base. Methods are available for the synthesis of a specific tetranucleotide and for binding oligonucleotides to an insoluble matrix such as cellulose. The implementation of this proposal is presented in the fourth chapter of this thesis.

1.6. Summary

In this introduction the central role of primary base sequences in nucleic acids and the essential nature of complementarity of these sequences in conveying and expressing this information have been discussed. The methods in synthetic nucleotide chemistry which were reviewed offerred a mechanism for duplicating or imitating nucleic acid structures as a probe for their function and/or structure.

Two proposals were made in which, although not new, were renewed in light of more recent advances. The second chapter contains a description of the experimental procedures, many of which were common to both proposed studies. Chapter three presents the results of the proposed synthesis of a messenger RNA-like molecule coding for N-formylmethionyl-

angiotensin. Chapter four presents the implementation of a proposal to demonstrate the affinity chromatographic retardation of isoleucine transfer RNA on an insoluble support containing a synthetic tetranucleotide.

Conclusions concerning the application of these synthetic methods are presented in the final chapter.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and Methods

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

Sephadex G10 and LH-20 were purchased from Pharmacia and Biogel P-2 from BioRad Products Ltd. respectively.

Bulk tRNA from yeast, ATP trisodium salt, CTP trisodium salt and isoleucine were purchased from Boehringer-Mannheim. ^{14}C (uniform label) isoleucine (305 mCi/mmol) and ^{3}H (4,5 label) isoleucine (60 - 80 Ci/mmol) were purchased from New England Nuclear. Pancreatic ribonuclease (RNase A), snake venom phosphodiesterase, and spleen phosphodiesterase, in highly purified form, were purchased from Worthington and stored at -20°.

Thin layer chromatograms of protected nucleosides and sequences were run on Analtech prescored Silica Gel G (250 microns thickness) plates with 10% methanol in methylene chloride unless otherwise stated. Detection was accomplished by spraying with 1% $H_4Ce(SO_4)_4$ in 10% sulfuric acid and heating to > 100°. Preparative t.l.c. was performed on Brinkman silica gel (20 X 20 cm, 500 microns thickness) plates. Compounds

containing triphenylmethyl (trityl) groupings appeared as bright yellow spots while those without appeared as brown or grey spots.

P.m.r. spectra were recorded on a Varian T-60 spectrometer with samples 5 - 10% (w/v) in D_6-DMSO (Stohler Isotopes Ltd.) and line positions are reported in p.p.m. from the tetramethylsilane (TMS) reference. Ultraviolet absorbance measurements and spectra were recorded on a Cary 118 recording spectrophotometer.

Melting points were recorded uncorrected from a Gallenkamp hot block apparatus or a Fisher-Johns stage type apparatus.

Completely deprotected sequences and enzymatic digests thereof were characterized by t.l.c. on Avicel F cellulose plates (5 X 20 cm with fluorescent backing) and detection by observation under short wave u.v., and by paper chromatography on Whatman 3MM or #1 paper; developed in these solvent systems: A, ethanol/ammonium acetate (1 M, pH 7.5) 7:3 (v/v); B, n-propanol/ammonium hydroxide/water 55:10:35 (v/v/v); C, ethanol/ammonium acetate (1 M, pH 7.5) 1:1 (v/v). Both t.l.c. and p.c. were carried out in closed chambers equilibrated with the running solvent before use.

Elemental analyses were performed by Galbraith Laboratories.

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 a slurry of CH_2Cl_2 and eluted with step gradients of CH_3OH or in a slurry of 40% hexane in ethyl acetate and eluted with step gradients of ethyl acetate.

Solutions were reduced in volume or to dryness where required by evaporation under reduced pressure at 30 - 40° except as noted. The anhydrous conditions essential to the phosphorylation and coupling reactions

were maintained after evaporation of the pyridine solutions under reduced pressure by restoring normal pressure with dry nitrogen, not air.

Protected nucleosides were synthesized and characterized by published procedures: HOUtOH (Griffin, Jarman, and Reese, 1968); $HOAt\overset{bz}{O}H$ and $HOCt\overset{bz}{O}H$ (Neilson and Werstiuk, 1971); $HOGt\overset{bz}{O}H$ (Neilson, Wastrodowski, and Werstiuk, 1973); TracUtOH (Werstiuk and Neilson, 1972).

2.2. N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenosine (II, $Trac At\overset{bz}{O}H$).

Sodium triphenylmethoxyacetate (Werstiuk and Neilson, 1972) (1.50 g, 0.0044 mol) was dissolved in anhydrous pyridine (50 ml) and evaporated to near dryness under reduced pressure. This was repeated twice, the second time reducing the volume to about half. TPS (0.74 g, 0.0024 mol) was added and the reaction sealed under dry nitrogen for 1 - 2 h. Low R_f diastereoisomer of N^6-benzoyl-2'-O-tetrahydropyranyladenosine (I, $HOAt\overset{bz}{O}H$) (1.0 g, 0.0022 mol) was similarly dried by repeated solution in anhydrous pyridine and evaporation under reduced pressure (3 X 50 ml), finally reducing the volume to ca. 20 ml. This solution was added to the trityloxyacetate - TPS complex prepared above and the reaction mixture stored at room temperature in darkness for 3 days. T.l.c. indicated ca. 90% conversion to a new product (black spot, R_f = 0.60 → yellow spot $R_f \geq 0.75$). Ice (12 g) was added and after 1 h, the mixture was poured into ice water (70 ml). The aqueous suspension was extracted with CH_2Cl_2 (3 X 20 ml), the combined organic extracts were washed with

water and evaporated to dryness under reduced pressure. The last traces of pyridine were removed by codistillation with toluene. The light brown glass obtained was purified by silica gel chromatography: elution with CH_2Cl_2 removed a small amount of yellow impurities, elution with 0.5% methanol gave a side product (dark yellow spot, R_f = 0.8) tentatively identified as the 3',5'-diesterfield derivative of I, and TracAtOH (II) was eluted with 1 - 1.5% CH_3OH in 73% yield. Its p.m.r. spectrum showed signals (δ TMS) at 11.41 p.p.m. (1 H broad singlet; N^6-amide proton); 8.66 p.p.m. (2 H two singlets; 2- and 8- protons of adenine); 8.02 p.p.m. (2 H multiplet; o-benzoate protons); 7.32 p.p.m. (18 H multiplet; trityl aromatic protons and remaining benzoate protons); 6.20 p.p.m. (1 H doublet; J = 6 Hz; 1'- anomeric proton); 4.79 p.p.m. (1 H singlet; tetrahydropyranyl acetal proton); 3.80 p.p.m. (2 H singlet; $-OCH_2CO-$ protons); 3.20 p.p.m. (2 H multiplet; $-OCH_2-$ tetrahydropyranyl protons) 1.55 p.p.m. (6 H broad multiplet; tetrahydropyranyl methylene protons); 1.20 p.p.m. (short multiplet; methyl protons of triisopropylbenzenesulfonate impurity).

The triisopropylbenzenesulfonyl derivative III was observed by t.l.c. on silica gel eluted with 15% hexane in ethyl acetate (III: brown spot, R_f = 0.45; II: yellow spot, R_f = 0.30) and was removed by silica gel column chromatography in the same solvent, giving 65% overall yield of pure II, indicated by the absence of the sharp multiplet at 1.20 p.p.m. (δ TMS) in the p.m.r. spectrum.

Elemental analysis calculated for $C_{43}H_{41}N_5O_8 \cdot H_2O$ (M.W. 773.8): C, 66.74; H, 5.60; N, 9.05%. Found: C, 66.64; H, 5.42; N, 8.81%.

2.3. Phosphorylation

Many different protected nucleoside and nucleotide derivatives were esterified at the 3'- or 5'-terminal hydroxyl to 2,2,2-trichloroethyl phosphate using the general procedure described here. In preparations subsequently described the terms phosphorylation or phosphorylated implies the application of this procedure.

Bis-cyclohexylammonium mono-2,2,2-trichloroethyl phosphate (Eckstein 1967a) (2 equivalents) was dissolved in anhydrous pyridine (20 - 30 ml/g) with heating. Repeated evaporation under reduced pressure and solution in anhydrous pyridine (5 - 6 X) converted the phosphomonoester to its pyridinium salt. TPS (4 equivalents) was added and after 1 h a pyridine solution of a nucleoside or oligonucleotide (1 equivalent), protected at all reactive sites except the desired site of phosphorylation, as required, was added to the former solution, now yellow in color.

The reaction mixture was sealed under dry nitrogen and stored at room temperature in the dark until t.l.c. indicated complete phosphorylation or no further reaction. If the reaction did not proceed to completion after one to two days, additional aliquots of TPS (0.25 equivalent) were added once or twice daily. In some cases an additional preparation of the activated phosphate (1 equivalent) was added. The rate of reaction was not reproducible and varied from about 80% complete after 2 - 4 h to less than 50% after several days.

Ice was added (about 0.2 g/ml) and after 0.5-1 hr the mixture was poured into five volumes of ice water and repeatedly extracted with methylene chloride (3 - 6 X 0.2 volume of the aqueous suspension). Emulsions frequently encountered at this point were resolved by the addition of several millilitres of saturated NaCl. Washing of the combined organic phases with water or dilute $NaHCO_3$ were not effective in removing the excess of triisopropylbenzenesulfonate salt carried in the organic phase until after removal of pyridine by co-distillation with toluene. Thus the combined extracts were evaporated to a small volume under reduced pressure, resuspended in toluene (a small volume of ethanol was sometimes added to facilitate suspension or solution) and evaporated to dryness (25 - 35°C, reduced pressure). Methylene chloride was added and a precipitate, if formed, was removed by filtration.

It was found that remaining traces of the sulfonate could be removed from this filtrate by extracting with water (1 - 2 X 0.2 volume).

The phosphorylated product in methylene chloride solution was purified by silica gel chromatography only if it was an "end" product [such as $-\overset{bz}{At}-Ut-\overset{bz}{Att}$ (XXVII)] or was contaminated by unreacted starting material [as found with $Trac\overset{bz}{At}-Ut-\overset{bz}{Ct}-$ (VIII after phosphorylation)]. Otherwise, it could be used directly in a coupling reaction. For examples of this procedure, see sections 2.10.3, 2.11 and 2.12.

2.4. Coupling

Esterification of the terminal phosphodiester, obtained in the phosphorylation procedure, to a protected nucleoside or other hydroxyl containing compound (the "incoming nucleoside") is termed coupling and in subsequent preparations where this term is used, the following procedure is implied.

Protected mono- or oligonucleotides as obtained above were rendered anhydrous by repeated solution in an hydrous pyridine and evaporation under reduced pressure and were activated with 1.1 - 1.5 equivalents of TPS as described for the activation of the phosphomonoester above. The incoming nucleoside (1.5 - 2 equivalents) with unprotected 5'-OH was added in a solution of anhydrous pyridine and the reaction was sealed under dry nitrogen as before.

After 1 - 2 days, if t.l.c. did not indicate nearly complete conversion into products ($R_f = 0 - 0.3 \rightarrow R_f \sim 0.7$) further aliquots of TPS were added once or twice daily (approximately 0.1 equivalents) until complete conversion or no further reaction was observed.

Ice (approximately 0.2 g/ml) was added and after 30 min the mixture was poured into approximately 5 volumes of ice water and extracted with methylene chloride (3 - 6 X 0.2 volume). The combined organic phases were evaporated to dryness under reduced pressure, the last traces of pyridine and water being removed by co-distillation with toluene, giving a brown glass.

The product was purified by silica gel chromatography: elution with 0 - 1.5% methanol gave small amounts of impurities and the protected sequence (product) eluted with 1.5 - 4% methanol, depending on the length and composition of the sequence. For examples of the coupling procedure see sections 2.10.3 and 2.11.

If the product co-eluted with the incoming nucleoside as usually occurred with cytidine as the incoming nucleoside, the mixture was dissolved in anhydrous pyridine and treated approximately 18 hour with 0.5-1 equivalents of monomethoxytrityl chloride. This mixture was quenched, extracted and evaporated to dryness as before. The incoming nucleotide, now present as the 5' monomethoxytrityl derivative, (R_f > 0.8, yellow-orange spot on t.l.c.) was removed by silica gel chromatography, being eluted with 0 - 1.5% metahnol. The pure coupled product was then eluted as before with 1.5%-4.0% methanol and evaporated to dryness under reduced pressure.

2.5. Block Coupling

2.5.1. Specific 5' Deprotection

Analytical scale reactions were carried out by dissolving 5 mg samples of protected oligonucleotides in 0.5 ml of (freshly prepared) 1% (v/v) (0.15 M) or 0.5 (v/v) (0.075 M) conc. ammonium hydroxide in methanol. Small aliquots (approximately 20 µl) were removed from the stirred reaction mixtures over a period of several hours; 0, 5, 10, 20, 30, 45 min; 1, 1.5, 2, 3, 4, and 5 h representing the usual schedule of samples taken for t.l.c. analysis.

From the developed t.l.c. plates of these samples, visual estimates were made of the amount of starting material (yellow spot, $R_f \sim 0.8$) remaining and the amounts of desired (black spot, $R_f \sim 0.7$) and debenzoylated products (yellow spot, $R_f \sim 0.7$ and black spots $R_f < 0.7$) formed with time. From the data obtained, the conditions to be used for the preparative scale reactions were decided.

TracUt-At-AtOH (0.250 g, 0.13 mmol) was dissolved in 25 ml of freshly prepared 0.5% (v/v) (0.075 M) NH_4OH in CH_3OH and the mixture was stirred at room temperature. After 2 h, t.l.c. indicated virtually complete removal of the Trac group (yellow spot, $R_f = 0.52 \rightarrow$ black spot, $R_f = 0.49$). The reaction was quenched by evaporation to dryness under reduced pressure (20°C) and the product was isolated by silica gel chromatography in 82% yield (0.174 g).

TracAt-Ut-Ct-CtOH (0.22 g, 0.087 mmol) was dissolved in 25 ml of freshly prepared 0.5% (v/v) (0.75 M) NH_4OH in CH_3OH with stirring. After 2 hr, t.l.c. indicated about 50% removal of the Trac group and traces of debenzoylation (yellow spot, $R_f = 0.51 \rightarrow$ black spot $R_f = 0.42$ + yellow spot (trace), $R_f = 0.35$ + black spot (trace), $R_f = 0.31$). The reaction was quenched by evaporation to dryness under reduced pressure (20°C) and the product isolated by silica gel chromatography giving a yield of 41% (0.08 g).

2.5.2. TracAt-Ut-Ut-Ct-Ut-At-AtOH (XXI)

Bis-cyclohexylammonium mono-2,2,2-trichloroethyl phosphate (0.16 mmol, 68.5 mg) was dissolved in anhydrous pyridine (25 ml) with warming

and converted to its pyridinium salt by repeated evaporation under reduced pressure and solution in anhydrous pyridine (5 x 25 ml). TPS (0.32 mmol, 97 mg) was then added to the pyridine salt in pyridine (1 ml). After 1 hr, a pyridine solution (10 ml) of TracAt^{bz}-Ut-Ut^{bz}-CtOH (XI, 0.08 mmol, 200 mg), previously prepared by the stepwise coupling procedure, was added to the yellow solution and the reaction mixture sealed under dry nitrogen and stored at room temperature in the dark. After two days, t.l.c. indicated complete conversion into products (R_f 0.65 → 0.3 - 0.45). Ice (0.3 g) was added and the quenched reaction left for 30 min, poured into ice water (50 ml) and repeatedly extracted with methylene chloride (3 x 20 ml). The combined organic phases were washed with 5% $NaHCO_3$ (50 ml) and water (2 x 50 ml) and evaporated to dryness under reduced pressure. Anhydrous pyridine was added (25 ml) and the solution was dried by repeated evaporation under reduced pressure and solution in anhydrous pyridine (3 x 25 ml). After finally reducing the volume to approximately 2 ml, TPS (0.12 mmol, 36 mg) was added and activation was allowed to proceed for 1 hr. HOUt-At^{bz}-At^{bz}OH (XVI, 0.12 mmol, 195 mg) was dissolved in anhydrous pyridine (10 ml) and dried by repeated evaporation and solution in anhydrous pyridine (3 x 10 ml). This solution (approximately 2 ml) was added to the activated complex of the phosphorylated tetranucleotide prepared above, and the volume of the mixture reduced by evaporation under reduced pressure to approximately 0.5 ml before sealing under dry nitorgen. The reaction was then stored in the dark at room temperature.

After one and two days, aliquots of TPS (20 mg) were added and the

reaction was monitored by t.l.c. After three days, t.l.c. indicated approximately 80% conversion to product (R_f = 0.3-0.45, yellow spot; + black-spot R_f = 0.49 → yellow spot R_f = 0.6-0.7). Ice (approximately 0.5 g) was added and after 30 min, the quenched reaction mixture was poured into ice water (50 ml). This solution was repeatedly extracted with methylene chloride (3 x 20 ml) and the combined organic phases were washed with 5% $NaHCO_3$ (50 ml) and water (2 x 50 ml). The methylene chloride solution was evaporated to dryness under reduced pressure, the last traces of pyridine being removed by co-distillation with toluene (2 x 20 ml). The brown glass obtained was redissolved in methylene chloride (5 ml) and applied to a column of silica gel (7 g). The column was washed successively with methylene chloride (50 ml), 1% CH_3OH/CH_2Cl_2 (100 ml), 2% CH_3OH/CH_2Cl_2 (100 ml) 3% CH_3OH/CH_2Cl_2 (100 ml), 4% CH_2OH/CH_2Cl_2 (200 ml). Elution with 5% CH_3OH/CH_2Cl_2 gave XXI (83 mg) 24%, XXI + XVI + traces of phosphorylated XI (167 mg) and unreacted phosphorylated XI (67 mg). The latter two fractions also contained significant contamination with triisopropylbenzenesulfonates.

A sample of the product (XXI, 20 mg) was completely deprotected as described generally in section 2.6. The sample was dissolved in DMF (1.6 ml) with stirring and Zn/Cu couple, previously prepared by the method of Le Goff, 1964, (approximately 50 mg, estimated) was added. Stirring was continued 48 hr at 40°C and for 24 hr more after addition of approximately 10 mg Zn/Cu couple. T.l.c. indicated virtually complete removal of the trichloroethyl groups (R_f → 0.0). Methanolic ammonium hydroxide (1:1, v/v; 2 ml) was added and stirring continued for 48 hr. at room temperature. This mixture was filtered and the solids rinsed thoroughly

with 1N NH_4OH (approximately 5 ml). The combined filtrates were stirred with 3 g of Dowex (NH_4^+ form) resin for 5 min, filtered, the resin washed with water (50 ml), and the combined filtrates reduced in volume to approximately 20 ml by evaporation under reduced pressure. Twenty drops of 2N HCl were required to adjust the pH to 2, and stirring was continued at room temperature for 3 days. This solution was neutralized (pH 7) with 1N NH_4OH, added dropwise, and the solution was reduced in volume under reduced pressure (10 ml).

The completely deprotected oligonucleotide in this solution was purified by paper chromatography using solvent C.

Examination of this paper chromatogram under u.v. light showed several overlapping bands (R_f < 0.25). The portion between R_f 0.1 - 0.05 was cut out and eluted with distilled water, giving 11.5 A_{260} units (approximately 5% of the theoretical yield).

Characterization of the crude deprotected material by DEAE cellulose chromatography (as described in section 2.8) gave five u.v. absorbing peaks eluted at salt concentrations less than 0.12 M, corresponding to molecules having zero through four charges, but none at higher salt concentration where the heptanucleotide ApUpUpCpUpApA, having six charges, was expected.

The zero yield obtained in the deprotection series of reactions was also encountered in subsequent attempts to deprotect samples of XXI from this and other preparations of XXI.

Gel filtration of the protected heptanucleotide (XXI, 0.5 mg) as described in section 2.9 gave an elution volume of 84-89 ml (slightly ahead of the elution position of a trinucleotide) while gel filtration of

a sample of the phosphorylated tetranucleotide XI gave an elution position of 50-56 ml, corresponding exactly to the elution position found for a protected tetranucleotide bearing a 3'-terminal phosphate. Retardation of XXI may be indicative of the strong affinity of aromatic compounds for this gel matrix in lower alcohol solvents.

2.5.3 $Trac\overset{bz}{At}\text{-}Ut\text{-}\overset{bz}{At}\text{-}\overset{bz}{Ct}\text{-}\overset{bz}{At}\text{-}Ut\text{-}\overset{bz}{Ct}\text{-}\overset{bz}{Ct}\text{-}OH$ (XXII)

$Trac\overset{bz}{At}\text{-}Ut\text{-}\overset{bz}{At}\text{-}\overset{bz}{Ct}OH$ (0.065 mmol, 167 mg), IX, was phosphorylated over a period of 3 days (R_f 0.69 → 0.3 - 0.45). The phosphorylated sequence was coupled to $HO\overset{bz}{At}\text{-}Ut\text{-}\overset{bz}{Ct}\text{-}CtOH$ (0.025 mmol, 55 mg). After 8 days (TPS, 5 mg, was added every second day) t.l.c. indicated no further reaction R_f 0.3-0.45 + R_f 0.42 → R_f 0.5 - 0.6 estimated 40%). Purification of the product by silica gel chromatography was not satisfactory: 60 mg of impure product was obtained on elution with 35% methanol. Purification of this fraction by preparative t.l.c. (30 mg on each of 2 20 cm x 20 cm plates; developed in 5% CH_3OH/CH_2Cl_2) gave 27 mg of product (R_f = 0.4 - 0.6 in 5% CH_3OH/CH_2Cl_2. A sample of the protected product (10 mg) was completely deprotected and subjected to paper chromatography (solvent C). No u.v. absorbing band at R_f less than 0.3 was observed. Elution of this portion of the paper chromatogram (R_f 0.0-0.3) with water gave a solution which had an ultraviolet spectrum increasing steadily from 320 nm to 250 nm. Thus the yield of deprotected octanucleotide ApUpApCpApUpCpCp obtained was 0%.

2.6. Complete Deprotection

Complete deprotection of sequences for characterization was usually carried out on samples of 10 mg. The sample was stirred in 1 ml of DMF,

and 20-30 mg (estimated) of Zn/Cu couple (prepared by the method of Le Goff (1964) and stored under anhydrous ether at 0°C) was added. Stirring was continued 18 h to 3 days at 30-50° (Eckstein, 1967a) (no heat was applied except the heat provided by the magnetic stirring motor). Removal of the trichloroethyl groups was indicated by t.l.c. (R_f → 0.0).

One to two volumes of 50/50 (v/v) NH_4OH/CH_3OH was added and stirring continued 18 h - 2 days. This treatment was adequate to remove the 5'-triphenylmethoxyacetyl and N-benzamido groups.

Filtration removed the Zn/Cu which was further washed with ~ 5 ml of 1 N aqueous NH_4OH. The combined filtrates were treated with Dowex - 50 W (NH_4^+) or Chelex (Na^+) ion exchange resins for 30 min and again filtered. The resin was washed with 10-20 ml of 1 N, NH_4OH and the combined filtrates evaporated under reduced pressure to a volume of 5-10 ml. 1 N HCl was added, lowering the pH to 2, and the solution was allowed to stand 2 days at room temperature. 1N NH_4OH was added to neutralize the solution. Solutions of deprotected sequences to be purified by paper chromatography were reduced in volume under reduced pressure to ~ 1 ml while those in later stages of the work which were purified directly by ion exchange chromatography were made up to ~ 7 M urea directly for application to the column.

Paper chromatographic purification of deprotected sequences was carried out on Whatman 3 MM paper eluted with solvent C containing 0.002 M EDTA. U.v. absorbing bands, observed on the developed paper under short wave irradiation, were marked and cut out. Usually, one major band of R_f < .25 (the lowest R_f band on the paper) was found to correspond to the

product. Each strip cut out was eluted with water (ethanol/water mixtures were not adequate) and the u.v. spectrum and volume of the eluate were recorded before freezing the solution at -20°. Yields were calcualted from the optical density of the eluted samples. For an example of this procedure, see section 2.10.3.6.

2.7. Enzymatic Digestion of Oligonucleotide Sequences.

Purified oligonucleotide sequences after deprotection were characterized by digestion with enzymes and identification of the products by thin layer and paper chromatography.

A measured aliquot of the oligonucleotide, in the range of 1 - 2 A_{260} units per nucleoside unit of the oligonucleotide, was evaporated to dryness under reduced pressure and redissolved in a buffer suitable for the enzyme to be used (100 µl). After withdrawing an aliquot (approximately 0.5 A_{260} units), a half volume of enzyme solution (1 mg/ml) in the same buffer was added and the mixture incubated overnight at 37°. The aliquot of undigested material was spotted on Whatman #1 chromatography paper (approximately 2/3) and two cellulose t.l.c. plates (approximately 1/6 each). The digested material was also spotted approximately 1/2 - 2/3 on the paper chromatogram and less than 1/4 on each t.l.c. plate. Standard samples of the expected nucleoside and nucleotide products were also spotted and these chromatograms were developed in the solvent systems described under Materials and Methods. After development, the materials were detected by observation under a short wave u.v. lamp. R_f values were calculated and converted to standard R_{Up} values where:

$$R_{Up} \text{ of } X = \frac{R_f \text{ of } X}{R_f \text{ of } Up}$$

The buffer used for alkaline phosphatase, pancreatic ribonuclease, (RNase A) and snake venom phosphodiesterase was Tric HCl (0.1 M, pH 7.5 - 7.8) while that used for spleen phosphodiesterase was sodium succinate (0.25 M pH 6.5).

Enzymatic hydrolysis of oligonucleotide linked to cellulose was carried out in the following way. Weighed samples of papers (containing the oligonucleotide) in the range of 0.02-0.03 g were cut into small pieces (smaller than 1 mm^2). A solution containing the enzyme to be used (0.2 mg/ml) in the prescribed buffer was then added (0.5 ml). The suspension was incubated overnight at 37°. The suspension was diluted with 0.9 ml distilled water, filtered and the u.v. spectrum of the filtrate was recorded against water and against the enzyme solution (also diluted by a factor of two). The control reaction or blank was a sample of cellulose (with oligonucleotide bound) digested with water only and 100% release of oligonucleotide was estimated by hydrolysis of a sample of cellulose (with oligonucleotide bound) with 0.2 N NaOH (0.5 ml) performed in the same way.

2.8. Diethylaminoethyl-cellulose Chromatography

Purification of deprotected sequences to be used for coupling to cellulose was accomplished by the method of Tomlinson and Tener (1963) as described below.

Solutions of urea (10 M) were deionized by passage through a mixed base ion exchanger MB-3 (Wm. Anderson and Co.) and used to prepare the low salt buffer: 0.02 M Tris pH 7.2-7.5, 0.001 M EDTA (optional) in 7 M urea; the high salt buffer: 0.02 M Tris pH 7.2-7.5, 0.001 M EDTA (optional), 0.3 M or 0.5 M NaCl in 7 M urea; and the "5x" buffer: 0.1 M Tris pH 7.2-7.5, 0.001 M EDTA (Optional) in 7 M Urea.

Dry DEAE-cellulose (Whatman DE 23, 4 g) was suspended in 150 ml 1 M NaCl for 30 min, filtered on a coarse sintered glass funnel, and washed with water (4 x 50 ml). The cellulose was resuspended in 0.5 N HCl (80 ml) for 30 min, filtered and washed with water until the pH of the filtrate was raised to about 4. The cellulose was resuspended in 0.5 N NaOH (80 ml) for 30 min, filtered and washed with water until the pH of the filtrate was below 8. Low salt "5x" buffer was used to equilibrate the ion exchanger by stirring for 30 min, filtering and washing thoroughly with five volumes of the regular low salt buffer. A slurry of the equilibrated ion exchanger in 1.5 x its settled volume (final volume ~70 ml) of low salt buffer was packed in a glass column (1.2 cm diam) under pressure using a peristaltic pump. The flow rate, after packing was approximately 50 ml/hr and the bed height, 25-30 cm.

Samples were applied in a convenient volume of 7 M urea solution containing less than 0.1 M salt. After pumping the sample into the column bed, a linear gradient of NaCl (0 - 0.35 M or 0 - 0.5 M) was started (total volume 500 ml or 1000 ml). Detection of ultraviolet absorbing material in the eluate was accomplished using an Isco UA-2 Ultraviolet Analyzer (254 nm) and chart recorder.

Fractions containing u.v. absorbing material were pooled for the major peaks and the volume and u.v. spectrum of each recorded for calculation of yields. In most cases, a major peak was eluted at the salt concentration expected for the desired product (separation is based on the total number of negative charges) and only this peak was kept for further treatment.

Urea and salts were removed from pooled product fractions by passage through Sephadex G-10 (2.5 x 50 cm) or Biogel P-2 (1.2 x 50 cm) and elution with deionized water. Dilute aqueous solutions containing u.v. absorbing material were reduced in volume by lyophilization. The resulting solutions or dried powders were stored at -20°C until use.

The ion exchanger was reused after removing a small portion of coloured material which appeared at the top of the bed, and washing the remainder with 1 M NaCl (in 7 M urea and buffered as before) either as a batch outside the column or *in situ*, followed by re-equilibration with the low salt buffer. Freshly prepared and degassed buffers were found to be preferable in this work.

2.9. Gel Filtration of Protected Oligonucleotides

Sephadex LH-20 (50 g) was suspended in CH_3OH (300 ml) overnight and packed by gravity flow into a glass column (90 cm x 0.25 cm) to give a final bed height of 70 cm and flow rate of approximately 2 ml/hr. Samples of protected oligonucleotides were dissolved in a small volume (less than 1 ml) of CH_3OH with 1 drop of CH_2Cl_2 and layered carefully on top of the gel bed. When the sample had been run into the bed, a further volume of CH_3OH (1 ml) was added to wash the sample in thoroughly. The solvent head was careuflly prepared by adding CH_3OH, without disturbing the gel bed, to a height of about 76 cm above the outlet of the column. An inverted round bottom flask of CH_3OH, with adapter, maintained this operating pressure during the elution. Fraction of 1 ml were collected and analyzed by t.l.c. or an Isco UA-2 Ultraviolet analyzer and chart recorder were used. The later method enabled greater senstivity of detection and greater precision in calculating the elution volumes. Standard plots of log M.W. v.s. elution volume were prepared for comparison of elution volumes of experimental samples.

2.10. 5'-Phosphorylated Sequences

2.10.1. N^6-benzoyl-2',3'-di-O-tetrahydropyranyl-5'-O-triphenylmethoxy-acetyladenosine (XXIII, Trac Att^{bz}).

p-Toluenesulfonic acid monohydrate (0.48 g, 0.25 mmol) was added to a mixture of p-dioxane (2 ml) and 2,3 dihydropyran (0.4 ml, 4.2 mmol)

and stirred with 4-5 grains of 4A° molecular seive at 4° until partially frozen. To the light pink solution, TracAtOH (II) (0.236 g, 0.321 mmol) was added. After several minutues, the pink color disappeared and the mixture was brought to room temperature. After 2 h, t.l.c. indicated to complete conversion (yellow spot, R_f = 0.75 → R_f = 0.82) (0.5 → 0.66 in 5% CH_3OH/CH_2Cl_2) to a new product. The reaction was quenched by the addition of 1% NH_4OH in methanol until the pH was approximately 7. The solution was reduced to a light yellow oil by evaporation under reduced pressure, resuspended in CH_2Cl_2 (10 ml), and filtered. The filtrate was diluted to 25 ml with CH_2Cl_2 and applied to a silica gel column (11 g SiO_2). Elution with CH_2Cl_2 removed traces of dioxane and dihydropyran. The product was eluted with 1% methanol in 77% yield. P.m.r. spectrum indicated the correct structure, with signals (δ TMS) at 8.60 p.p.m. (2 H 2 singlets; C-2 and C-8 protons of adenosine); 8.02 p.p.m. (2 H multiplet; o-benzoate protons); 7.32 p.p.m. (18 H multiplet; trityl aromatic protons and remaining benzoate protons); 6.20 p.p.m. (1 H doublet; J = 6 Hz; C-1' anomeric proton; 5.10 p.p.m. (1 H multiplet; C-3' proton); 4.95 p.p.m. (1 H triplet, J = 5Hz; C-4' proton); 4.79 p.p.m. (2 H singlet; tetrahydropyranyl acetal protons); 4.31 p.p.m. (3 H multiplet; C-2' and C-5' protons); 3.80 p.p.m. (2 H singlet; $-OCH_2CO-$); 3.20 p.p.m. (4 H multiplet; $-OCH_2-$ of tetrahydropyranyls); 1.55 p.p.m. (12 H broad miltiplet; tetrahydropyranyl methylene protons). Elemental analysis calculated for $C_{48}H_{49}O_9N_5$ (M.W. 839.95): C, 68.64; H, 5.88; N, 8.34. Found: C,68.63; H, 6.01; N, 8.27.

2.10.2. N^6-benzoyl-2',3'-di-O-tetrahydropyranyladenosine (XXIV, $HOAtt^{bz}$).

$TracAtt^{bz}$ (XXIII) (125 mg, 0.149 mmol) was dissolved in 1% (v/v) NH_4OH in CH_3OH (0.15 M) (10 ml) and stirred at room temperature. After 6 h, t.l.c. indicated complete removal of the Trac group (yellow spot, R_f = 0.61 → black spot, R_f = 0.29 in 5% CH_3OH/CH_2Cl_2), and a small amount of debenzoylated material (black spot, R_f = 0.125 in 5% CH_3OH/CH_2Cl_2). The reaction was quenched by evaporation to dryness under reduced pressure. Purification by silica gel chromatography gave XXIV in 90% yield on elution with 2% CH_3OH in CH_2Cl_2. P.m.r. spectrum gave signals (δ TMS) at 8.60 p.p.m. (2 H 2 singlets; C-2 and C-8 protons of adenosine) 8.05 p.p.m. (2 H multiplet; o-benzoate protons); 7.30 p.p.m. (3 H multiplet; remaining benzoate protons); 6.24 p.p.m. (1 H doublet; J = 6 Hz; C-1' anomeric proton); 5.20 p.p.m. - 4.20 p.p.m. (7 H overlapping multiplets; C-3', tetrahydropyranyl acetal, C-2' and C-5' protons); 3.75 p.p.m., 3.20 p.p.m. (2 X 2 H, overlapping multiplets; $-OCH_2$-tetrahydropyranyl protons); 1.55 p.p.m. (12 H broad multiplet; tetrahydropyranyl methylene protons). Elemental analysis calculated for $C_{27}H_{33}N_5 \cdot H_2O$ (M.W. 557.60) C, 58.16; H, 6.33; N, 12.56. Found C, 58.12; H, 6.21; N, 12.08.

2.10.3. 5' Phosphoryladenylyl-(3'-5')-Uridylyl-(3'-5')adenosine

2.10.3.1. N^6-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenylyl-[3'-(2,2,2-trichloroethyl)-5']2'-O-tetrahydropyranyluridine ($TracAt^{bz}$-UtOH, IV).

Bis-cyclohexylammonium mono-2,2,2-trichloroethyl phosphate (1.31 g, 2.92 mmol) was dissolved in anhydrous pyridine (20 ml) with warming, and converted to its pyridinium salt by repeated evaporation under reduced pressure and solution in pyridine (5 x 50 ml). TPS (1.77 g, 5.84 mmol) was then added to the pyridine salt in pyridine (10 ml). After 1 h a pyridine solution (10 ml) of N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenosine ($TracAt^{bz}OH$, II) (1.10 g, 1.46 mmol; R_f = 0.75) was added to the yellow solution and the reaction mixture sealed under dry nitrogen and stored at room temperature in the dark. After 2 days t.l.c. indicated complete conversion into products of R_f = 0.0-0.20. Ice (5 g) was added and the quenched reaction left for 30 min, poured into ice-water (150 ml) and repeatedly extracted with methylene chloride (4 x 40 ml). The combined organic phases were washed with water (4 x 40 ml) and methylene chloride removed by evaporation under reduced pressure and replaced by anhydrous pyridine (10 ml) by repeated evaporation under reduced pressure and solution in pyridine (3 x 20 ml). TPS (443 mg, 1.46 mmol) was added to the blocked adenylate derivative and activation allowed to proceed for 30 min at room temperature. The high R_f diastereoisomer of 2'-O-tetrahydropyranyluridine (Griffin, Jarman, and Reese, 1968) (705 mg, 2.19 mmol; R_f = 0.45) dissolved in

anhydrous pyridine (10 ml) was added, and the reaction mixture sealed under dry nitrogen and stored at room temperature in the dark. On the second day, a further portion of TPS (100 mg) was added to ensure complete coupling. After 3 days t.l.c. indicated complete conversion to a product of R_f = 0.70. Ice (10 g) was added to the mixture and stirring continued for a further 30 min. The reaction mixture was poured into ice-water (150 ml) and repeatedly extracted with methylene chloride (6 x 50 ml). The combined organic extracts were washed with water (5 x 40 ml) and evaporated to dryness under reduced pressure, the last traces of pyridine being removed by co-distillation with toluene (3 x 20 ml). The light brown residue was dissolved in methylene chloride (50 ml) and the bulk of triisopropylbenzenesulfonic acid separated and was filtered off. The filtrate was subjected to chromatography on silica gel (50 g) in methylene chloride. Elution with 2% CH_3OH-CH_2Cl_2 gave the desired product (IV) (1.4 g, 75%; R_f = 0.66) on removal of solvent. The p.m.r. spectrum showed signals (δ TMS) at 12.4 (1 H-broad multiplet; N^6-proton of A); 8.65 (2 H-overlapping singlets; 2- and 8-protons of A); 8.1 (2 H-multiplet; o-benzoate protons); 7.4 (19 H-broad multiplet; other aromatic protons and 6-proton of U); 6.28 (1 H-doublet; J = 5Hz; 1'-proton of A); 5.9 (1 H-doublet; J = 5Hz; 1'-proton of U); 3.85 (2 H-singlet; OCH_2CO); 1.6 p.p.m. (12 H-broad multiplet; C-CH_2 protons of thp). Since IV occurs as a diastereoisomeric pair (P atom is chiral centre), its spectrum is not as sharp as that of II.

2.10.3.2. N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxy-acetyladenylyl[3'-(2,2,2-trichloroethyl)-5']2'-O-tetrahydro-pyranyluridylyl[3'-(2,2,2-trichloroethyl)-5']-N^6-benzoyl-2'-O-tetrahydropyranyladenosine (TracAt-Ut^{bz}-At^{bz}OH, V).

Phosphorylation of TracAt-Ut^{bz}OH (IV) (720 mg, 0.564 mmol; R_f = 0.66) and subsequent coupling with high R_f isomer of N^6-benzoyl-2'-O-tetrahydro-pyranyladenosine (I),(Werstiuk and Neilson, 1971a) (385 mg, 0.846 mmol; R_f 0.48) gave the desired product as a solid foam (730 mg; R_f 0.64 in 66% yield. Complete deprotection gave ApUpA in 86% yield. R_{Up} 0.59 (0.60 [Lohrmann et al., 1966]) in solvent A. ApUpA was completely digested by snake venom diesterase to give A, pU, and pA in the ratio of 1:1:1 and by pancreatic RNase and, subsequent alkaline phosphatase treatment to give ApU and A.

2.10.3.3. N^6-Benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxy-acetyladenylyl[3'-(2,2,2-trichloroethyl)-5']2'-O-tetrahydro-pyranyluridylyl[3'-(2,2,2-trichloroethyl)-5'] N^6-benzoyl-2',3'-O-ditetrahydropyranyladenosine (TracAt-Ut^{bz}-Att^{bz}, XXV).

A solution of p-toluenesulfonic acid monohydrate (24 mg, 0.013 mmol) in dry dioxan (2 ml) containing molecular sieves (10 mgs) were cooled in ice untill solidified. Dihydropyran (184 mg, 0.22 mmol) was then added drop-wise with stirring, followed by a dioxan solution (1 ml) of TracAt-Ut^{bz}-At^{bz}-OH (IV) (300 mg, 0.156 mmol; R_f = 0.38 in 5% CH_3OH-CH_2Cl_2). The reaction

mixture was allowed to warm up to room temperature after 30 min and stirring was continued a further 4 h. T.l.c. indicated complete conversion into a product R_f = 0.50 in 5% CH_3OH-CH_2Cl_2 and the reaction mixture was neutralized with 0.15 N methanolic ammonia. Evaporation under reduced pressure of the solution gave a yellow oil which was purified by chromatography on silica gel (15 g) in methylene chloride. Elution with 2.5% CH_3OH-CH_2Cl_2 gave the desired product as a light brown foam (261 mg, R_f = 0.50 in 5% CH_3OH-CH_2Cl_2 and 0.73 in 10% CH_3OH-CH_2Cl_2).

2.10.3.4. N^6-Benzoyl-2'-O-tetrahydropyranyladenylyl[3'-(2,2,2-trichloroethyl)-5'] - 2'-O-tetrahydropyranyluridylyl[3'-(2,2,2-trichloroethyl)-5'] - N^6-benzoyl-2',3'-O-bis-tetrahydropyranyladenosine(HOAt-Ut-Att) (XXVI).

TracAt-Ut-Att (XXV) (261 mg, R_f 0.73) was dissolved in 0.15 N methanolic ammonia (35 ml) and stirred for 2.5 h. T.l.c. indicated almost complete cleavage of the Trac group (yellow spot, R_f = 0.73 → black spot R_f = 0.62), and the reaction was quenched by evaporation. The residue was subjected to chromatography on silica gel (15 g) in methylene chloride. Elution with 4% CH_3OH-CH_2Cl_2 gave the desired product (131 mg), in 59% yield. Complete deprotection gave ApUpA in 93% yield.

2.10.3.5. N^6-benzoyl-2'-O-tetrahydropyranyl-5'-(2,2,2-trichloroethyl-phosphoryl)adenylyl-[3'-(2,2,2-trichloroethyl)-5']2'-O-tetrahydropyranyluridylyl[3'-(2,2,2-trichloroethyl)-5']-N^6-benzoyl-2',3'-O-bis-tetrahydropyranyladenosine (-At^{bz}-Ut-Att^{bz}), XXVII).

Bis-cyclohexylammonium mono-2,2,2-trichloroethylphosphate (59 mg, 0.14 mmol) (11) was converted to its corresponding pyridinium salt as described earlier. TPS (85 mg, 0.28 mmol) was added to the anhydrous pyridine solution (2 ml) and activation allowed to proceed for 2 h under dry nitrogen. HOAt^{bz}-Ut-Att^{bz} (XXVI) (131 mg, 0.077 mmol, R_f = 0.62) was dissolved in anhydrous pyridine (1 ml) and added to the TPS-complex. The volume of the reaction mixture was reduced to 1 ml by gentle evaporation under reduced pressure. T.l.c. indicated phosphorylation was complete in 2 h, black spot, R_f = 0.62 → black spot, R_f = 0.2-0.3). Ice (0.5 g) was added and the quenched reaction left for 30 min, poured into ice-water (20 ml), and repeatedly extracted with methylene chloride (6 x 5 ml). The combined organic phases were washed with water (2 x 25 ml) and evaporated to dryness under reduced pressure, the last traces of pyridine being removed by co-distillation with toluene (2 x 10 ml). The residue was purified by column chromatography on silica gel (8 g) in methylene chloride. Elution with 4% CH_3OH-CH_2Cl_2 gave the desired product as a glass (98 mg, 70% R_f = 0.2). Yield over the last three steps (insertion of the 5'-phosphate) was 29%.

2.10.3.6. 5'-Phosphoryladenylyl(3'-5')uridylyl(3'-5')adenosine (pApUpA)

A sample of -At^{bz}-Ut-Att^{bz} (XXVII) (10 mg, 0.005 mmol) was dissolved in DMF (0.8 ml) and Cu/Zu couple (ca 10 mg) was added and the mixture stirred overnight. T.l.c. indicated loss of all 2,2,2-trichloroethyl groups (R_f = 0.2 → 0.0). Conc. ammonia (half diluted with methanol) (1 ml) was added and stirring continued a further day. Loss of N^6-benzoyl groups was indicated by conversion to a major species (R_f 0.7 → 0.4, cellulose plates in Solvent A). Dowex-50 (NH_4^+ form) (ca. 1 g) was added and gentle stirring continued for 30 min. The resin was removed by filtration. The filtrate was reduced to ca. 5 ml by evaporation under reduced pressure and its pH adjusted to 2 with 2N-hydrochloric acid. Overnight stirring removed the tetrahydropyranyl groups as indicated by t.l.c. (R_f 0.4 → 0.0, cellulose plates in Solvent A). pH was adjusted to 7 with N-ammonium hydroxide and the solution reduced to ca. 2 ml by evaporation under reduced pressure. pApUpA was purified by paper chromatography on Whatman 3 MM developed for 18 hours in solvent C. The most intense band (R_f 0.2) was eluted with distilled water and eluate stored as a frozen solution. Yield was 128 A_{260} units, 66%. Alkaline phosphatase digestion gave a single product ApUpA, R_{Up} 0.59; in solvent A. Snake venom diesterase digestion gave pA and pU in the expected ratio of 2:1.

2.11. Protected Nucleotides with 3' Linked Decane-diol Spacer

2.11.1. 2'-O-Tetrahydropyranyl-5'-O-triphenylmethoxyacetyluridylyl-(3'-[2,2,2-trichloroethyl]-10)-decan-1-ol (XXIX, TracUt-dOH)

TracUtOH (XXVIII) (1.0 g, 1.59 mmol) was phosphorylated in the usual manner. The phosphorylated intermediate was dried and activated with TPS (0.694 g, 2.29 mmol) in anhydrous pyridine for one hour exactly as described for the first stage of the coupling procedure. Decane-1, 10-diol (2.64 g, 15.2 mmol) was dissolved in anhydrous pyridine and repeatedly evaporated to dryness under reduced pressure and redissolved in anhydrous pyridine (3 x 50 ml), on the last evaporation, reducing the volume to ca. 20 ml. This solution was added to the TPS-activated uridine derivative prepared above. After 4 h, t.l.c. indicated that about 25% of the phosphorylated XXVIII had reacted (yellow spot, R_f = 0.2 → yellow spot, R_f = 0.7) but no further reaction was apparent after one and two days. The reaction was quenched by the addition of 3 g ice. After 1 h the quenched mixture was poured into ice water (125 ml) and extracted with CH_2Cl_2 (3 x 20 ml). The combined organic phases were washed with water and evaporated to dryness under reduced pressure. A precipitate obtained on resuspension of the dried foam in CH_2Cl_2 was removed by filtration. The filtrate was purified by silica gel chromatography. The putative product and residual unreacted decane diol co-eluted with 1-1.5% methanol.

Gel filtration of the impure product obtained, on Sephadex LH-20 did not permit separation of the decane diol.

T.l.c. in 20% hexane/ethyl acetate indicated identical R_f values for the two components (0.5). As the elution patterns on t.l.c. and in silica gel columns in the same solvent system often differ, this system was used to separate the decane-diol by column chromatography. The diol eluted first and was separated from the TracUt-dOH (XXIX). Rechromotography of the purified product in the CH_2Cl_2-CH_3OH system usually employed was necessary to remove less volatile and foul smelling contaminants found in ethyl acetate. The yield obtained was 154 mg (8%).

P.m.r. spectrum gave signals (δ TMS) at 7.37 p.p.m. (16 H multiplet; trityl aromatic protons and C-6 proton of uracil); 5.95 p.p.m. (1 H doublet; J = 6 Hz; 1'-anomeric proton); 5.54 p.p.m. (1 H doublet; J = 8 Hz; C-5 proton of uracil); 4.70 p.p.m. (1 H singlet; tetrahydropyranyl acetal proton proton); 3.80 p.p.m. (2 H singlet; $-OCH_2CO-$ protons); 1.55 p.p.m. (26 H multiplet; tetrahydropyranyl and decyl methylene protons).

2.11.2. N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyl-(3'-[2,2,2-trichloroethyl]-10)-decan-1-ol ($TracAt^{bz}$-dOH, XXXI).

$TracAt^{bz}OH$ (II) (.500 g, 0.663 mmol) was phosphorylated by the previously described procedure. The phosphorylated intermediate was activated with TPS (0.300 g, 0.998 mmol) in anhydrous pyridine as described for coupling reactions. 1,10-decane-diol (1.16g, 6.63 mmol) was dissolved in anhydrous pyridine and after repeated evaporation to dryness and resolution in anhydrous pyridine (3 X 50 ml) was added to the TPS-activated intermediate prepared above. After one day, t.l.c. indicated

about 50% reaction (yellow spot, R_f = 0 → 0.2 → yellow spot, R_f = 0.7) and no further progress was seen after one more day. The reaction was quenched with ice (3 g) and after 1 h poured into ice water (50 ml and extracted with CH_2Cl_2) (4 X 10 ml). After the combined organic phases were washed with water, they were evaporated to dryness under reduced pressure, the last traces of pyridine being removed by co-distillation with toluene. Some of the excess of decane diol was removed by filtration after resuspension of the dried extract in methylene chloride.

Purification by silica gel chromatography gave 200 mg (27%) of XXXI contaminated with traces of decane diol. P.m.r. spectrum showed signals (δ TMS) at 8.63 p.p.m. (2 H 2 singlets; C-2 and C-8 protons of adenosine); 8.12 p.p.m. (2 H multiplet; o-benzoate protons); 7.40 p.p.m. (18 H multiplet; aromatic protons of trityl and remaining benzoate protons); 6.32 p.p.m. (1 H doublet; J = 5 Hz; C-1' anomeric proton); 4.79 p.p.m. (1 H singlet; tetrahydropyranyl acetal proton); 3.87 p.p.m. (2 H singlet $-OCH_2CO-$ protons); 1.40 p.p.m. (26 H multiplet; tetrahydropyranyl and decyl protons).

Elemental analysis calculated for $C_{55}H_{63}N_5O_{12}PCl_3$ (M.W. 1123.47): N, 6.23; P, 2.76. Found: N, 6.12; P, 2.79.

2.11.3. Protected Affinity Site with spacer: N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenylyl-(3'-[2,2,2-trichloroethyl]-5')-2'-O-tetrahydropyranyluridylyl-(3'-[2,2,2-trichloroethyl]-5')-N^4-benzoyl-2'-O-tetrahydropyranylcytidylyl-(3'-[2,2,2-trichloroethyl]-5')-N^6-benzoyl-2'-O-tetrahydropyranyladenylyl-(3'-[2,2,2-trichloroethyl]-10)decyl-(2,2,2 trichloroethyl)-1-phosphate. ($TracAt^{bz}$-Ut^{bz}-Ct^{bz}-At-d-, XXXV).

$TracAt^{bz}$-Ut^{bz}-Ct^{bz}-AtOH (XXIII) (0.56 g, 0.22 mmol), previously synthesized by the stepwise phosphorylation and coupling procedures described, was phosphorylated as before and activated with TPS (0.099 g, 0.33 mmol) as for coupling. After 1 h, 1,10-decane diol (0.303 g, 2.20 mmol), dried by repeated solution in anhydrous pyridine and evaporation to a small volume under reduced pressure (3 x 25 ml), was added and the combined volume of the reaction mixture reduced to ca. 5 ml by evaporation under reduced pressure. After 2 days no further change in the ratio of product to starting material (approximately 1:2) was observed and the reaction was quenched with 1 g ice. After 1 h, the quenched mixture was poured into ice water (50 ml) and extracted repeatedly with methylene chloride (4 x 10 ml). The combined extracts were evaporated to dryness under reduced pressure and on resuspension in methylene chloride, decane diol precipitated and was removed by filtration.

The filtrate was purified by chromatography on silica gel (10 g). Decane diol eluted with 2% methanol and $TracAt^{bz}$-Ut^{bz}-Ct^{bz}-At-dOH (XXXIV) eluted with 2.5-3% methanol, yielding (0.180 g, 28%, yellow spot, R_f = 0.57) after evaporation to dryness.

Phosphorylation of XXXIV with a 2 fold excess of phosphorylating agent was incomplete as indicated by t.l.c. (yellow spot, R_f = 0.57 → yellow spot, R_f 0.2) after one day but no further reaction was observed. The reaction was quenched with 0.5 g ice and after 1 h poured into 5 ml ice water and repeatedly extracted with methylene chloride (3 x 5 ml). The combined extracts were evaporated to dryness under reduced pressure, the final traces of pyridine being removed by co-distillation with toluene.

On resuspension of the residue in methylene chloride (10 ml), triisopropybenzene sulfonate precipitated and was removed by filtration.

The filtrate was subjected to chromatography on silica gel (10 g), unphosphorylated material eluting with 2-3% methanol, and the product TracAt-Ut-Ct-At-d- (XXXV) and triisopropylbenzenesulfonic acid co-eluting with 3-5% methanol. Calculation of yield was not practical due to the extent of contamination of the product by the sulfonic acid indicated by t.l.c.

2.11.4. ApUpCpApdOH

TracAt-Ut-Ct-At-dOH (XXXIV, 15 mg) was stirred overnight with 1 ml DMF and approximately 20 mg of Zn/Cu couple. Then 2 ml of methanolic ammonium hydroxide (1:1, v/v) was added and stirring was continued 24 hr. The mixture was filtered and the Zn/Cu couple was washed with 1 N NH_4OH (10 ml). The combined filtrates were stirred with Dowex 50 (NH_4^+) (approximately 2 g) for 0.5 hr, filtered, and the Dowex resin was washed with 1 M NH_4OH (approximately 20 ml). The volume was reduced to approximately 3 ml by evaporation under reduced pressure. The pH was adjusted to 2 with 2 N HCl and after 24 hr, this solution was neutralized by the addition of 1 M NH_4OH. After reducing the volume to approximately 0.5 ml by evaporation

under reduced pressure. This material was applied to Whatman 3 MM for paper chromatography using solvent C after development. The u.v. absorbing band (R_f 0.4-0.5) was cut out and eluted with distilled water, giving 69 A_{260} units (27%).

One aliquot of 10 A_{260} units was evaporated to dryness under reduced pressure and redissolved in Tris HCl (0.1 N, pH 7.8, 100 µl). Pancreatic RNase (1 mg/ml, 50 µl) was added and the mixture was incubated overnight at 37°C. A second aliquot of 10 A_{260} units was similarly dissolved in 100 µl of sodium succinate buffer (0.25 M, pH 6.5) and spleen phosphodiesterase (1 mg/ml, 50 µl) was added and the mixture incubated at 37° overnight.

Chromatography data for these digests and the corresponding standards are shown in table 10 (p 172), showing the correct structure.

2.11.5. ApUpCpApdp

TracAtUt-At-Ct-d- (XXXV, 15 mg) was completely deprotected exactly as described in the preceeding section for XXIV. Paper chromatographic purification of the deprotected material gave 42 A_{260} units (16%) (R_f 0.13, solvent C) of ApUpCpApdp

Aliquots of 10 A_{260} units were digested with spleen phosphodiesterase, 10 A_{260} units with pancreatic RNase, and 2 A_{260} units with alkaline phosphatase and the data, presented in table 10, p 172, confirm the identity of the product.

DEAE cellulose chromatography of this and subsequent preparations indicated the correct number of charges, the product being eluted at 0.21-0.22 M NaCl.

2.12. Coupling of Nucleotide Sequences to Cellulose

2.12.1. Coupling of Protected Sequences via Phosphotriester Linkages.

TracAt-Ut-Ct-At-d- (XXXV), synthesized by the methods described, (0.100 mmol) was activated with TPS (90 mg 0.300 mmol) in anhydrous pyridine (3 ml) in the usual manner. After one hour, this solution was added to cellulose (1.0 g, Sigma cellulose 99.5%) which had been oven dried and the mixture sealed under dry nitrogen. After nine days the reaction was quenched by addition of methanol/water (9:1 v/v) (2 ml). After 1 h the mixture was filtered and washed thoroughly with the same

solvent. The combined filtrates were evaporated to dryness under reduced pressure. T.l.c. analysis of this residue indicated a large amount of unreacted nucleotide sequence. Ultraviolet spectra of this recovered material and the starting material at the same dilution (from a solution of 50 ml, 0.025 ml was removed and diluted to 2.5 ml with methanol) indicated that little or no coupling had occurred.

An aliquot of the washed cellulose (100 mg dry wt.) was treated with 1% NH_4OH in methanol (5 ml) for 2 h and filtered. The filtrate was evaporated to dryness and redissolved in several drops of CH_2Cl_2. T.l.c. of this material indicated only traces of yellow after developing and spraying with ceric sulfate.

TracAt^{bz}-dOH (XXXI) (74 mg, 0.066 mmol) was phosphorylated in the prescribed manner. The phosphorylated intermediate (XXXII) was dried and activated with TPS as for a coupling reaction in a volume of 20 ml of anhydrous pyridine, and this solution was added to cellulose (Sigma) (1 g) previously dried by repeated suspension in anhydrous pyridine and evaporation under reduced pressure (6 X 50 ml). The reaction volume was reduced to ca 10 ml. After 2 days, the suspension was filtered and the cellulose washed thoroughly with pyridine. The filtrates were evaporated to a small volume under reduced pressure, dried and activated with TPS once again and recombined with the cellulose for a further period of two days. Again the mixture was filtered and the cellulose washed with pyridine, then methanol, and finally methylene chloride. T.l.c. analysis of the filtrates indicated much unreacted XXXII.

An aliquot of cellulose (100 mg) was stirred for 3 h in 1% NH_4OH in methanol (2 ml). This mixture was filtered and the filtrate was

spotted entirely on one t.l.c. plate beside a standard consisting of 20 µl of TracUtOH (5 mg/ml in 1% NH_4OH in methanol for 3 h) and the plate was eluted with pure CH_2Cl_2. After development, the standard, containing about 10% of the theoretical yield of triphenylmethoxyacetyl groups bound to 100 mg of cellulose, showed a very strong spot (R_f = 0.7) while the cellulose hydrolysate indicated only a trace suggesting that less than 1% coupling had occurred.

Cellulose (Sigma) was prepared by stirring 18 h in pyridine, 24 h in 50% NH_4OH/CH_3OH and 24 h in 0.1 N HCl. This was followed by thorough washing with water and drying overnight at 100° under reduced pressure.

TracUtOH (XXVII) (100 mg, 0.112 mmol) was phosphorylated, isolated and activated for coupling to the cellulose prepared above (1 g). This coupling reaction was carried out in a volume of 10 ml. A second reaction identical to the first in every respect was prepared and its volume reduced by evaporation under reduced pressure until the cellulose appeared as a dampened powder rather than a mobile slurry. After 2 days, each batch was filtered and thoroughly washed with pyridine, methanol, and finally methylene chloride.

Aliquots of each batch (100 mg dry wt.) were treated with 1% NH_4OH in methanol and the filtrates analyzed as before. Yields of coupling estimated by this method, were about 1% for the "wet" reaction and about 2% for the "damp powder" reaction. Other spots were also observed in these hydrolysate t.l.c. analyses indicating that the washing procedure was not completely effective.

TracAt^{bz}-dOH (XXXI) (250 mg, 0.331 mmol) was phosphorylated, isolated and coupled to cellulose (Whatman CF-11, 500 mg) by the "damp powder" method described above. Analysis of the cellulose indicated less than 1% yield coupled to cellulose.

2.12.2. Coupling of Protected Sequences via Phosphodiester Linkage

TracAt^{bz}-dOH (XXXI) (74 mg, 0.066 mmol) was phosphorylated and isolated as before. The phosphorylated intermediate (XXXII) was dissolved in DMF (2 ml) and Zn/Cu couple, prepared by the method of LeGoff (1964), (~ 100 mg) was added. After one day, t.l.c. indicated loss of the phosphate protecting groups ($R_f \rightarrow 0.0$). The mixture was filtered and reduced to a small volume under reduced pressure. This material was dried by repeated solution in anhydrous pyridine and evaporation to a small volume under reduced pressure and activated with TPS as before. This material was added to 1 g of cellulose (Sigma), similarly dried, and allowed to react for two days. The cellulose was then filtered and washed with anhydrous pyridine. The combined filtrates were evaporated to a small volume under reduced pressure dried and activated with TPS as before and recombined with the same batch of cellulose. After a further two days, the mixture was filtered and washed with pyridine, methanol and finally methylene chloride.

Analysis of this cellulose by hydrolysis with 1% NH_4OH in methanol indicated about 2% coupling.

TracAt^{bz}-dOH (XXXI) (200 mg 0.265 mmol) was phosphorylated and the phosphate protecting groups removed with Zn/Cu couple in DMF as described

above. One quarter of this material was dried by repeated solution in anhydrous pyridine (6 X 25 ml) and evaporation to a small volume under reduced pressure, activated with TPS (115 mg, 0.0381 mmol) and added to cellulose (Whatman CF-11, 500 mg) previously dried by treatment with pyridine as before.

After 1 day, the reaction was quenched by addition of aqueous pyridine (50/50, v/v) for one h. The suspension was filtered and the cellulose washed with methanol and finally methylene chloride.

Analysis of the cellulose as previously described indicated 4-5% yield of nucleotide bound.

2.12.3. Coupling of Deprotected Sequences

Synthetic sequences to be coupled to cellulose were deprotected by the three step procedure and purified by DEAE cellulose chromatography.

This procedure was presented by Astell and Smith (1972) as a modification of the method of Gilham (1968).

Whatman 3 MM paper, cut in strips (5 cm X 18 cm), was treated with EDTA (10^{-3} M, pH 7.0) for 3 h; water, 3 h; and after drying in air, 1% HCl in absolute methanol for 3 days. The paper strips were then rinsed thoroughly with water and allowed to stand in water (changed 3 X 0.5 hr intervals) before drying in air. They were then allowed to stand in MES buffer (0.05 M, pH 6.0) for 4 h before finally washing by descending chromatography with pure water overnight. These were dried and stored in a sealed container at 4° for future use.

Coupling reactions were performed by preparing a solution (500 µl)

containing 10 - 50 A_{260} units of deprotected nucleotide, and 50 mg of CMC, in MES buffer (0.04 M, pH 6.0), and spreading evenly on one end (5 cm X 12 cm, ~ 1 g cellulose) of a paper strip as prepared above. A further 500 µl MES buffer was used to wash the sample onto the paper before it was allowed to dry in the laboratory atmosphere. After two days, a further portion of CMC (50 mg) in MES buffer was applied as above and allowed to dry for a further two days. At the end of this period, the paper was washed by descending chromatography with sodium phosphate buffer (0.5 M, pH 7.0, 20 - 30 ml). After drying, this paper was stored at 4° until used.

The eluate from each paper was diluted to 100 ml with water and ultraviolet spectra were recorded against a blank containing either the buffer alone, or eluate from a blank reaction containing no nucleotide. In some cases, an aliquot of the reaction mixture before coupling was retained and diluted to the same concentration, after adding an amount of CMC corresponding to the second addition to the coupling reaction, and this solution was placed in the sample beam and the eluate from the reaction was placed in the reference. The difference spectrum corresponded directly to the quantity of nucleotide bound to the cellulose.

Yields of nucleotide coupled were determined independently from each of the three spectra and compared. Contributions of these spectra at 260 nm from CMC could readily be eliminated by arithmetic methods as the spectrum of CMC has a strong maximum and a minimum at 261 and 258 nm respectively. Assuming that the absorbance due to the nucleotide present is the same at these two wavelengths and using values obtained from a spectrum of CMC alone a formula was derived for calculation of the

absorbance due to nucleotide alone in a spectrum of a mixture:

$$A_o = 4.33\ A_{258} - 3.33\ A_{261}$$

where A_o is the absorbance due to nucleotide and A_{258} is the total absorbance at 258 nm and A_{261} is the total absorbance at 261 nm.

Yields calculated by these subtractive methods were also compared with direct analysis of the cellulose. Samples of 100 - 200 mg of cellulose were submerged in a measured volume (5 - 10 ml) of 1 N HCl or 0.2 N NaOH overnight and the ultraviolet spectrum of the supernatant indicated the quantity of nucleotide material which had been bound.

2.12.4. Coupling of ApUpCpApdp to Cellulose

The deprotected oligonucleotide with its attached spacer group (ApUpCpApdp) was coupled to cellulose using the method of Astell and Smith (1972) as follows. The deprotected oligonucleotide with its attached spacer group (ApUpCpApdp, 21 A_{260} units) was dissolved in MES buffer (0.04 M, pH 6.0, 250 µl) and CMC (25 mg) was added. An aliquot of 10 µl was removed and diluted to 2.5 ml for later comparison. This solution was spread evenly on a strip of Whatman 3 MM paper (2.5 x 12 am) previously prepared as described in the preceeding section. A further portion of MES buffer (0.04 M, pH 6.0, 250 µl) was used to rinse the container used for the oligonucleotide coupling solution, and this was also spread evenly on the paper strip. The strip was allowed to dry at room temperature in the laboratory atmosphere for 48 hr when a further application of CMC (25 mg CMC in MES, 0.04 M pH 6.0, 250 µl). Again the container used to prepare this solution

was rinsed with 250 μl of the same buffer and the rinse was also applied to the paper.

After a further 48 hr the paper strip was eluted with sodium phosphate (0.5 M, pH 7.0) by descending chromatography (20 ml of eluate was collected) overnight before drying the paper and diluting the eluate to 62.5 ml.

To the aliquot of the original coupling reaction 0.5 mg CMC was added (corresponding to the second application of CMC in the coupling procedure) and the ultraviolet spectra of this solution was recorded against the diluted eluate obtained above. From this difference spectrum, the amount of oligonucleotide bound can be calculated: A_{260} x volume of eluate = 0.078 A_{260} units/ml x 62.5 ml = 4.9 A_{260} units. From the ultraviolet spectra of the eluate recorded against water and against CMC (1 mg/2.5 ml) the amount of unbound oligonucleotide was calculated using the formula described in the preceeding section (A_{260} due to nucleotide = 4.33 A_{258} - 3.33 A_{261}) and multiplying this absorbance by the volume. These calculations indicated that 16 A_{260} units of oligonucleotide were eluted, unbound. Thus the results obtained by these calculations agree: 24% of the oligonucleotide was bound to the paper.

Alkaline hydrolysis of 0.2 g of the paper containing the bound oligonucleotide was carried out in 5 ml of 2N NaOH overnight at room temperature. This treatment released 1.06 A_{260} units of u.v. absorbing material, indicating that 5.3 A_{260} units of oligonucleotide (25%) had been bound in this experiment.

Samples of ApUpCpApdp - cellulose were digested with spleen phosphodiesterase and with pancreatic RNase (in addition to the water and NaOH "digests") and samples of cellulose-pApUpA were digested with snake venom phosphodiesterase and pancreatic RNase (in addition to the water and NaOH "digests").

Amounts of u.v. absorbing material released are presented in table 12 (p 193). It was observed that the u.v. spectra obtained in all of these experiments were noisy and had high background levels of absorbance ($A_{300} \sim 25\% A_{260}$) which differed for each reaction. The presence of phenol as a preservative in the pancreatic RNase preparation contributed to high absorbance in samples digested with this enzyme. Eliminating the absorbance in the blank and that due to the enzyme solutions did not eliminate these high background levels. Thus the values recorded must be considered maximum estimates.

2.13. Enzymatic Acylation of tRNA

2.13.1. Preparation of Isoleucine:tRNA Ligase

A crude preparation containing this enzyme was prepared by a method adapted from Hoskinson and Khorana (1965) and Nishimura (1967).

Commercial bakers Yeast, (Lallemand, Montreal, 1 lb) was purchased locally. 100 g yeast was suspended in 100 ml Tris HCl (0.05 M, pH 7.5), $MgCl_2$ (0.01 M) and mercaptoethanol (0.02 M) and glass beads (3 M Co. "Superbrite" type 130-5005, 400 g) were added. This mixture was homogenized in a VirTis homogenizer (4 X 2 min top speed, with 3 min cooling periods between). All subsequent operations were carried out at 0-4°. The suspension was centrifuged at 2000 X g (10 min) and the pellet, consisting mostly of glass beads, was washed with 100 ml of the same buffer solution. The combined supernatants were centrifuged at 10,000 X g for 20 min and the supernatant centrifuged again at 30,000 X g. A portion of this supernatant (35 ml) was applied to a column of DEAE

cellulose (1.5 X 14 cm) previously equilibrated with Tris HCl (pH 7.5, 0.05 M), containing $MgCl_2$ (0.01 M), and mercaptoethanol (0.006 M), KCl (0.06 M), and NaCl (0.15 M), and was eluted with the same buffer.

A volume of 120 ml was collected and made up to 85% saturation with solid ammonium sulfate. After one hour of gentle stirring, the precipitate was collected by centrifugation at 5000 X g for 15 min and the precipitate redissolved in 10 ml of Tris HCl (0.1 M, pH 7.8), $MgCl_2$ (0.01 M), KCl (0.01 M) and mercaptoethanol (0.006 M). After dialyzing overnight against a large volume of the same buffer, an equal volume of glycerol was added and the preparation was stored at -20° until required. No loss of activity was observed after 6 months.

2.13.2. tRNA Acylation Assay

This procedure was adapted from a preparative scale procedure used by Goss and Parkhurst (1974).

A 5 X stock buffer containing Tris HCl (0.5 M, pH 7.5), $Mg(OAc)_2$ (150 mM), KCl (50 mM) was prepared and stored at 4° until freshly diluted for use. Solutions of triisodium ATP (12.5 mM), bulk tRNA (4 mg/ml), and ^{14}C isoleucine (4 µCi/ml) or ^{3}H isoleucine (100 µCi/ml) were prepared in this diluted buffer.

Assays were performed by incubating together 1 volume of each of the three solutions above at 37° for 5 min before adding 2 volumes of the enzyme solution prepared previously. Aliquots (10 - 100 µl) were withdrawn over a period of 0 - 30 min and spread on Whatman 3MM paper discs (2.5 cm). These were immediately plunged into ice cold TCA (5%) and

washed with cold TCA (3 X 15 min) and with ethanol/ether (50/50, ice cold) 15 min and finally with ether, before drying. The dried discs were placed in scintillation vials containing 10 ml of xylene/triton X-114 based scintillation cocktail (Anderson and McClure, 1974) and counted in a Beckman LCS 230 counter.

Incorporation of radioactivity into acid precipitable form increased until a plateau was reached after 10 - 15 min. Other variations on these conditions, including the method of Hoskinson and Khorana (1965) were less satisfactory. Once these conditions had been established, levels of incorporation were measured by taking only one sample at 10 - 15 min after addition of the enzyme.

2.13.3. Preparative Acylation of tRNA

In the preparative scale reactions, the proportions of each component are the same as described above. However the reactions were quenched after 20-30 min by the addition of one volume of ice cold sodium acetate (0.2 M, pH 4.5, with 1 M NaCl) and cooling to 0°. Acylated tRNA was precipitated by addition of 2 volumes of ice cold absolute ethanol. The precipitated was collected by centrifugation for 15 min at top speed in a desk top clinical centrifuge. The supernatant was decanted and the pellet stored at -20° or redissolved in the desired solution.

In the earlier stages of this work, the pellet was resuspended in 1 M NaCl and used directly in the affinity chromatography studies. The high level of protein remaining in these preparations, evidenced by cloudiness of the suspension, made additional purification desirable.

This was carried out by suspending the ethanol pellet in 0.3 M NaCl (buffered with 0.1 M Na acetate, pH 4.5) and applying the solution to a small column of DEAE cellulose (0.5 cm X 5 cm), previously equilibrated with the same solvent. After applying the sample and washing thoroughly with this buffer, the salt concentration in the buffer was raised to 1.0 M and several fractions of small volume were collected and tested for ultraviolet absorbance. The major ultraviolet absorbing fractions were combined and stored or again precipitated with 2 volumes of cold ethanol.

Presence of radioactivity in these purified fractions was confirmed by measuring the acid insoluble radioactivity as described for the assay procedure or by placing an aliquot of the solution directly in the scintillation vial with the scintillation fluid.

2.13.4. Spontaneous Hydrolysis of Aminoacyl-tRNA

Sodium phosphate buffers (0.01 M, pH 5.5, 6.0, 6.5, 7.0 in 1 M NaCl and 10 mm Mg $(OAc)_2$ were prepared. Into each buffer (400 µl) tRNA labelled with ^{14}C isoleucine (5800 cpm/A_{260}, 200 µl containing approximately 2500 cpm [acid precipitable]) in 1 M NaCl was added. Aliquots of 100 µ were assayed for acid precipitable radioactivity as described previously at 0, 8, 24, 48 and 72 hr. (These data are plotted in figure 22, p 184).

2.14. Affinity Chromatography

2.14.1. Column Preparation

Whatman 3MM paper (0.5 g) as used for the oligonucleotide coupling reactions was cut into small squares (~ 1 mm^2) and suspended in 20 ml of buffer (0.01 M sodium phosphate, pH 7.0, 1 M NaCl) and stirred rapidly overnight at 4°. The resulting slurry was not completely homogeneous and on attempting to pack this material into a glass tube (0.5 cm ID) considerable layering and void formation were observed.

Sonication of the paper chips (6 X 1 min, 2 - 3 min cooling between using a Bronson Sonifier with a broad tip) in an ice bath gave a smoother slurry but packing problems persisted until the following pro-

cedure was employed.

The slurry was placed in a test tube. A glass column was prepared by drawing a fine tip on a soft glass tube (1 m X 0.5 cm ID) and fire polished at the ends. A small wad of glass wool was placed firmly into the drawn tip from the opposite end and the tube was filled with water or buffer. A syringe was attached to the drawn tip. The open end of the column was inverted into the test tube containing the slurry and the slurry was drawn up into the tube with the syringe. Several steps were required as the syringe could not contain the whole volume with one aspiration.

When all of the slurry had been drawn into the column, it was righted and mounted with a tightly fitting top having a connection to 3/32 in. (OD) Tygon tubing. Buffer was pumped through the column using a peristaltic pump, as slowly as possible. Faster pumping resulted in tight packing at the bottom of the column, and none at the top, causing the back pressure to rise and the fittings to burst or leak. Gaps could be eliminated by gentle tamping with a glass rod. After 3-6 h the bed had reached a stable volume, the tube remaining above the bed was removed, and the end fire-polished before refitting the top.

When temperatures below ambient were required, the column was jacketted by wrapping with Tygon tubing (3/8 in. OD) after mounting, and the cooling solution passed through this tube by gravity flow. Temperatures of -4 -0° were maintained in this way with the help of glass wool wrapped around the cooling system for insulation.

2.14.2. Experimental Affinity Chromatography

Samples were applied by removing the top and withdrawing the solvent over the cellulose bed, and gently adding the sample solution (< 0.5 ml) with a fine tipped micropipette. Replacement of the column top created enough pressure to force the sample gently into the bed and solvent pumping was begun directly or after adding another layer of solvent above the bed. Flow rates of < 1 ml/h were obtained.

Fraction sizes of 10 - 25 drops each were found to be suitable depending on the temperature and salt concentration.

Samples of 0.1 - 5 A_{260} units of tRNA were measured from a solution containing the tRNA in 1 - 1.5 M NaCl and in some experiments, 10 mM Mg $(OAc)_2$ also and which had previously been measured for concentration of ultraviolet absorbing material and radioactivity.

After application of the sample, fractions were collected in pre-weighed tubes. These fractions were diluted with a measured volume of water (600 - 800 µl), weighed again and their ultraviolet absorbance at 260 nm recorded. Each sample was then washed into a scintillation vial with an equal volume of water and 10 ml scintillation fluid (Anderson and McClure, 1974) added. These were counted as before.

From these data, the total recovery of A_{260} units and radioactivity as well as the shape of the elution profile for each were obtained. These results are reported in c.p.m. as counting efficiencies remained constant (± 3%) over each series of samples. This was indicated by the external standard ratios measured for each sample.

3. SYNTHETIC MESSENGER RNA

3.1. Introduction

The use of synthetic oligo- and polynucleotides in solving the genetic code and its punctuation is well known. It is less well understood how these punctuation signals are actually read by the ribosome, as initiation signals do not appear at the 5' end, nor do termination signals appear at the 3' end of the messenger RNA sequence. Isolation of pure, natural mRNA and its subsequent characterization of sequence, secondary, and tertiary structure has not yet been accomplished although many such undertakings are in progress. The study of how initiation and termination of translation are dependent on these signals is, in turn, dependent on the availability of well characterized sequences. Availability of synthetic messages mimicking specific features of native sequences would be a tremendous asset in such studies.

The first step in achieving this goal should be the synthesis of a very simple mRNA, having only the basic required features: initiator codon, terminator codon and several internal codons. Once such a sequence has been synthesized, characterized, and shown to serve as a proper template for translation, further additions or modifications can be introduced in order to study their effects on initiation and termination. The object of this work was to design and synthesize such a message.

Any sequence of nucleotides, or codons, separating the initiator and terminator codons could be employed, but as repeating sequences have already been studied and are less "natural", a unique sequence would be preferable. The peptide coded would also have to be characterized. In this respect, a naturally occuring, biologically active peptide sequence would perhaps be the easiest to detect, analyze and characterize. Obviously, to chose a peptide such as insulin or ribonuclease would be too ambitious: accordingly, a brief survey of short, native peptides of known sequence was undertaken and the results are shown in table 5.

Many of these sequences contain modifications such as acetylation of the amino terminal residue, amidation of the carboxyl terminal, residue sulfonylation of a tyrosyl residue, or cyclization of the amino terminal glutamic acid. Although some of the processing steps leading to these modifications _in vivo_ are understood, the method of biosynthesis of the amino acid sequence has not been proven. It is believed that longer sequences are synthesized on ribosomes as part of a larger precursor, such as is insulin (see Tager and Steiner, 1974, for review), but this is much less certain for shorter sequences. Some incorporation of amino acids into peptide antibiotics is accomplished enzymatically, and proceeds by activation of the amino acids _via_ enzyme bound adenylate and thioester intermediates (see Kuruhashi, 1974, for review). Other amino acids are incorporated into bacterial peptidoglycans enzymatically _via_ tRNA but without ribosomal participation (Gauss _et al._, 1974). However, it must be emphasized that this study is not intended to bear on the problem of how these peptides are synthesized _in vivo_.

Table 5.

Amino acid sequences of some short, naturally occurring peptides having biological activity.

Name	Sequence	Reference
1. Angiotensin I (Bovine)	asp-arg-val-tyr-val-his-pro-phe-his-leu	Handbook of Biochemistry, 1970
I (equine)	asp-arg-val-tyr-ile-his-pro-phe-his-leu	"
II	asp-arg-val-tyr-ile-his-pro-phe	"
2. Arg-vasopressin	cys-tyr-phe-gln-asn-cys-pro-arg-gly-NH_2	"
3. Arg-vasotocin	cys-tyr-ile-gln-asn-cys-pro-arg-gly-NH_2	"
4. Bradykinin	arg-pro-pro-gly-phe-ser-pro-phe-arg	"
5. Caerulin	pyroglu-gln-asp-tyr(SO_3)-thr-gly-trp-met-asp-phe-NH_2	Dayhoff and Eck, 1969
6. Elidoisin	pyroglu-pro-ser-lys-asp-ala-phe-ile-gly-leu-met-NH_2	Handbook of Biochemistry, 1970
7. Glumitocin	cys-tyr-ile-ser-asn-cys-pro-gln-gly-NH_2	Dayhoff and Eck, 1969
8. Isotocin	cys-tyr-ile-ser-asn-cys-pro-ile-gly-NH_2	Handbook of Biochemistry, 1970
9. Kallidin	lys-arg-pro-pro-gly-phe-ser-pro-phe-arg	"
10. Kininogen	met-lys-arg-pro-pro-gly-phe-ser-pro-phe-arg	"
11. Lys-vasopressin	cys-tyr-phe-gln-asn-cys-pro-lys-gly-NH_2	"

Table 5 (continued)

Name	Sequence	Reference
12. Mesotocin	cys-tyr-ile-gln-asn-cys-pro-ile-gly-NH_2	Handbook of Biochemistry, 1970
13. MSH Release Inhibiting Hormone	pro-leu-gly-NH_2	Nair *et al.*, 1971
14. Oxytocin	cys-tyr-ile-gln-asn-cys-pro-leu-gly-NH_2	Handbook of Biochemistry, 1970
15. Phylokinin	arg-pro-pro-gly-phe-ser-pro-phe-arg-ile-tyr-NH_2	Dayhoff and Eck, 1969
16. Physalaemin	pyroglu-ala-asp-pro-asn-lys-phe-tyr-gly-leu-met-NH_2	Handbook of Biochemistry, 1970
17. Porcine LH-FSH Releasing Hormone	pyroglu-his-trp-ser-tyr-gly-leu-arg-pro-gly-NH_2	Matsuo *et al.*, 1971
18. Ser^3-Ile^8-oxytocin	cys-tyr-ser-gln-asn-cys-pro-ile-gly-NH_2	Handbook of Biochemistry, 1970
19. Substance P	arg-pro-lys-pro-gln-gln-phe-phe-gly-leu-met-NH_2	Chang *et al.*, 1971
20. Scotophobin	ser-asp-asn-asn-glu-gln-gly-lys-ser-gln-gly-gly-gln-tyr	Anon. 1970
" (corrected)	ser-asp-asn-asn-gln-gln-gly-lys-ser-ala-gln-gln-gly-gly-tyr-NH_2	Ungar *et al.*, 1972

Introduction of modifications to the peptide after its release from the ribosome in vitro would be an additional complication, so in this study, a sequence not requiring such modifications was chosen. Obviously, synthesis of shorter sequences would be more suitable than synthesis of longer ones.

The degeneracy of the genetic code would allow a multiplicity of RNA sequences to code for the same peptide. The final choice of sequence was made by examining the possible RNA sequences for each peptide. Although the amino acid sequences of the bradykinin series and the angiotensin series generated the most favorable sequences, all the peptides shown in table 4 were examined by the method shown in figure 7 for angiotensin II (equine).

The sequence of amino acids was written across the page and beneath each amino acid, possible codons were written as shown. Attempts were made to specify blocks of nucleotides in such a way that as few as possible different blocks would be required.

Although one sequence derived in this way for kininogen is slightly more repetitive (A U G,A A G,C G C,C C G,C C A,G G U,U U U,U C G,C C U,U U U,A G A,U G A), at the time this work was begun, work was in progress on obtaining a suitably protected guanosine residue (Wastrodowski, 1972) and protected cytidine was not available in large quantities.. Thus the shorter sequence coding for N-formylmethionylangiotensin was selected as the target sequence. This sequence of thirty nucleotides can be assembled from the eight protected blocks having the sequences shown in figure 7. Two of these tetranucleotide blocks are identical and a third differs only in the last nucleotide. Three more of these blocks differ in the last two

Formylmethionylangiotensin II (Equine):

Amino acid

Sequence: fmet - asp - arg - val - tyr - ile - his - pro - phe - term.

Codon: A U G G A U A G A G U U U A U A U U C A U C C U U U U U A G
C G C C C C C G A
U C G U A A A A A
C G
A
G

A U A Blocks: A U A G A U A C A U A C

A U Blocks: A U G G A U C C A U U C

Others: A G U U A A

Nucleotide

Sequence: A U G G A U A G A G U A U A C A U A C A U C C A U U C U A A

Figure 7. Method for optimizing the nucleic acid sequence coding for formylmethionylangiotensin: the peptide sequence is written to allow the various codons common to each amino acid to be specified beneath it. Visual examination allows the selection of a nucleotide sequence having a maximum of common segments which in turn allows simplification of the proposed synthesis.

nucleotides: only the two trinucleotide blocks are unique.

Synthesis of these protected blocks was to be accomplished by the phosphotriester method of Neilson: stepwise incorporation of each protected nucleoside proceeding from the 5'- to the 3'-end. This approach depended on the availability of the selectively removable triphenylmethoxyacetyl group; and protection of the 5' terminal adenosine residue with this group was to be the first step in the synthesis of each block except the last. The synthesis of the corresponding uridine derivative for the last block has been described (Werstiuk and Neilson, 1972). A sample of each block completed was completely deprotected and digested enzymatically to demonstrate the fidelity of 3'-5' linkages and correctness of the sequence.

The remaining steps of assembly of the protected blocks could be accomplished by many different routes. The route chosen is shown in figure 8. Every second block must be specifically deprotected at the 5' end and subsequently coupled to the preceding block. This binary coupling procedure is repeated until the final sequence is assembled. The approximate doubling of size of the product with respect to the two reactants at each step offers the maximum difference between precursors and products in order to facilitate purification at each coupling step and the separation of the final product from any side products or "error sequences" arising during synthesis by incomplete reactions and/or faulty purification at intermediate stages.

The goal of this work was production of the proposed synthetic mRNA-like sequence by the phosphotriester method. In working towards this goal, the utility of this synthetic approach would be extended to

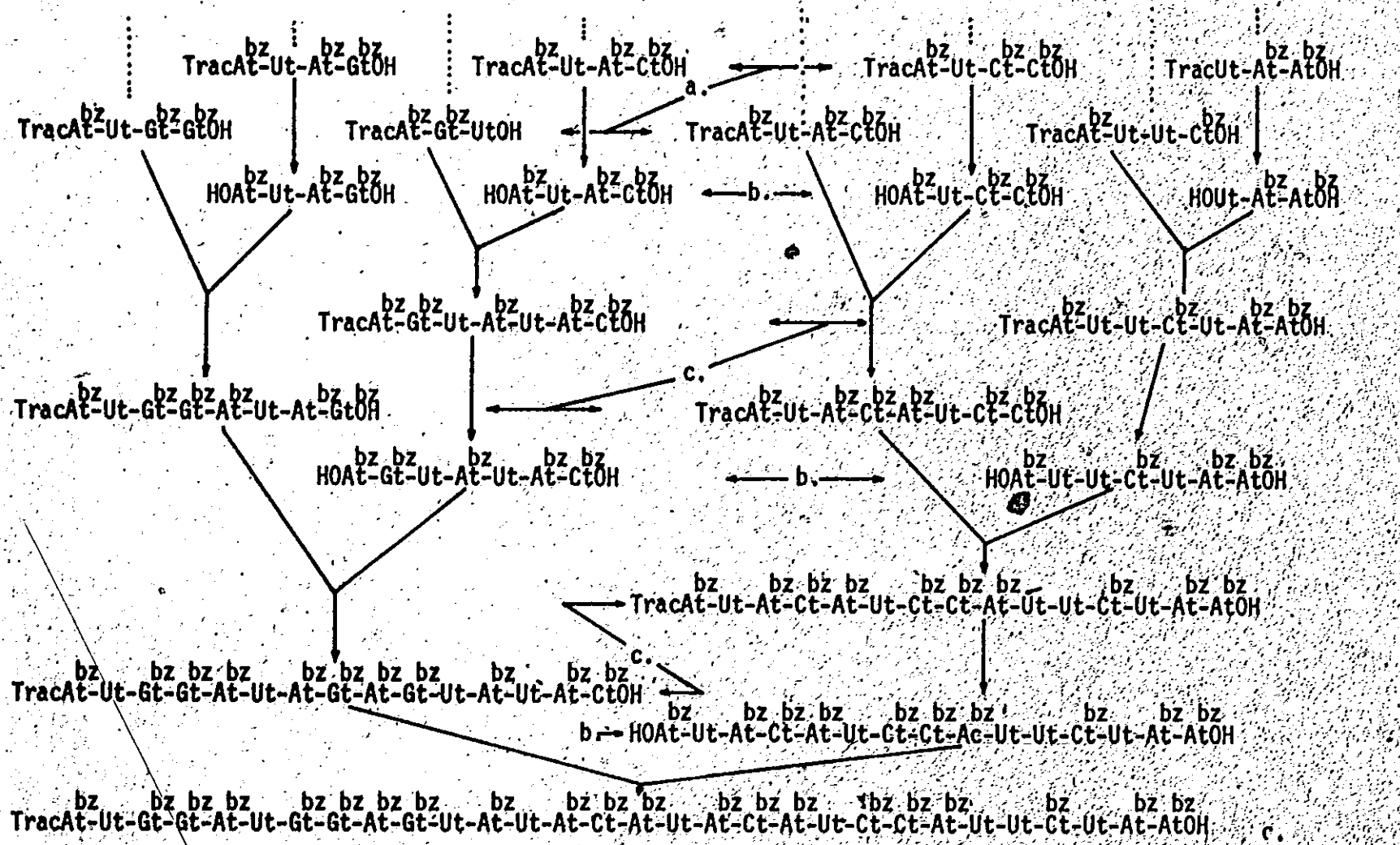

Figure 8. Logistics of assembling N-formylmethionylangiotensin messenger: a, blocks synthesized by the stepwise addition of nucleosides; b, 5′ deprotected blocks; and c, coupled products.

longer sequences, and in reaching this goal, a suitable basic sequence for research on the punctuation of translation would be provided. Thus the synthesis, as well as the final product, is of importance. After this work was undertaken, a similar project was described by Koster (1974) for the synthesis of a DNA duplex, one strand of which corresponded to an RNA code for this same peptide. The sequence of nucleotides and the synthetic approach used were different from those used here.

3.2. Results and Discussion

3.2.1. Synthesis and Characterization of N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenosine ($TracAtOH^{bz}$*, II).

Six of the seven protected oligonucleotide blocks required have adenosine as their 5'-terminal nucleoside. Since this method of synthesis

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* In order to condense the complex names and formulae of protected nucleosides and oligonucleotides into a more clearly readable form, the following system of abbreviations has been used. Nucleosides and protecting groups are presented as recommended (IUPAC -IUB Commission on Biochemical Nomenclature, 1970), Trac represents the triphenylmethoxyacetyl group, and a hyphen represents an internal or terminal 2,2,2-trichloroethyl phosphate group. 5'-substituents are placed to the left of a nucleoside symbol, 2'- and 3'-substituents are placed to the right in succession, and amino substituents as superscripts. Thus $TracAt^{bz}$-UtOH would represent N^6-benzoyl-2'-O-tetrahydropyranyl-5'-O-triphenylmethoxyacetyladenylyl-[3'-(2,2,2-trichloroethyl)-5']-2'-O-tetrahydropyranyluridine.

proceeds in the 5' to 3' direction, the synthesis of TracA^{bz}tOH is prerequisite for synthesis of these blocks. The synthesis of TracUtOH, which is required for the last block, has been described (Werstiuk and Neilson, 1972) and its use in block synthesis has been found satisfactory.

The same method was used for synthesis of TracA^{bz}tOH (Neilson et al. 1975) as outlined in figure 9. Low R_f diastereoisomer of N^6-benzoyl-2'-O-tetrahydropyranyladenosine (I), prepared by the method of Neilson and Werstiuk (1971), was treated with sodium triphenylmethoxyacetate (Werstiuk and Neilson, 1972) and triisopropylbenzenesulfonyl chloride (TPS) in anhydrous pyridine, giving one major product (yellow spot, R_f = 0.75 in the standard t.l.c. system described in the experimental procedures) in a yield of 73%. Attempts to crystalize this material from ethanol, ethyl acetate, and hexane, or methylene chloride and hexane were unsuccessful. P.m.r. spectrum of this product was consistent with the desired structure except for the presence of a sharp multiplet at ~ 1.2 p.p.m. as shown in figure 10. These resonances may be attributed to isopropyl methyl protons of the 5'-triisopropylbenzenesulfonyl derivative (III) of I. This contaminant was not separated from II in the methanol and methylene chloride systems, the principal solvent mixtures used for column and thin layer and column chromatography throughout this work.

Several other systems were tried on silica gel thin layer plates: ether, 2% methanol in ether, 5% methanol in benzene and 15% hexane in ethyl acetate. The last system appeared to give the best separation (II: yellow spot, R_f = 0.3; III: brown spot, R_f = 0.45).

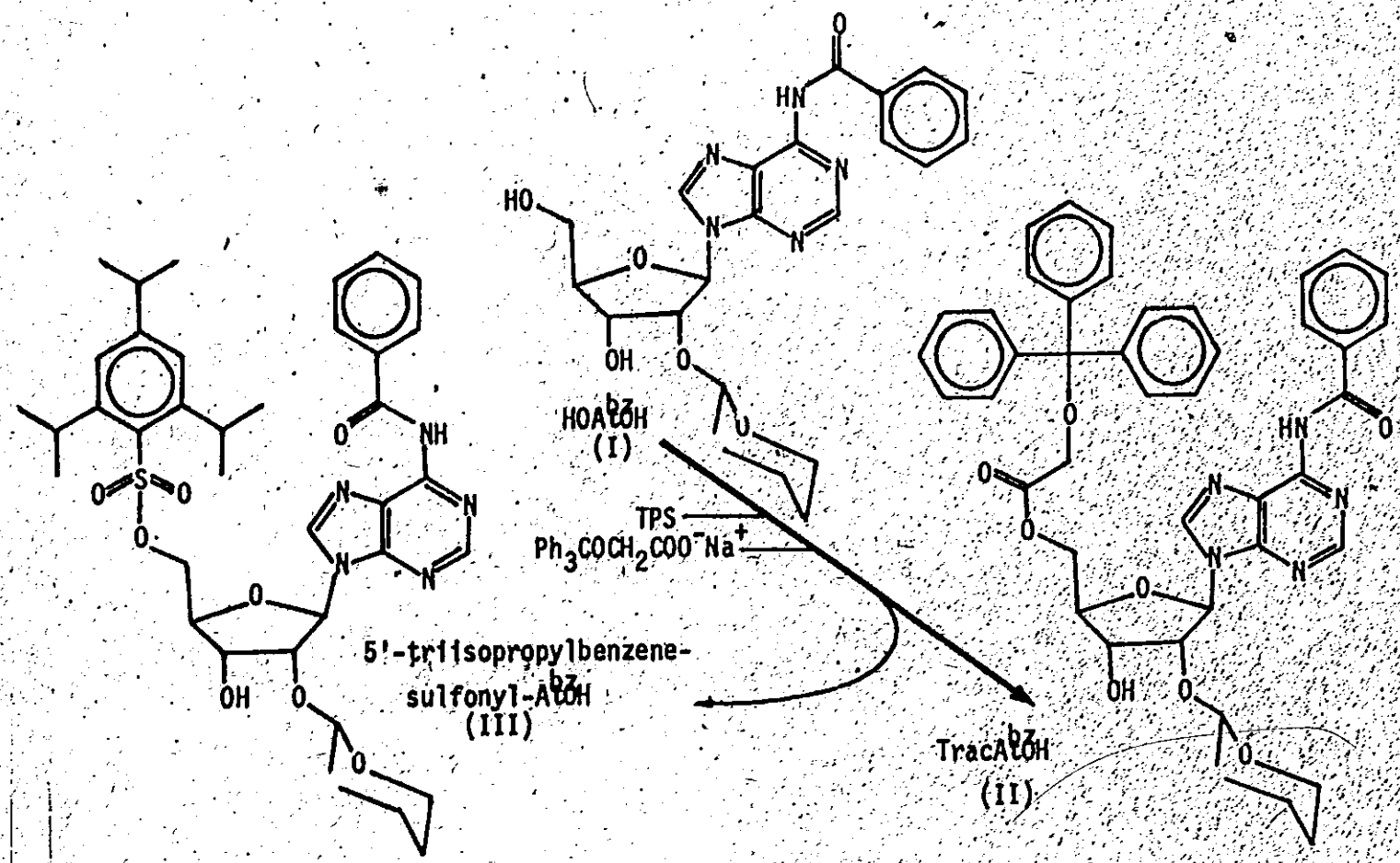

Figure 9. Synthesis of TracA^{bz}COH (II).

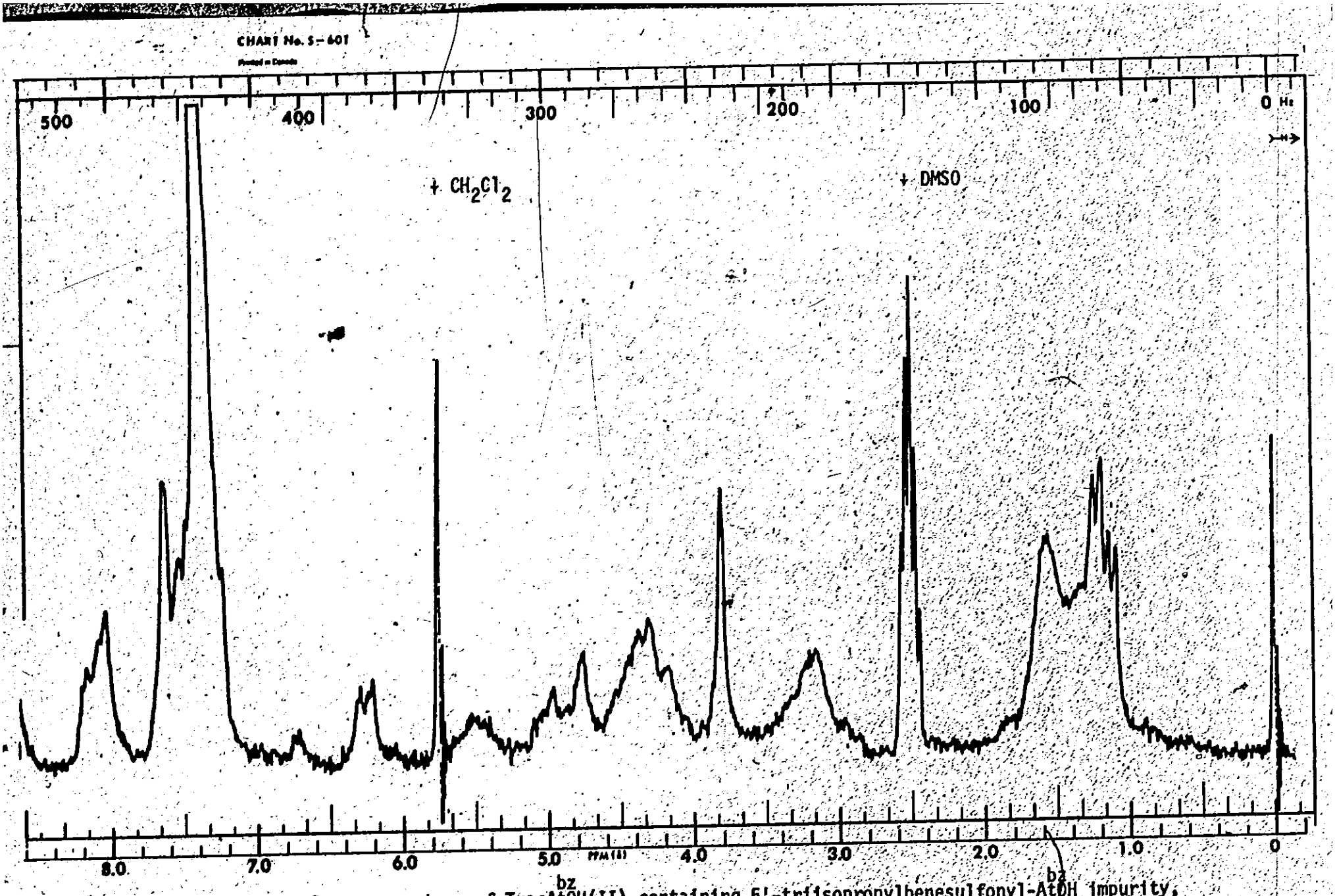

Figure 10: P.m.r. spectrum of TracAtOH(II) containing 5'-triisopropylbenesulfonyl-AtOH impurity, indicated by the sharp signals at δ = 1.2 p.p.m.

Purification of the mixture of II and III on a silica gel column eluted 60-80% ethyl acetate and 40-20% hexane gave a satisfactory separation of the two species but a small amount of mixed material remained. The p.m.r. spectrum of the desired product confirmed that it was free of contaminant and the p.m.r. spectrum of the contaminant was consistent with the triisopropylbenzenesulfonyl derivative (III) (see figures 11 and 12 respectively).

Attempts to increase the yield of II in this reaction by increasing the amount of TPS and/or triphenylmethoxyacetate resulted in formation of the 3',5' disubstituted product (yellow spot, R_f = 0.8) in larger quantities with little or no increase in overall yield of II.

Treatment of II with 1% concentrated ammonium hydroxide (v/v) (0.15N) in methanol regenerated I in yield greater than 95% (estimated by t.l.c.) in 2.5 hours and showed only traces of debenzoylated material. The different rates of hydrolysis of the N-benzamido function and the 5'-triphenylmethoxyacetyl function observed should allow selective removal of the 5' group under these conditions from oligoribonucleotides prepared with an adenosine residue in the 5' position.

A purified sample of II could not be crystalized; however a sample of the thoroughly dried foam gave a satisfactory elemental analysis for C, H, and N when calculated for the inclusion of one mole of water.

Preparations of TracAt^{bz}OH were routinely purified first in the ethyl acetate solvent system and then in the methylene chloride system giving yields of 60-75% in the form of a light yellow foam. The corresponding derivatives of cytidine and guanosine have been reported also (Werstiuk and Neilson, 1973; Neilson, Wastrodowski and Werstiuk, 1973).

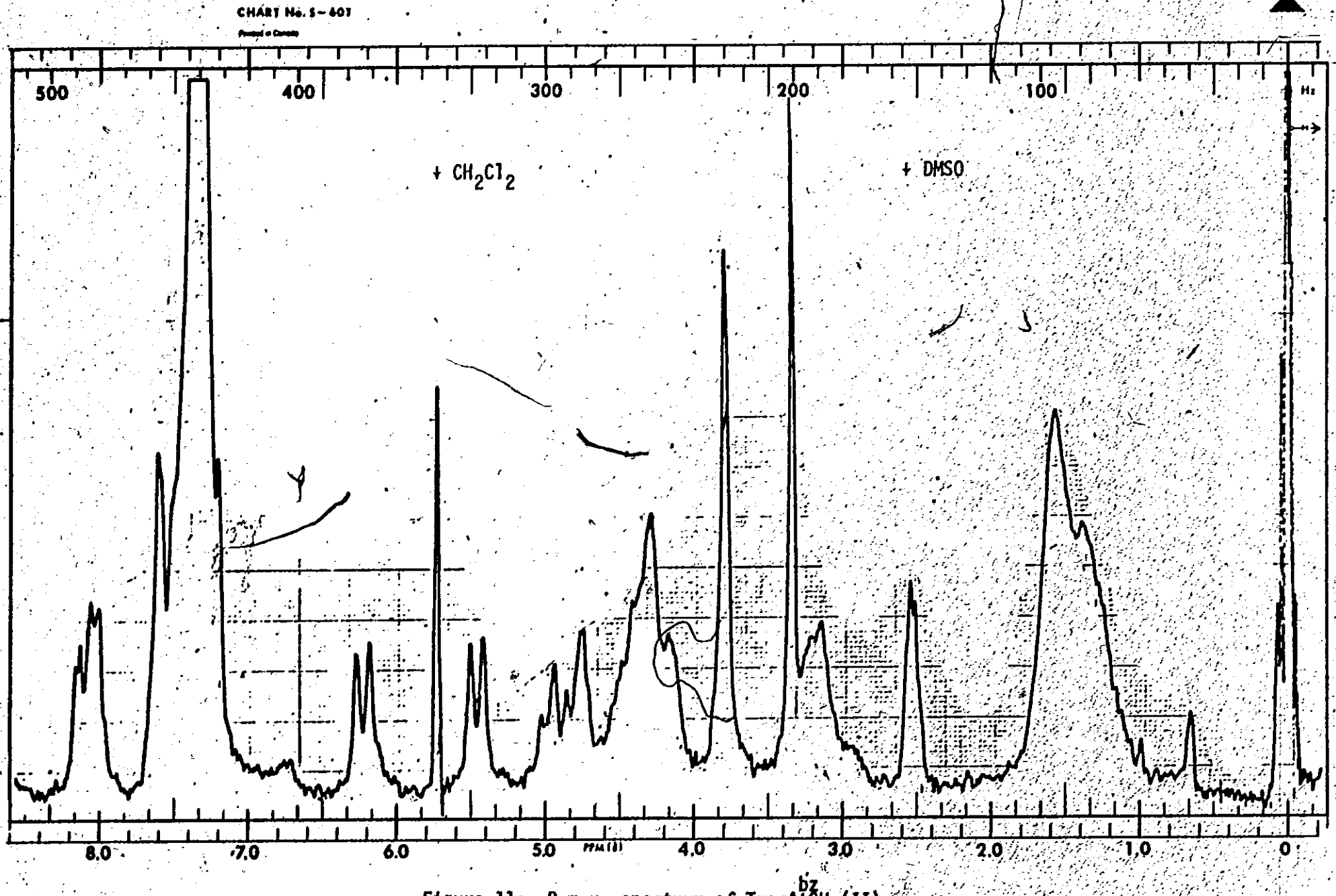

Figure 11: P.m.r. spectrum of TracAtOH (II).

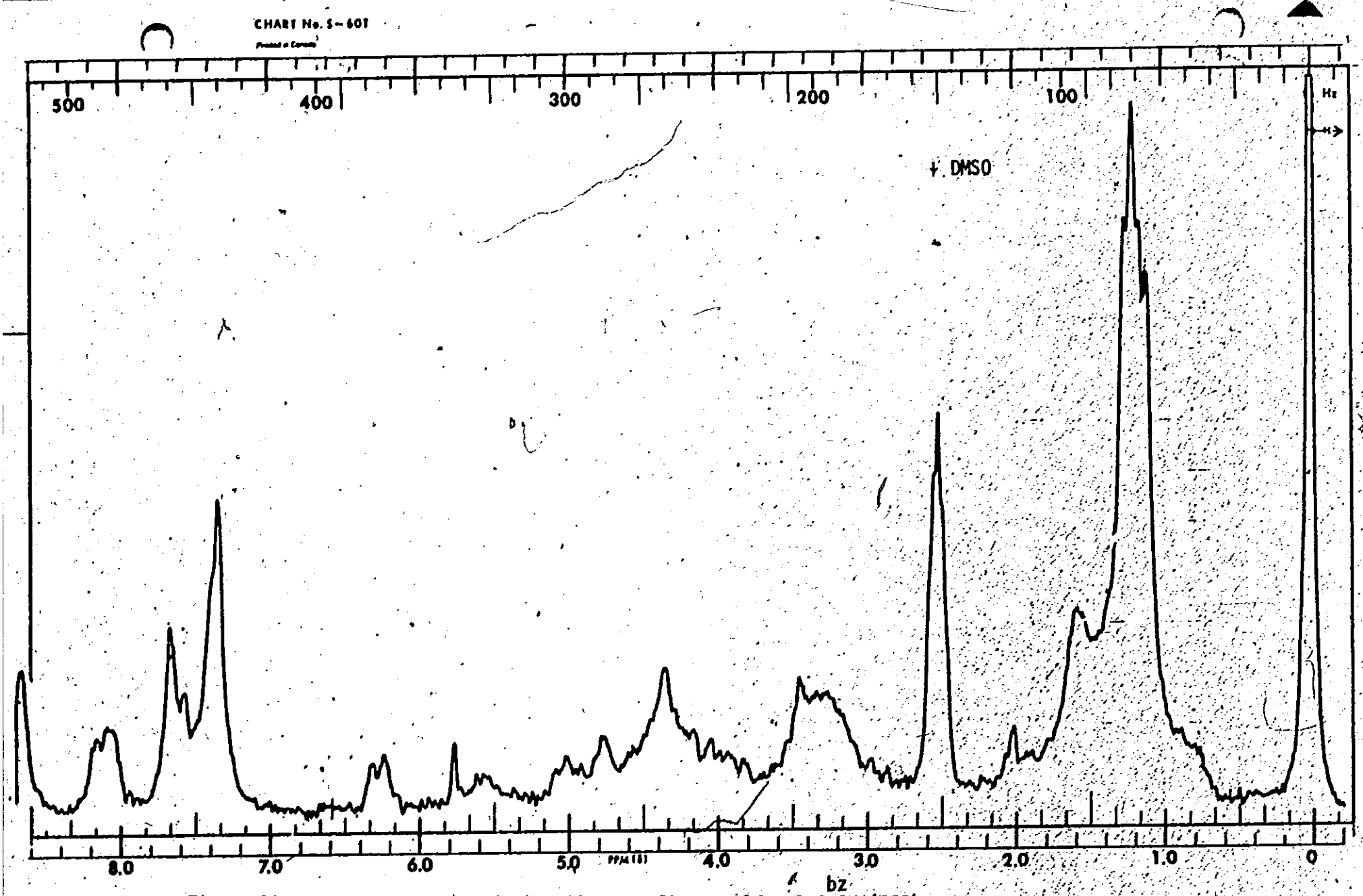

Figure 12: P.m.r. spectrum of 5'-triisopropylbenzesulfonyl-AtOH (III).

3.2.2. Stepwise Synthesis of Oligonucleotides

Assembly of the required protected tri- and tetranucleotide blocks proceeded by stepwise addition of suitably protected nucleosides to the 3' ends of sequences. Each addition required the formation of a protected 3'-5' phosphate linkage which was accomplished in two steps as outlined in figure 13. The 5' terminal nucleoside bearing the 5'-triphenylmethoxyacetate group as described above, a 2'-O-tetrahydropyranyl group and, except for uridine, an N-benzamido group, was treated with 2,2,2-trichloroethyl phosphate in anhydrous pyridine using TPS as the activating agent. The 3'-(2,2,2-trichloroethyl) phosphate derivative formed was extracted from a quenched reaction mixture with methylene chloride and the extract was evaporated to dryness under reduced pressure. Repeated co-distillation with toluene was required to remove residual water and pyridine.

Residual triisopropylbenzenesulfonate decreased the efficiency of subsequent coupling reactions but aqueous washing of methylene chloride extracts prior to removal of pyridine did not extract the sulfonate effectively. After removal of pyridine, much of the sulfonate precipitated from dry methylene chloride solution. It has subsequently been found that the remainder could be removed by aqueous extraction at this point.

The 3' phosphorylated intermediate thus isolated was used directly in the subsequent coupling reaction without purification by silica gel

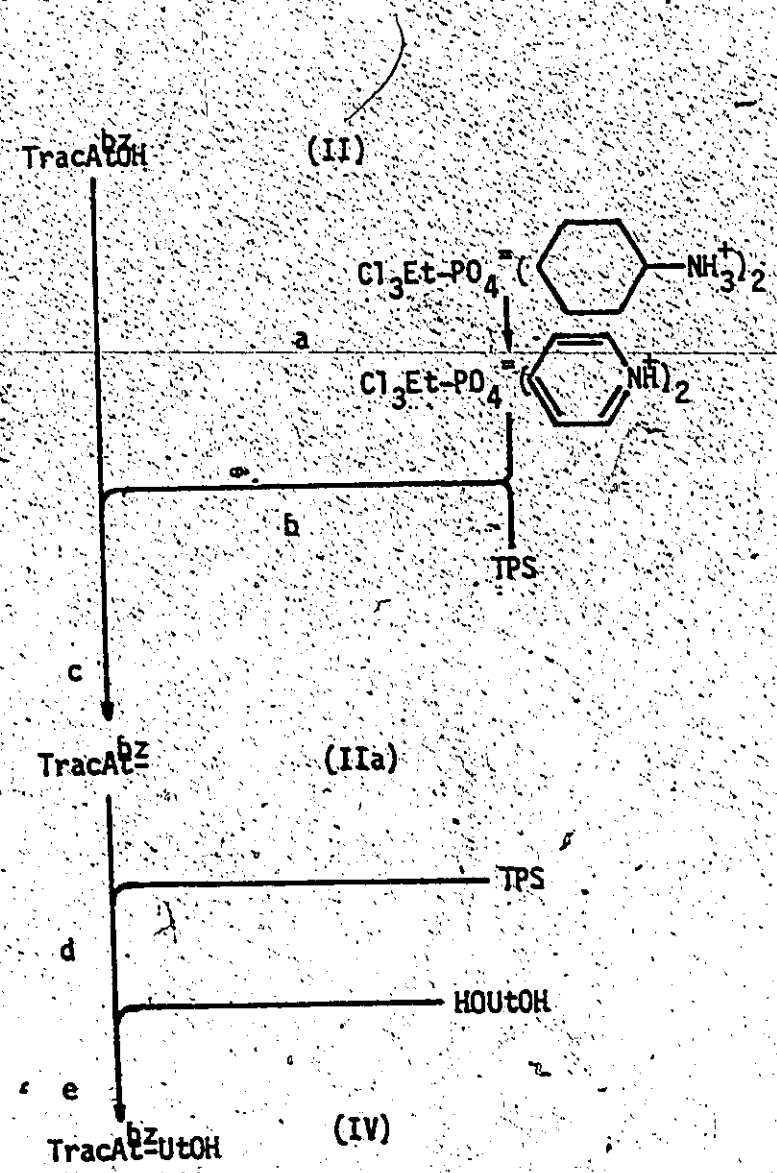

Figure 13: Stepwise synthesis of oligonucleotides:

a) Conversion of cyclohexylammonium salt to pyridinium salt;

b) activation of 2,2,2-trichloroethylphosphate with TPS;

c) phosphorylation of unprotected 3'-hydroxyl group ;

d) activation of the phosphorylated intermediate with TPS;

e) coupling of the activated terminal phosphate selectively with the unprotected 5'-hydroxyl of the "incoming nucleoside".

chromatography*. The progress of phosphorylation was readily followed by t.l.c.; as the phosphorylated sequence holds a full anionic charge, so its R_f is much lower than the unchanged starting material.

Activation of this inserted terminal phosphate with TPS in anhydrous pyridine was followed by addition of a 0.1 - 1.0 molar excess of nucleoside, protected by a 2'-O-tetrahydropyranyl group, and except for uridine, an N-benzamido group. This nucleoside will be referred to as the "incoming" nucleoside. The coupling reaction is also followed conveniently by t.l.c. The phosphate shows as a yellow spot of low R_f and the incoming nucleoside, lacking a trityl group, shows as brown or grey spot of intermediate R_f. The coupled product appears as a yellow spot of higher R_f.

In the presence of the unprotected 3'-hydroxyl of the incoming nucleoside, some 3'-3' coupling might be expected. However, nucleotide linkages obtained by this two step procedure are exclusively 3'-5' and not 3'-3', because of steric hindrance of both 3'-hydroxyl and activated phosphate by their respective, adjacent, 2'-O-tetrahydropyranyl groups, and because of the lesser reactivity of the secondary 3'-hydroxyl compared to the primary 5'-hydroxyl.

Table 6 presents data pertinent to the stepwise synthesis of

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* In some cases, phosphorylation did not appear to be complete as shown by t.l.c. analysis of the reaction mixture. Silica gel chromatography was then used to purify the phosphorylated material before use in the coupling reaction to prevent contamination of the coupled product with the original starting sequence.

Table 6

Data[a] on stepwise synthesis of oligonucleotides required for the assembly of a messenger coding for N-formylmethionylangiotensin.

	Sequence[b]	R_f	Yield:%	Scale: mmol	Scale: mg	Phosphorylation: t(days)	Phosphorylation: V(ml)	Coupling: t(days)	Coupling: V(ml)	Remarks
IV	AU	0.75	60	1.33	1000	2	20	4	30	e
V	AUA	0.64	53	0.98	1250	2	11	8	25	f
VI	AUG	0.61	22	0.78	1000	2	15	4	20	e
VII	AUU	0.63	58	0.59	750	2	15	4	20	
VIII	AUC	0.68	57	0.59	750	2.5	10	5	15	c
IX	AUAC	0.69	72	0.34	650	3.5	5	4.5	10	e
X	AUAG	0.57	48	0.26	500	3	5	9	10	e
XI	AUUC	0.65	68	0.29	520	2	10	7	10	e
XII	AUCC	0.72	32	0.25	500	4.5	15	10	10	c,d,e
XIII	AG	0.60	23	0.99	500	7	10	14	10	c,d
XIV	UA	0.72	68	2.07	1333	3	15	3	30	

(continued)

Table 6 (continued)

	Sequence	R_f	Yield:%	Scale: mmol	Scale: mg	Phosphorylation: t(days)	Phosphorylation: V(ml)	Coupling: t(days)	Coupling: V(ml)	Remarks
XV	UAA	0.67	68	0.98	1300	2.5	25	3	40	c

a. Values are averages of several preparations: the range of values was small as was the number of preparations.

b. The abbreviations used refer to the fully protected sequence of nucleotides, as the product.

c. Reactions requiring "back-tritylation" to remove residual incoming nucleoside.

d. Reactions requiring purification of the phosphorylated intermediate before coupling.

e. Characterization by complete deprotection and enzymatic digestion shown in table 7.

f. Characterization by complete deprotection and enzymatic digestion shown in table 10.

Table 6

Specific Examples of Stepwise Synthesis of Oligonucleotides

Growing Chain (1 equiv)		Incoming Nucleoside (1.5 equiv)			Product		Yield	
II	(2.0 g)	+ HOUtOH	(1.20 g)	→	TracAt-UtOH (bz)	(IV)	2.42 g	72%
IV	(2.0 g)	+ HOAtOH (bz)	(970 mg)	→	TracAt-Ut-AtOH (bz bz)	(V)	1.74 g	58%
IV	(1.0 g)	+ HOGtOH (bz)	(559 mg)	→	TracAt-Ut-GtOH (bz bz)	(VI)	350 mg	22%
IV	(750 mg)	+ HOUtOH	(289 mg)	→	TracAt-Ut-UtOH (bz)	(VII)	560 mg	53%
IV	(1.0 g)	+ HOCtOH (bz)	(508 mg)	→	TracAt-Ut-CtOH (bz bz)	(VIII)	940 mg	63%
V	(300 mg)	+ HOCtOH (bz)	(101 mg)	→	TracAt-Ut-At-CtOH (bz bz bz)	(IX)	300 mg	76%
V	(500 mg)	+ HOGtOH (bz)	(183 mg)	→	TracAt-Ut-At-GtOH (bz bz bz)	(X)	320 mg	48%
VII	(500 mg)	+ HOCtOH (bz)	(179 mg)	→	TracAt-Ut-Ut-CtOH (bz bz)	(XI)	458 mg	68%
VIII	(550 mg)	+ HOCtOH (bz)	(162 mg)	→	TracAt-Ut-Ct-CtOH (bz bz bz)	(XII)	220 mg	30%
II	(1.0 g)	+ HOGtOH (bz)	(468 mg)	→	TracAt-GtOH (bz)	(XIII)	200 mg	23%
TracUtOH	(1.5 g)	+ HOAtOH (bz)	(1.64 g)	→	TracUt-AtOH (bz)	(XIV)	2.12 g	70%
XIV	(1.0 g)	+ HOAtOH (bz)	(532 mg)	→	TracUt-At-AtOH (bz bz)	(XV)	1.02 g	72%

protected oligoribonucleotides. Many of the values reported are averages of several preparations of the same product.

The yields from these two step coupling reactions ranged between 20% and 75% with most yields in the upper part of this range. Yields have tended to decrease with increasing chain length in oligonucleotide synthesis by both the diester (Jacob and Khorana, 1965) and triester (Smrt, 1972a) methods but no such correlation is indicated by these data. Letsinger (and Ogilvie, 1969) and Cusack et al. (1973) also did not observe a decrease with chain length.

The stepwise coupling of guanosine residues gave lower yields than those of other nucleosides. This problem has also been encountered in doexyribonucleotide synthesis (Ralph et al., 1963).

While the yield of TracAt^{bz}-Ut-Gt^{bz}OH (VI) initially appeared to be high, this material could not be purified by silica gel chromatography. Attempts at purification resulted only in loss of material and the unidentified impurities (of similar R_f on t.l.c.) remained.

The yield of the protected dinucleotide TracAt^{bz}-Gt^{bz}OH (XIII) was also low, but a similar coupling reaction of HOGt^{bz}OH to a protected adenosine residue in the sequence TracAt^{bz}-Ut-At^{bz}OH (V) gave a higher yield of the tetranucleotide X than the yield of XIII. While synthesis of the tetranucleotide has not been repeated, synthesis of the dinucleotide XIII by Mr. T. England (1975) gave a yield of about 30%. Yields in a given coupling reaction tend to be consistent over several preparations but the difference between yields of tetramer X and dimer XIII appears to be greater than is attributable to the normal range of variations. This suggests that reactivity of these molecules may be significantly influenced by their sequence and length.

Yields obtained in preparations of TracAt^{bz}-Ut-Ct^{bz}-CtO^{bz}H (XII) were consistently low: 26%-35% over 6 preparations. Phosphorylation of TracAt^{bz}-Ut-CtO^{bz}H (VIII) proceeded very slowly and was incomplete. The phosphorylated intermediate was purified by silica gel chromatography before coupling to the next nucleoside. Apparently unreacted starting material was also isolated but is subsequent treatment with activated trichloroethyl phosphate gave little additional phosphorylation. It seems unlikely that this material resulted from coupling an activated 3'-phosphate to the unreacted 3'-hydroxyl during the original phosphorylation but this has not been disproven.

Condensation of two phosphorylated molecules could occur to give a tetrasubstituted pyrophosphate. However, these may be hydrolyzed during the workup procedure as some tetrasubstituted pyrophosphates are phosphorylating agents in their own right (Khorana, 1961).

Attempts to improve the yields of many of these coupling reactions by keeping the reactions anhydrous and using various excesses of phosphorylating or coupling reagents had only marginal success.

In coupling reactions involving HOCtO^{bz}H the unreacted excess co-chromatographed with the product during purification. As oligonucleotides were usually eluted from silica gel by higher concentrations of methanol than were required to elute the monomer HOCtO^{bz}H singly, there was apparently some degree of association occurring. Partial co-elution was also observed with other incoming nucleosides.

This contamination was removed by treating the mixture with p-anisyldiphenylchloromethane (monomethoxytrityl chloride) in anhydrous pyridine overnight, which readily tritylated the 5'-hydroxyl of the

nucleoside but did not react with the 5' protected oligomer. The derivatized monomer could be eluted from silica gel with 0-1.5% methanol. This procedure is termed "back tritylation".

Most of the protected oligonucleotides appeared as an oval shaped spot on t.l.c. and the longer the sequence, the longer the oval. This has been attributed to the existence of chiral centers in phosphotriesters (Miller, et al., 1971; Werstiuk and Neilson, 1972). Thus there would be 2^n diastereoisomers of a sequence containing n + 1 nucleosides linked by n phosphotriesters.

Amounts of each diastereomer formed with each linkage may differ but no attempt was made to separate these species as chirality is lost with removal of the trichloroethyl protecting groups. As a result, the proportions of each isomer in these sequences remain unknown. Werstiuk and Neilson (1972) observed an unequal proportion of the two isomers of TracUt-A^{bz}tOH and were able to isolate an enriched fraction of the major component. The different proportions of the two isomers were attributed to "steric restrictions towards the reacting species in the second step of internucleotide link formation".

The absolute stereochemistry of adenosine and uridine 2'-O-tetrahydropyranyl derivatives are known (Kennard et al., 1971; Stothart, Brown, and Neilson, 1973) but the influence on the efficiency and stereochemistry of coupling reactions has not been investigated. Both the high R_f (R configuration in A and U derivatives) and the low R_f (S configuration in the A and U derivatives) of the four protected nucleosides have been used in coupling reactions in this laboratory and others. No obvious effects are yet attributable to this stereoisomerism.

Initially the low R_f isomers had been used to prepare 5' protected derivatives because without the additional lipophilic protecting group, they were less soluble than the high R_f derivatives. In the major part of this work, the incoming nucleoside was the high R_f diastereomer and low R_f diastereomers were used for the 5' protected nucleosides.

3.2.3. Complete Deprotection and Analysis of Oligonucleotide Blocks

The identity of protected oligonucleotide blocks and the fidelity of synthesis of the internucleotide linkages was confirmed by complete deprotection and enzymatic digestion of small samples. These procedures are outlined in figures 14 and 15.

Treatment of samples with Zn/Cu couple in DMF for 18-24 hours at 30-40° (Eckstein 1967a) removed the trichloroethyl groups. Two volumes of ammonium hydroxide in methanol (1:1, v/v) were added and stirring was continued for one or two days at room temperature to remove the 5' triphenylmethoxyacetate group and N-benzamido groups. The mixture was then filtered and the filtrate treated with Dowex or Chelex ion exchange resins in order to remove zinc and copper ions solubilized by ammonium hydroxide treatment. This step was taken for two reasons: these ions may interfere with subsequent enzymatic steps or they may contribute directly to unwanted hydrolysis of phosphodiesters (Butzow and Eichhorn, 1965). After removal of the Dowex, the pH of the solution was adjusted to 2. After one or two days at room temperature, the tetrahydropyranyl groups had been removed. The solution was neutralized and the deprotected

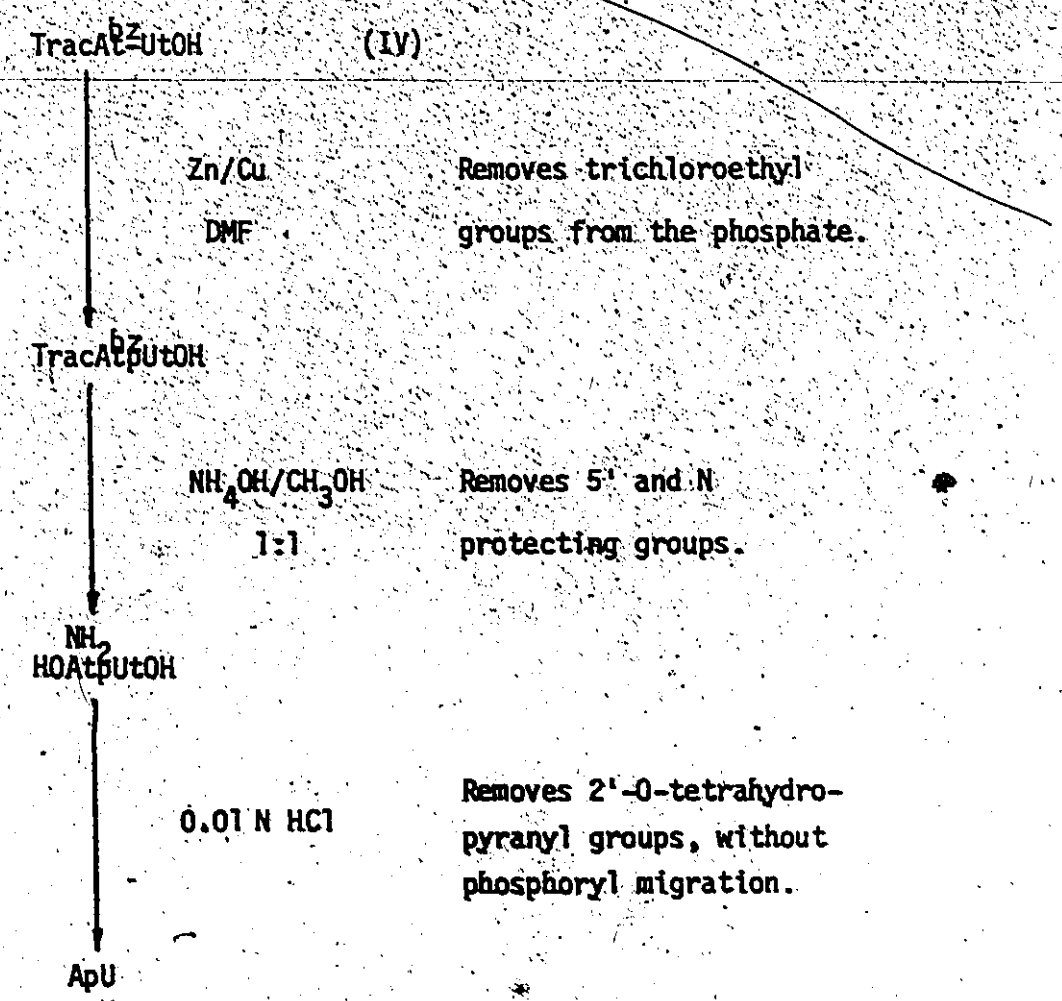

Figure 14. Three step procedure for complete deprotection of synthetic oligoribonucleotides.

A p U p↓U p↓C	Pancreatic RNase cleaves after pyrimidines, giving	ApUp, Up, C
A↓p U↓p A↓p C	Snake venom diesterase cleaves sequentially from the 3' end (in the absence of 3' phosphate) to give 5' nucleotides and 5' terminal nucleoside.	A, pU, pA, pC
A p↓U p↓C p↓A p↓d OH	Spleen phosphodiesterase cleaves sequentially from the 5' end (in the absence of 5' phosphate) to give 3' nucleotides.	Ap, Up, Cp, Ap, HOdOH
A p U↓p	Alkaline phosphatase removes terminal phosphate.	ApU, p

Chromatographic analysis of the products may give information on base composition (e.g. Spleen digest), terminal residues (e.g. SVD digest), and site of Cleavage (e.g. Pan digest). Absence of 3'-3' linkages shown by SVD).

Figure 15. Enzymatic digestion for characterization of deprotected oligonucleotides showing cleavages introduced by enzymes used.

oligonucleotide purified by paper chromatography.

Ultraviolet absorbing bands observed on paper chromatograms, after development and drying, were cut out and eluted with water. Materials eluted from paper in this way were stored at -20° after recording the volume and ultraviolet spectrum. Measured aliquots in the range of five to ten A_{260} units could be removed, after thawing, for enzymatic digestion.

The measured aliquots were reduced to dryness under reduced pressure and made up to a final concentration of 50 A_{260} units per ml in the buffer suitable for the enzyme to be used. Then half this volume of the appropriate enzyme solution was added and the mixture incubated at 37° overnight.

Digestion products and samples of undigested materials were analyzed by paper and cellulose thin layer chromatography as indicated in table 7. Standard samples of the expected products were run concurrently, with two exceptions: R_{Up} for ApA (expected from pancreatic RNase digestion of UpApA) was compared with a published value (Lohrmann et al., 1966) and R_{Up} values for ApUp (expected from pancreatic RNase digestion of sequences beginning ApUp...) obtained from different digests were compared with each other. Complete conversion to the expected products in every case indicated that the sequences are correct and that 3'-3' linkages are not present.

The fact that yields from the deprotection procedure are not quantitative might suggest either or both of two alternatives: one, that the protected sequence is impure, (i.e. has suffered modification, degradation or incomplete synthesis) or two, that deprotection reactions have

Table 7

Data on complete deprotection and enzymatic characterization of oligonucleotides.

Sequence	Yield:%	R_{Up}[b] A	R_{Up}[b] B	R_{Up}[b] C	Digest[a]	Products	R_{Up}[b] A Std.	A Dig.	B Std.	B Dig.	C Std.	C Dig.
ApU	86	1.31	1.20	-	SVD	A	3.03	2.95	1.67	1.51		
						pU	.76	.82	.95	.93		
ApUpG	29	0.44	0.85	-	SVD	A	3.62	3.60	1.78	1.75		
		0.42[c]				pU	.77	.77	.97	.97		
						pG	.42	.48	.58	.61		
						ApU	1.44	1.44	.97	1.19		
						G	2.36	2.28	1.32	1.22		
UpApA	74	0.51	1.10	0.63	SVD	U	3.64	3.54	1.51	1.53	1.76	1.59
		0.60[c]				pA	.69	.68	1.20	1.17	.69	.62
					Pan	Up	1.00	.96	1.00	.99	1.00	.92
						ApA	1.14[c]	1.19	-	1.29	-	.89
					+ SVD	A	3.30	3.44	1.88	1.84	1.57	1.46
						pA	.64	.67	1.31	1.28	.68	.65
ApUpApC	31	0.20	0.96	0.29	SVD	A	2.93	2.95	1.86	1.62	1.65	1.53
						pU	.75	0.45-.80	1.06	.96	.87	.85
						pA	.65	0.45-.80	1.29	1.29	.70	.68
						pC	.47	0.45-.80	.94	.96	.70	.68
					Pan	ApC	1.12[c]	1.22	1.42	1.35	.94	.94
						ApUp	-	.33	-	.77	-	.52
					+ Pase	ApU	1.47	1.22	1.33	1.35	1.00	1.00

(continued)

Table 7 (continued)

Sequence	Yield:%	R_{Up} A	R_{Up} B	R_{Up} C	Digest[a]	Products	R_{Up}[b] A Std.	A Dig.	B Std.	B Dig.	C Std.	C Dig.
ApUpUpC	43	0.22	0.98	0.40	SVD	A	3.11	3.08	1.87	1.66	1.46	1.39
						pU	.74	.71	.94	.94	.84	.86
						pC	.46	.50	.90	.94	.70	.71
					Pan	ApUp	-	.29	-	.89	-	.45
						Up	1.00	1.06	1.00	1.00	1.00	1.03
						C	3.21	3.10	1.63	1.60	1.70	1.64
ApUpCpC	28	0.39	1.38	0.63	SVD	A	3.12	3.07	2.00	1.97	1.64	1.69
						pU	.79	.81	.96	.98	.88	.91
						pC	.61	.57	.94	.98	.73	.77
					Pan	ApUp	-	.20	-	.62	-	.49
						Cp	.91	.95	1.07	1.03	.80	.84
						C	3.34	3.18	1.89	1.82	1.96	1.93
ApUpApG	13	0.18	0.91	0.27	SVD	A	3.40	3.12	1.91	1.78	1.44	1.54
						pU	.75	.68	.83	.90	.95	.91
						pA	.66	.68	1.24	1.32	.64	.52-.61
						pG	.40	.43	.59	.56	.49	.52-.61

a. The "Digest" column indicates the enzyme used (SVD for snake venom phosphodiesterase, Pan for pancreatic ribonuclease and Pase for alkaline phosphatase) where + indicates reaction with completed digest described in the line immediately above. The products expected from each digest are listed in the adjacent column. R_f values for each product and the corresponding standard, run concurrently, are given on the same line for three different solvent systems.

b. A = Whatman #1 paper eluted with Solvent A, B and C; cellulose t.l.c. eluted with solvents B and C respectively.

c. Standard values, Lohrmann et al., 1966.

caused degradation or are incomplete. These explanations will be discussed in depth after further data on synthetic aspects of this work have been presented.

3.2.4. Specific 5' Deprotection

Conditions required for effective removal of the 5'-triphenylmethoxyacetate group without removing the N-benzamido groups have been shown to vary, depending on the nature of the sequence (Werstiuk and Neilson, 1973). Therefore, small pilot reactions were performed for each complete oligonucleotide block. Small samples of each protected block were dissolved in 1% (0.15 N) and 0.5 (0.075 N) ammonium hydroxide in methanol and small aliquots were taken for t.l.c. analysis over a period of several hours.

From these t.l.c. plates visual estimates for the disappearance of starting material (yellow spot, $R_f \sim 0.8$), appearance of the desired product (brown spot, $R_f \sim 0.7$) and appearance of de-N-benzoylated material (brown and yellow spots, $R_f < 0.7$) were made. These observations are presented in table 8. Selection of the conditions to be used for the preparative scale 5' deprotection of a given sequence and estimation of the optimal length of treatment for selective removal of the triphenylmethoxyacetate group were made on the basis of these data.

Two of the protected sequences, TracAt^{bz}-Ut-Ct^{bz}-Ct^{bz}OH (XII) and TracUt^{bz}-At^{bz}-AtOH (XV), were treated on a preparative scale, in preparation for block coupling.

Table 8

Data on selective 5' deprotection.

Sequence*		R_f	Yield:%	Removal in 0.5% NH_4OH/CH_3OH: Trac 99%	N-benzoyl 1%	N-benzoyl 5%	Removal in 1% NH_4OH/CH_3OH: Trac 99%	N-benzoyl 1%	N-benzoyl 5%	Preparative Conditions:
XVI	UAA	0.49	82	4 h	2 h	4 h	2 h	40 min	1 h	½%, 4 h
XVII	AUAC	0.55	-	4	2	3	3	1	1.5	-
XVIII	AUUC	0.57	-	6	12	-	4	7	12	-
XIX	AUCC	0.42	41	8	6	8	6	5	7	½%, 1-6 h
XX	AUAG	0.54		6	5	6	5	2	4	-

*. Sequences referred to are the 5' drportected products expected. R_f values reported in this table were taken from t.l.c. plates which were thoroughly dried before development, and as a result are lower than values discussed in the text by about 0.1. Data refers to the amount of time required to obtain the estimated percentage of cleavage of group as named in the column headings.

The trimer XV was treated with 0.5 ammonium hydroxide in methanol for four hours. Progress of the reaction was followed by t.l.c. as before, and after quenching the reaction by evaporation under reduced pressure at room temperature, the product XVI was isolated by silica gel chromatography in 82% yield. Only traces of starting material were recovered, the remainder of the material was a mixture of de-N-benzoylated materials.

Specific deprotection of the tetramer XII proved less satisfactory. Trial hydrolysis indicated that the desired product could not be expected in high yield and, in fact, yields obtained from several preparative scale reactions were both low and variable, averaging 41%.

This small difference between rates of hydrolysis of one or more benzamido groups and the triphenylmethoxyacetate group has been observed for several other sequences: TracAt-Ut-At-CtOH (IX) in the test scale reactions and other sequences containing adjacent adenosine and cytidine residues (Neilson and Werstiuk, 1974; Werstiuk and Neilson, 1973).

Previously published conclusions on the stability of amides of the heterocyclic bases indicated a ranking of stability to alkaline hydrolysis (0.1 N sodium hydroxide) of G > A > C (Ralph and Khorana, 1961). This is consistent with the $t_{\frac{1}{2}}$ values found for removal of the N-benzoyl groups of these three protected nucleosides in ammonium hydroxide/methanol (1:1, v/v): C, $t_{\frac{1}{2}}$ = 16.5 min; A, $t_{\frac{1}{2}}$ = 2 h, 50 min; G, $t_{\frac{1}{2}}$ = 27 h 50 min. These data readily explain the preference for the more stable p-anisoyl group for protection of the cytidine amino group (Khorana et al. 1961), and use of the less stable isobutyroyl group as a less bulky protecting group for the guanosine amino group (Kumar and Khorana, 1969)

in the synthesis of deoxynucleotide sequences.

However, selective deprotection carried out under milder conditions (ammonium hydroxide/methanol 1:100, v/v) gave quite different results (Werstiuk and Neilson, 1973), the order of stability found being C > A > G for the N-benzamido groups of the mononucleosides. Subsequent studies showed that the 3'-hydroxyl substituent of a protected oligonucleotide tended to control the rates of hydrolysis (Neilson *et al.*, 1975) but there is still insufficient data to explain the changes in rates of hydrolysis of the N-benzamido groups relative to the 5'-triphenylmethoxyacetate group and relative to each other.

An investigation to define the mechanisms and rates of hydrolysis by sodium hydroxide or ammonium hydroxide in these different sequences was not considered practical. Thus the comparative similarity in stability among the nucleoside N-benzamides, reversal from the order of stability to stronger alkaline conditions, and effects of the particular sequence of nucleotides on these stabilities have created a matrix of anomalous observations from which extrapolations cannot reliably be made. Appropriate conditions for selective deprotection of new sequences must still be determined empirically.

A sufficient quantity of $HOA\overset{bz}{t}-Ut-C\overset{bz}{t}-C\overset{bz}{t}OH$ (XIX) was obtained by these methods to proceed with block coupling but on a smaller scale than had originally been planned.

3.2.5. Block Coupling

Two block couplings were attempted: TracAt-Ut-Ut-Ct-OH + HOUt-At-At-OH and TracAt-Ut-At-Ct-OH + HOAt-Ut-Ct-Ct-OH (XI + XVI), and IX + XIX respectively). The phosphorylation and coupling steps were identical to those used in stepwise synthesis except that the incoming nucleoside was a 5'-deprotected oligomer, and that the scales of the reactions were in the 10-100 μmol range.

The former coupling to give the protected heptamer XXI was attempted several times. In each case, phosphorylation of the protected tetramer XI proceeded slowly but steadily to near completion in 2-3 days. The coupling reactions with XVI proceeded smoothly to 75%-80% completion as estimated by t.l.c. (yellow spot, R_f, 0.3 - 0.45 + black spot, R_f = 0.49 yellow spot, R_f = 0.6 - 0.7) over a period of 4 days.

After quenching, extracting and purifying the mixture, yields of about 25% were obtained. Weight loss with respect to the tetramer component of 20 - 30% was also noted. The small scale of these reactions may account for both of these observations as inhibition of the coupling by small amounts of moisture and mechanical losses are both magnified in small scale reactions.

Although the reactants and products should in principle be separated by the t.l.c. system used, this was often not the case. The R_f of each successively longer oligomer was slightly less, and the R_f of the corresponding 3'-phosphorylated oligomers was slightly greater than the one before. Since each sequence of length n + 1 nucleotides is

a series of 2^n diastereoisomers because of the chirality of phosphotriesters as discussed previously, the R_f values of longer sequences become more diffuse. These two factors and the variability of R_f on an absolute scale resulted in some confusion, at times, about whether there were two spots (tetramer phosphate and heptamer) or only one (which one?).

Gel filtration chromatography on a column of Sephadex LH-20 (1.2 x 70 cm in pure methanol) indicated that the tetramer phosphate standard eluted at the same position as did the "tetramer phosphate" or lower R_f material recovered from the coupling reaction. The unexpected result obtained for the "heptamer" or higher R_f material, was that it was eluted significantly later, suggesting that the molecular weight of this material was less than that of the tetramer. Plots of log (M.W.) vs. elution volume for several protected sequences are shown in figure 16.

Since Sephadex LH-20 has the capacity to adsorb aromatic moieties, particularly in lower alcohol solvents, and anionic species tend to be excluded from the matrix, these results must be considered with caution.

Chromatography of completely deprotected samples of the higher and lower R_f materials, tested above, on DEAE cellulose in 7M area with an ascending gradient of NaCl (0-0.5 M) by the method of Tomlinson and Tener (1961) supported the conclusion that the lower R_f material was tetramer phosphate. Peaks eluting at positions corresponding to three and four charges were smaller than the last eluted peak which corresponded to five charges as expected for the tetramer phosphate. The elution

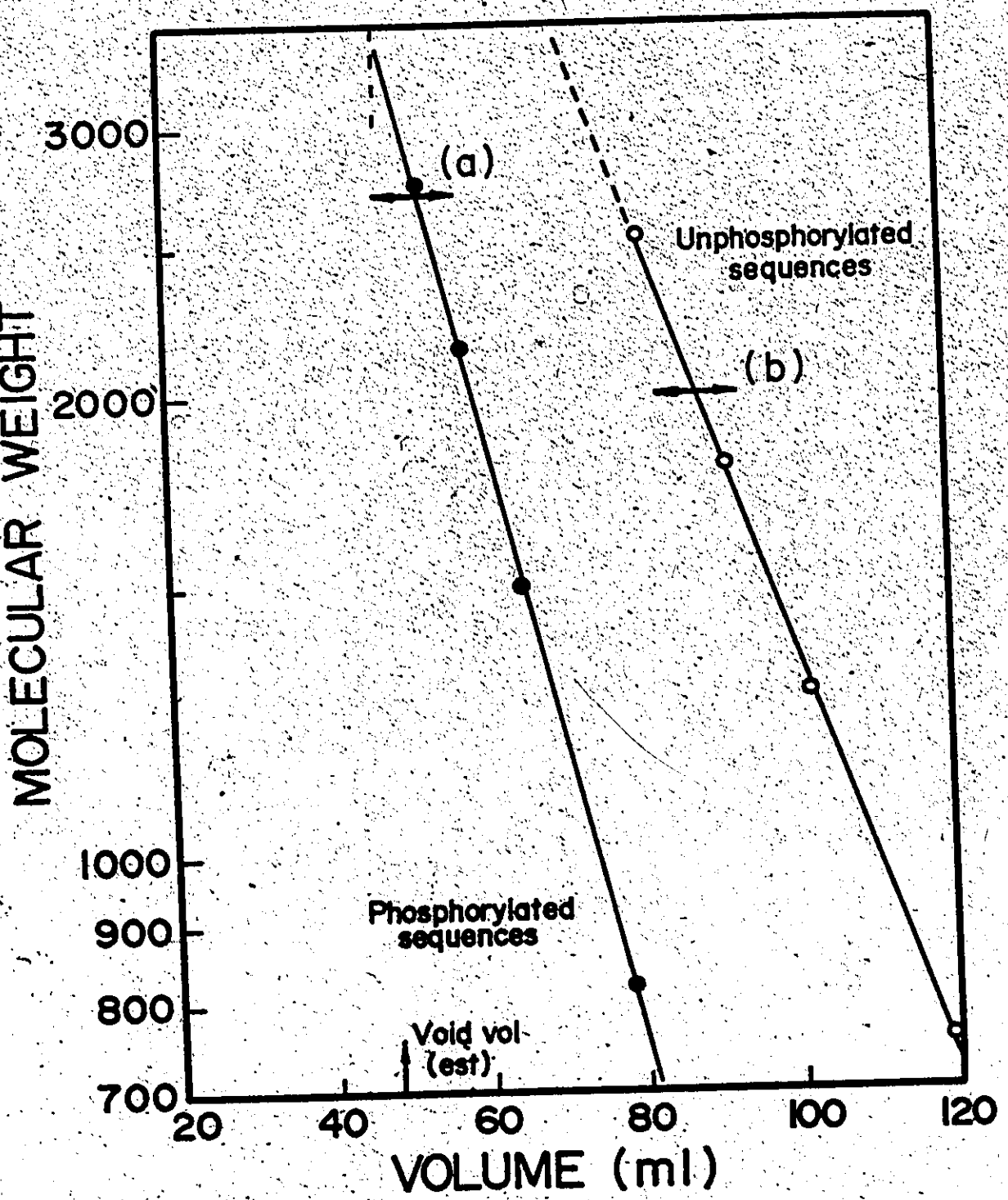

Figure 16: Gel filtration of protected oligoribonucleotides on Sephadex LH-20:

a. lower R_f, b. higher R_f material from block coupling.

pattern for the deprotected higher R_f material showed no peaks of u.v. absorbing material having charges greater than three or four. This result, while not conclusive because of the evidence of degradation in both profiles, supports the conclusion that the high R_f material is smaller than the tetranucleotide and is not the heptamer as expected.

The low yield (approximately 5%) obtained from complete deprotection of the supposed heptamer or high R_f material after purification by paper chromatography is also indicative of severe degradation, loss, or near absence of the coupled product. Quantitative enzymatic analysis of the deprotected material was not feasible.

Before the problems discussed above were fully realized, an attempt was made to couple XIX to IX to give the octanucleotide XXII. TracAt^{bz}-Ut-At^{bz}-Ct^{bz}OH (IX) had been synthesized previously in good yield and the difficulties encountered in obtaining HOAt^{ba}-Ut-Ct-Ct^{bz}O^{bz}H (XIX) have been discussed.

The reaction was of a smaller scale than the heptamer reaction but the results were very similar: phosphorylation of IX was slow but eventually nearly complete, and coupling appeared slow but finally appeared to give a reasonable yield as estimated by t.l.c. Column chromatography was not satisfactory for purification of the coupled product but preparative t.l.c. gave 24% of the supposed octamer.

Mass recovery was again poor as discussed for the heptamer. Paper chromatography of the completely deprotected material gave no u.v. absorbing band at low R_f which could correspond to the octanucleotide. Elution of this part of the paper chromatogram (R_f0-0.25) gave a solution whose u.v. spectrum did not resemble nucleotide material. Chromatography

on DEAE cellulose in 7M Urea of this material as before gave only one peak at very low salt concentration. The yields of high R_f "coupled" material isolated by column chromatography are similar to yields of 20-25% reported by Neilson and Werstiuk (1974)* for block couplings, although, failure to demonstrate any further evidence for block coupled products here is not consistent with their data.

Why it has not been possible to obtain more definite evidence for the existence of the block coupled products is not clear. There was no reason to doubt the structure of the protected nucleotide blocks used: their structures were demonstrated by complete deprotection and analysis by enzymatic digestion. The possibility of errors in preparing and carrying out these reactions could be considered but these difficulties occurred consistently with three attempts at the heptamer and one attempt at the octamer, and did not occur with other coupling reactions carried out during the same time period. Errors in the characterization procedures or misinterpretation of the data obtained appear unlikely as the same methods were used subsequently, virtually unchanged, for studies on other sequences. Familiarity with the methods and interpretation of the data strengthened rather than weakened the interpretations given above.

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* The higher yield (39%) reported for coupling of HO$\overset{bz}{At}$-C$\overset{bz}{t}$OH to Trac$\overset{bz}{At}$-U$\overset{bz}{t}$-AtOH support the contention that larger scale reactions give better yields: the scale of this reaction was about 750 µmol, the others were near to the 100 µmol scale used in the work reported above.

The fate of these reactions could not be determined conclusively with the small amounts of synthetic oligonucleotides available, and the extensive amount of synthesis required to begin such an investigation anew was considered beyond the scope and capabilities of the proposed project. Instead, a careful analytical consideration of the experimental procedures and theory was considered a more profitable approach to the definition and solution of the problems encountered.

3.2.6. Analysis

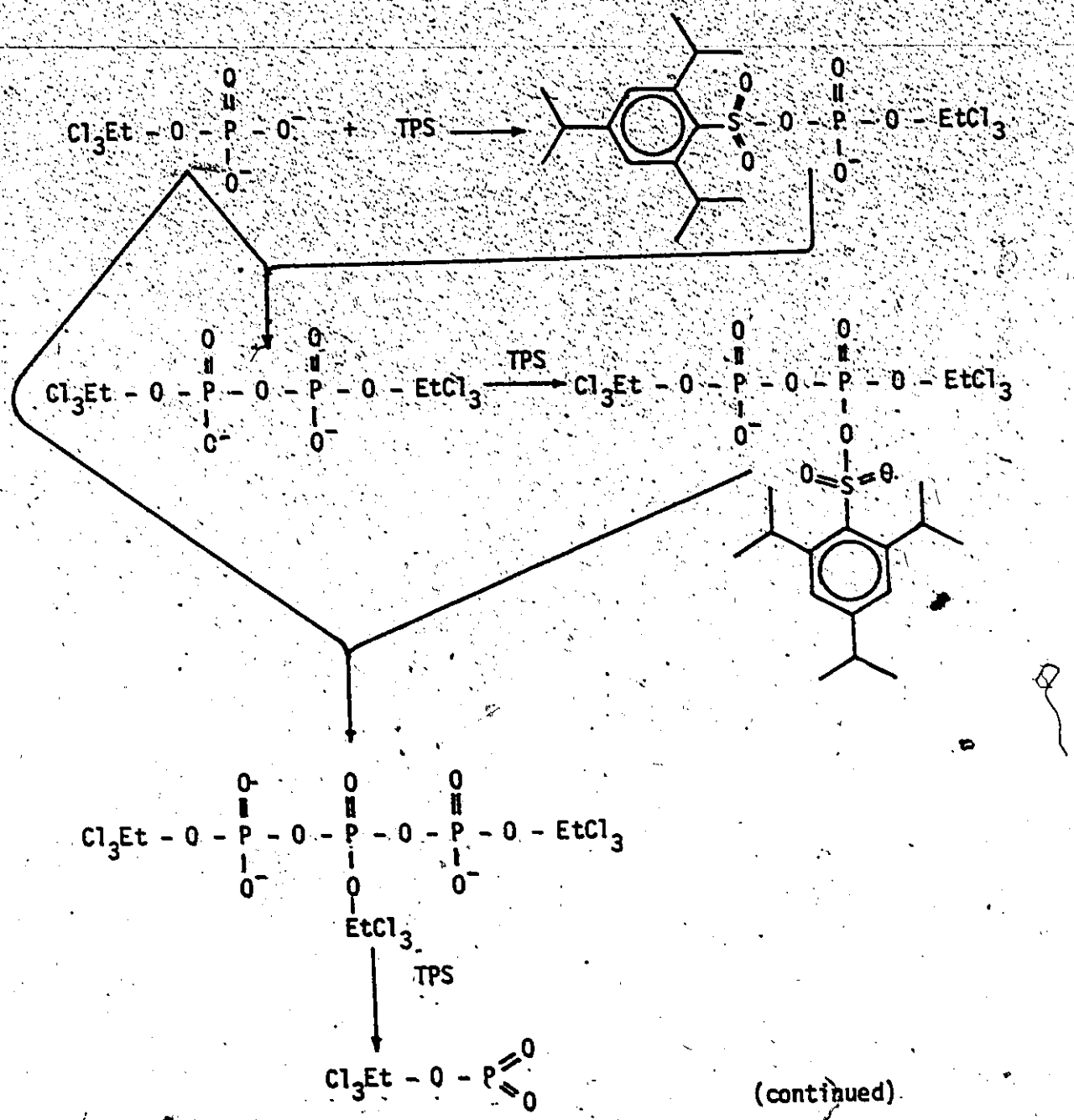
With the clarity of vision afforded by hindsight, and by the availability of theoretical and empirical data obtained recently by other authors, a more thorough understanding of the problems encountered and their potential solutions becomes apparent.

At the time these studies were begun, the mechanisms operating in the activation of phosphate and its esters by TPS and subsequent reaction of the active intermediate with nucleoside hydroxyl groups were not fully understood. Khorana's original studies of arenesulfonyl chlorides in synthesis of the internucleotide bond (Jacob and Khorana, 1964) suggested various forms of mixed anhydride, or possibly metaphosphate, as possible candidates for the activated intermediate of phosphomonoesters. It was shown that this mechanism differs from the mechanism of activation of phosphomonoesters by DCC, which gave the trimetaphosphate as the active intermediate (Weimann and Khorana, 1962b). In Eckstein's application of TPS to the phosphotriester method of synthesis, the active form of the phosphodiester was concluded to be the tetrasubstituted pyrophosphate

rather than the sulfonate-phosphate anhydride on the basis of ^{31}P n.m.r. data (Eckstein and Rizk, 1969). Knorre and Zarytova (1974) reviewed the state of understanding of mechanisms of phosphate activation and presented their ^{31}P n.m.r. studies on the activation of phosphate esters by TPS as applied in oligonucleotide synthesis.

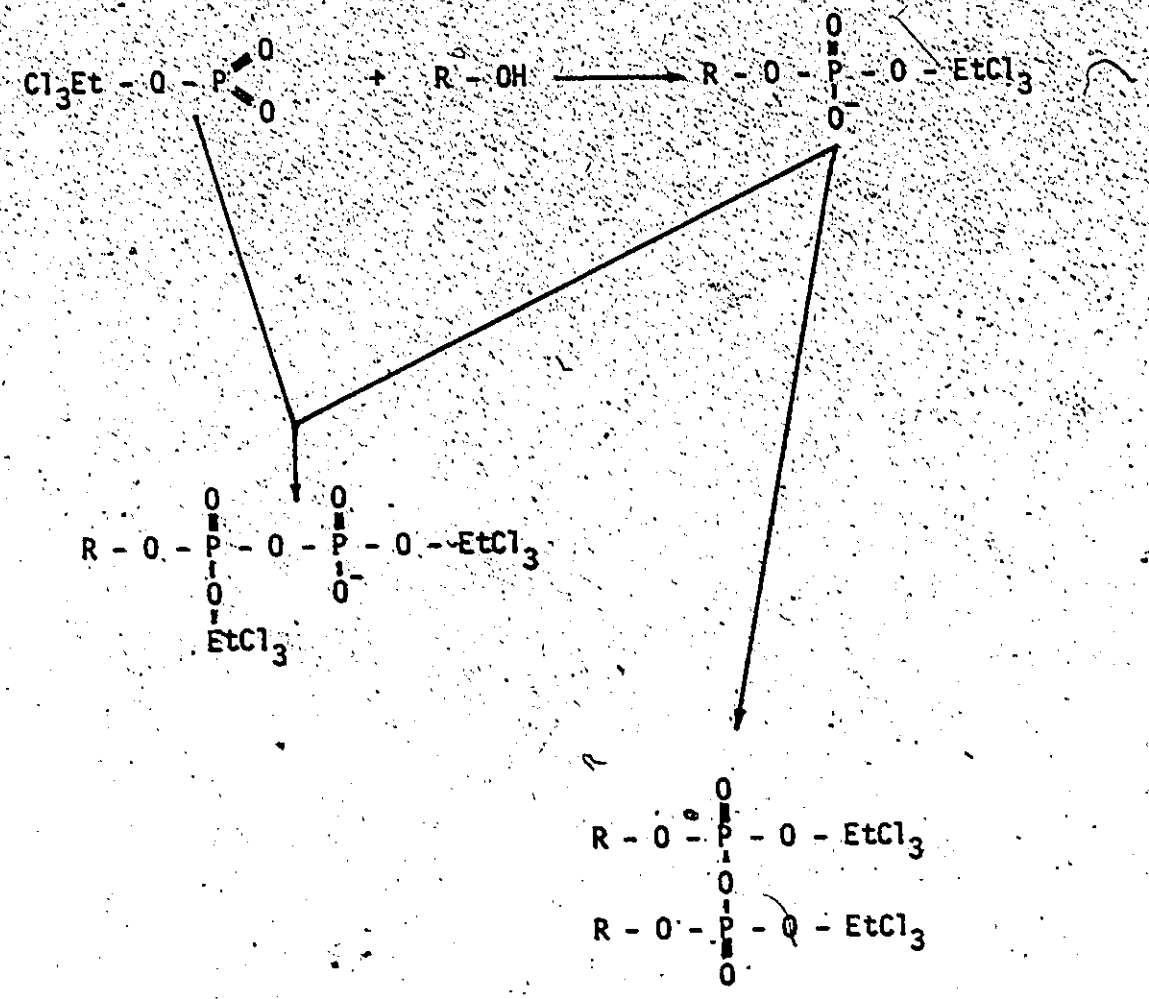
They demonstrated a series of reactions going from monophosphate monoester, to symmetrical pyrophosphate diester, to linear triphosphate triester, and finally, to the monosubstituted metaphosphate. Subsequent reaction of this metaphosphate with a nucleoside hydroxyl group resulted in formation of the phosphodiester which, being a stronger nucleophile than the hydroxyl group of a nucleoside, would then readily form a trisubstituted pyrophosphate by reaction with another metaphosphate. (Stoichiometry: two phosphomonoesters plus two TPS plus one nucleoside gives one phosphodiester linkage). Reaction of the phosphodiester itself with TPS and subsequent reaction with another diester gives the symmetrical pyrophosphate and this method could be used to drive the equilibrium of the previous reaction in favor of the metaphosphate (Stoichiometry: two phosphomonoester plus three TPS plus two nucleosides gives two phosphodiester linkages). This scheme is shown in figure 17.

Since the equilibrium of the reaction linking the two schemes favors the former, much more TPS would have to be used. In order to drive the phosphorylation reaction itself to near completion, usually an excess of one equivalent or more is desirable. Thus it would seem that two to four equivalents of phosphate (as monoester) and three to four or more equivalents of TPS for each equivalent of nucleoside would be desirable.



(continued)

Figure 17. Mechanism of activation of phosphate esters by TPS as proposed by Knorre and Zarytova (1974).



where R refers to a protected nucleoside or oligonucleotide

Figure 17. (continued)

The phosphorylation procedures used in this work used two equivalents of trichlorethyl phosphate and four equivalents of TPS, and in most cases these proportions were adequate. They could, perhaps, be improved by using still greater proportions.

The increased proportions of phosphate and TPS are proposed as there may be some loss of utilizable phosphate and TPS by formation of phosphoramidates of residual cyclohexylamine. The bis-cyclohexylammonium salt of trichloroethyl phosphate is transformed into the pyridinium salt before the activation step by repeated solution in anhydrous pyridine and evaporation to near dryness under reduced pressure. Since the vapor pressure of cyclohexylamine is less and its basicity (and therefore its extent of protonation) is greater, than those of pyridine this co-distillation method would appear to be an inefficient method of removal of the cyclohexylamine. Any traces remaining would react readily with the activated phopshate, thus removing it from the desired reaction pathway.

More recent work in this laboratory has found the use of trichloroethyl phosphoric acid rather than the cyclohexylamine salt satisfacory, thus eliminating this problem.

Activation of the phosphodiester by TPS (as required for the coupling reaction) was found (Knorre and Zarytova, 1974) to proceed via formation of the tetrasubstituted pyrophosphate (no further reactions leading to the metaphosphate can occur because of the second ester group on each phosphate). This supports the same conclusion drawn earlier (Eckstein and Rizk, 1969). While these studies were carried out with

deoxynucleotides, the same arguments may well apply to the ribonucleotides used here, sterically hindered by the 2'-O-tetrahydropyranyl groups.

The natural consequences of this steric hindrance in the ribonucleotides were slower rates of reaction with the incoming hydroxyl group. Furthermore, the success of a nucleophilic attack by the incoming nucleoside results in displacement of a non-activated 3'-terminal phosphate. Thus it must be considered that only half of the phosphorylated species is activated at any given time.

If steric hindrance is sufficient to prevent significant formation of a pyrophosphate, then the actual intermediate must be the mixed sulfonicphosphoric anhydride. Since the triisopropylbenzenesulfonyl chloride reagent was chosen for its sterically hindered active end, the activated complex will also be highly hindered.

In either case, a less sterically hindered activating agent such as toluenesulfonyl chloride or mesitylenesulfonyl chloride would be desirable even at the expense of a significant extent of sulfonylation of the 5' hydroxyl of the incoming nucleoside. Khorana has shown that the use of an excess of the nucleosidic component in the coupling reaction can reduce this problem to insignificance. (Lohrmann and Khorana, 1966).

Another alternative which would appear advantageous is the revival of an old principle of synthetic chemistry. The formation of a phosphotriester may be looked upon as the substitution of a phosphate group with three ester functions of different sizes. The advantages of adding the smallest group last are several. Formation of a phosphodiester as discussed previously can be driven to completion reasonably well and com-

paratively quickly because of the lesser influence of steric factors on that mechanism and the greater reactivity of the metaphosphate compared to the symmetrical phosphoric anhydride as the active intermediate. Then addition of the third ester group would involve a small and less hindered nucleophile, which should result in a greater efficiency and rate of formation of the triester product.

Since activation of a dinucleoside phosphodiester would be less favorable, though, perhaps a better method would be to activate the hydroxyl of the third group (trichloroethanol) for attack by the naked diesterified phosphate anion. This cannot be done profitably with nucleosides because of possible internal cyclization by attack of ring constituents such as the N-3 of adenosine on the activated 5'-site (Khorana, 1961). However the trichloroethyl group could be so activated, thus eliminating the necessity of using highly hindered 3'-3' pyrophosphates or mixed anhydrides as activated intermediates.

In any case, addition of the phosphate protecting group last would allow vast excesses of the reagent to be used to drive the reaction without vast expense, as would be the case if similar excesses of nucleoside components were used in the same context.

Such an approach, using the diester coupling method followed by the addition of the triester protecting group has been termed a "mixed" approach to oligonucleotide synthesis in studies reported by Smrt (1972b).

While this new-found understanding of the mechanism may explain why some coupling reactions do not go to completion and, in part, the low yields obtained on block coupling reactions, it does not shed

light on the problem of nonquantitative yields obtained of deprotected sequences, especially the apparent nil yields from the block coupled products. The scheme of complete deprotection and purification by paper chromatography used in earlier studies on di- and trinucleotides in this laboratory were established and apparently satisfactory. However, a thorough consideration of these methods and comparison of the results obtained with those of other investigators reveal possible pitfalls in working with longer sequences.

Reductive cleavage of the trichloroethyl protecting group by a zinc-copper couple in DMF (and several other conditions) was reported by Eckstein (1967a,b; and Eckstein and Rizk, 1969) in 42% - 90% yields. Alteration of N-benzoylcytosine under conditions required for removal of the trichloroethyl group was reported by Cook et al. (1969a,b) and other authors have reported varying degrees of success with this protecting group and its removal. Letsinger (1975) expressed distrust of this method for removal of the trichloroethyl group and presented successful preliminary results using a different method: sodium or lithium with naphthalene in hexamethylphosphoric triamide gave 95% cleavage without apparent side reactions. Experimental details have not yet been published.

The β-elimination mechanism at work in the removal of trichloroethyl (also the cyanoethyl) protecting groups should not result in chain cleavage but the effect of zinc and copper in solid or solvated form on phosphotriester linkages has not been investigated. The effect of zinc and copper ions on phosphodiester linkages has been pointed out previously (Butzow and Eichhorn, 1965).

After removal of the phosphate protecting groups, the sequences are polyanionic. Subsequent removal of the N-protecting groups in methanolic ammonium hydroxide renders the sequences still more polar, and this treatment did not entirely solubilize the metal couple. Small sequences such as the mono- and dinucleotides used in earlier studies may be bound to the surface of the metal but may be washed off with the 1 N ammonium hydroxide treatment used. Increasing the number of anionic charges (by increasing the chain length) will tend to increase the strength of binding to the metal surface in a cooperative manner so that quantitative recovery may require a very extensive wash under optimal conditions. Obviously, if longer (ie. intact) sequences are more firmly bound to the zinc-copper surface while shorter (i.e. degraded) sequences are readily washed off, a very poor picture of the relative yields of intact and degraded sequences emerges. Total yields of u.v. absorbance are subject to variations in extinction coefficients at any given wavelength between the N-protected and deprotected sequences and in the solvents of different pH used as well as changes due to hypochromicity, making determination of recovery of material from the deprotection reaction unreliable.

The basic deprotection step is methanolic ammonium hydroxide and should not be sufficiently drastic to cause significant amounts of chain cleavage if removal of the phosphate protecting groups has been completed. Similarly, conditions for removal of the 2'-O-tetrahydropyranyl groups have been established as mild enough to cause only a few percent migration of the phosphate from 3' to 2', at the very worst. Thus chain cleavage should be almost nonexistent, in these two steps.

The possibility of degradation by nucleases is extremely small until the completely deprotected product is obtained. Deprotected sequences could be stored and handled without apparent degradation after their initial purification, strongly suggesting that degradation must have occurred during or prior to deprotection.

Therefore the problem must lie in the first step of the deprotection procedure: removal of the trichloroethyl groups with a zinc-copper couple in DMF. How and why chain cleavage should occur at this step is unclear but the correlation of these data with the findings of other investigators supports this interpretation.

3.2.7. Summary

Synthesis of a ribonucleotide sequence coding for N-formylmethionylangiotensin was proposed as the first thrust into exploration of mechanisms of genetic code punctuation by structural features of a messenger or messenger-like sequence. The approach to be used in this synthesis was stepwise synthesis of protected tri- and tetranucleotide blocks by the triester method and subsequent coupling of adjacent pairs of blocks after selective deprotection of the 5' hydroxyl of the second member of the pair. Complete deprotection and characterization of each block assembled provided evidence of the fidelity of synthesis.

The methods to be used had been established for several shorter sequences. Synthesis of most of the tri- and tetranucleotide blocks required were completed, though on a smaller scale than initially anticipated. Attempts to obtain the necessary 5'-deprotected blocks were

partially successful, allowing the coupling of two pairs of blocks to be investigated. Preliminary evidence suggested modest success of these block couplings but characterization of the products after complete deprotection was uniformly unsuccessful. Due to the length of the synthetic pathways and difficulties encountered with selective deprotection, block coupling and complete deprotection of block coupled products, further progress towards the objective was not possible.

Instead, a thorough analysis of the progress made and difficulties encountered was undertaken in order to define the problems and indicate potential solutions. In doing so, a basis for future work along the same lines was established.

The first of the three problem areas, selective deprotection of the 5'-hydroxyl was promptly recognized and its solution is dependent on empirical studies to optimize conditions for the particular sequence to be used.

The second difficulty, low efficiency of block coupling was explained in light of recent studies on the mechanism of activation of phosphate esters by TPS. Several potential improvements were suggested on the basis of this theory, which would avoid the highly sterically hindered intermediates presumably formed in the reactions now used.

For the third problem, a plausible explanation for the lack of evidence for the existence of block coupled products was offered. This degradation and binding of larger sequences with zinc-copper couple has been discussed as a possibility (rather than a proven conclusion because of the lack of sufficient information) in view of its potential im-

portance for future work along these lines.

Finally, a descision was made to extend the stepwise synthesis methods to another area of interest in protein biosynthesis: the isolation of a transfer RNA species by oligonucleotide affinity chromatography. The conclusions reached in this study and the following one will be presented in detail in the last chapter.

4. AFFINITY CHROMATOGRAPHY OF ISOLEUCINE TRANSFER RNA

4.1. Introduction

The anticodon of a tRNA molecule is, by necessity a unique feature of each species of tRNA. The present proposal, as introduced previously, would employ the strength and specificity of interaction of a synthetic tetranucleotide with the complementary anticodon region of isoleucine tRNA for separating this species from bulk tRNA by affinity chromatography.

The selective nature of this interaction was demonstrated by the binding of specific tRNA species to ribosomes in the presence of a trinucleotide or codon by Nirenberg and Leder (1964) in deriving the genetic code. The specificity and strength of complementary interaction, in the absence of ribosomes, of trinucleotides with tRNA have been measured using gel filtration chromatography (Hogenaur, 1967) and equilibrium dialysis (Hogenaur, 1970) and of tri- and tetranucleotides with single stranded regions of methionyl tRNA (Uhlenbeck, Baller, and Doty, 1970), 5S RNA (Lewis and Doty, 1970), isoleucine tRNA (Schimmel *et al.*, 1972), and tyrosine tRNA (Uhlenbeck, 1972).

Selective retardation of tRNA on cellulose columns containing oligodeoxynucleotides (Gilham, 1964; Erhan, Northrup and Leach, 1965) and on polyacrylamide containing purified phenylalanine tRNA (Grosjean, Takada and Petre, 1973) also strongly support this proposal. The advantages of being able to use a specific synthetic ribonucleotide sequence as the affinity site should allow improvement on these previous

studies and permit the development of a general method for separating isoaccepting tRNA species from each other or from bulk tRNA.

Since the binding of synthetic tri- and tetranucleotides to isoleucine tRNA has been studied and one of the isoleucine codons (ApUpC) is available in the protected form from previous work, these were the logical choice for this affinity chromatography study. The fact that the nucleotide adjacent to the anticodon on the 5' side in tRNA, regardless of species is uridine suggests that the affinity site should be the tetranucleotide ApUpCpA.

Cellulose was chosen for the insoluble matrix because it had been used successfully in similar applications and because it can undergo coupling procedures in either pyridine or aqueous media. This offers the opportunity of coupling the affinity site to cellulose either at the protected stage or after deprotection.

For the actual linkage between the synthetic oligonucleotides and cellulose, phosphodiester linkages as used in earlier studies would by convenient and stable. The present proposal is complicated by consideration of the point of attachment and probable requirement for a spacer group as discussed in the introduction.

In previous oligonucleotide affinity studies, spacer groups were not used. However the data of Astell and Smith (1972) indicate that binding sites of oligonucleotides attached by a 5'-phosphodiester are available for binding up to within one half nucleotide of the point of attachment. No data is available on the accessibility of 3'-bound affinity sites. Since the whole tetranucleotide binding site must be

available to the tRNA anticodon, a spacer group would be advisable regardless of the site of attachment of the oligonucleotide.

If the spacer group had two hydroxyl groups as its reactive termini, and no internal linkages succeptible to cleavage under the reaction conditions used for deprotection of oligonucleotides, it could be introduced at the protected stage of oligonucleotide synthesis. Its incorporation would be analogous to coupling a protected nucleoside residue. Decane-1,10 diol was chosen as the simplest possible analog, having sufficient length to serve as an adequate spacer group. This group could be coupled to a protected sequence bearing an activated 3' terminal trichloroethyl phosphate as in the normal nucleoside coupling reaction. Coupling to both ends of the bifunctional diol was avoided by using a ten-fold excess of decane diol. Phosphorylation of the purified oligonucleotide with the coupled diol would give a terminal protected phosphate, which could be used as the cellulose attachment site either before or after deprotection.

The other alternative considered was the use of a 5' phosphodiester linkage as used by Gilham and by Smith. The preparation of 5' phosphorylated synthetic oligoribonucleotides is also important in several other applications of biochemical and biological interest and a suitable method has been developed in this laboratory (Neilson et al., 1975). A spacer group could also be introduced at the 5' end, if required. Model studies were conducted to ensure that decane diol could be coupled and that 5' phosphorylated sequences could be prepared.

The design of the affinity chromatography experiment after preparation and characterization of the cellulose linked deprotected sequence

must also be considered. The time and expense required for preparation of purified species of tRNA would make it inconvenient and impractical to use these in order to demonstrate selective retardation of the isoleucine tRNA species. Instead, if bulk tRNA, enzymatically acylated with radioactively labelled isoleucine were used, selective retardation could be observed as an increase in the ratio of radioactivity to ultraviolet absorbance in material eluted later from the column. Procedures are available for enzymatically acylating tRNA.

Actual conditions for affinity chromatography may have to be examined experimentally but sufficient data is available from previous studies to suggest a likely set of initial conditions: 1.0 M NaCl, 10 mM Mg^{++} pH 4.5 - 7.5, 0 - 5° (e.g. Uhlenbeck, 1972).

In summary, there are four basic parts of this proposed separation of isoleucine transfer RNA by affinity chromatography. First, the protected tetranucleotide, $TracAt^{bz}$-Ut^{bz}-Ct^{bz}-AtOH, must be synthesized and extended on the basis of model preparations to a protected sequence bearing a 3' decyl group with a terminal phosphate. Attachment of the sequence to cellulose and characterization of the deprotected material would follow directly. The third prerequisite is the preparation and characterization of labelled aminoacyl tRNA by established methods. Finally, affinity chromatography may be attempted.

4.2. Results and Discussion

4.2.1. Model Compounds Containing Decane Diol Spacer

The feasibility of incorporating a 3' terminal decyl group and its subsequent phosphorylation was demonstrated by preparing these derivatives of TracAtOH and TracUtOH, as outlined in figure 18, using the established coupling and phosphorylation procedures.

TracUtOH, previously synthesized, was phosphorylated and coupled to 1,10-decane diol using the TPS activating agent as before. A tenfold excess of decane-diol was used to ensure that the major product would be the monoesterified diol. This reaction was expected to proceed more rapidly than other coupling reactions because the hydroxyl groups of the decane-diol are less sterically hindered than those of an incoming nucleoside. For this same reason, the coupling reaction could not be driven by treatment with additional TPS because of the possibility of reaction of TPS with the diol hydroxyl groups. After four hours, t.l.c. indicated about 25% conversion of protected mononucleotide to the coupled product (XXIX) and no further reaction was observed after one and two days.

Excess decane diol co-eluted with the product during purification by silica gel chromatography. While some of this material precipitated from methylene chloride solution, it was found that chromatography using the ethyl acetate and hexane solvent system used to purify TracAtOH separated these two species effectively. The final yield obtained was 8%.

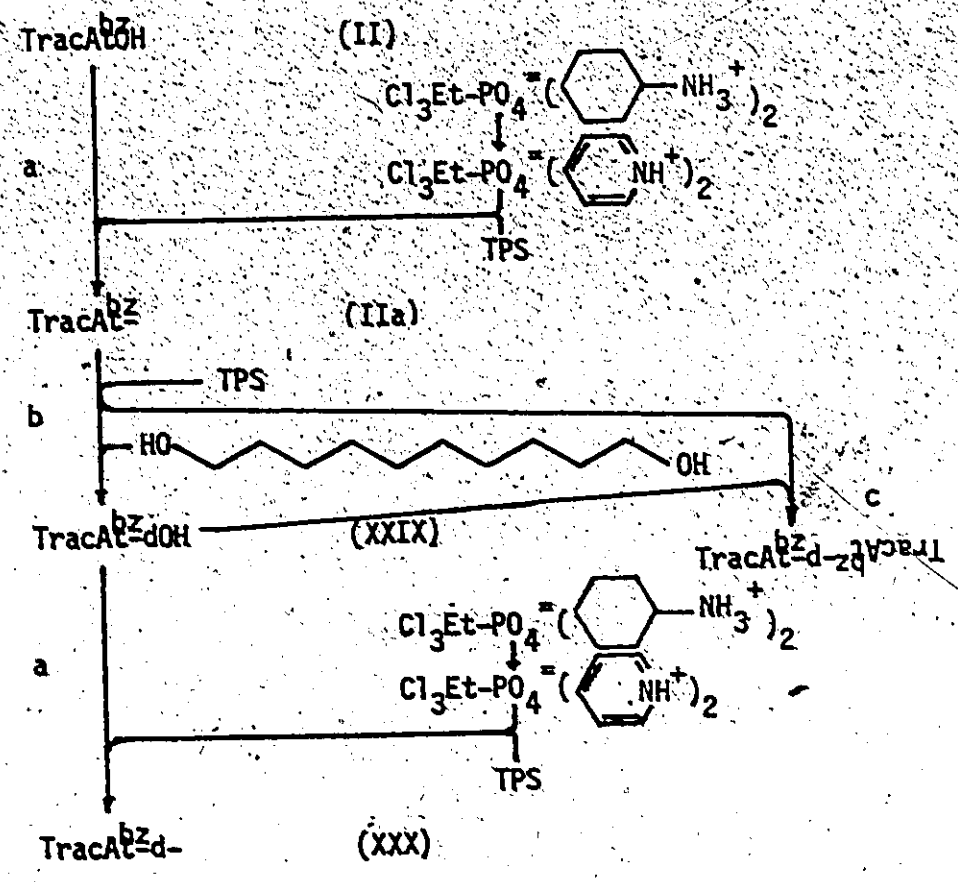


Figure 18: Synthesis of TracAt^{bz}-d- (XXXII).

a) Phosphorylation (as before for coupling).

b) Coupling with 10 fold amount of decane 1,10 diol (the decyl group is abbreviated "d". Thus decane diol is abbreviated HOdOH and N^6-benzoyl-2'-O-tetrahydropyranyl 5'-o-triphenylmethoxyacetyladenylyl (3'-[2,2,2-trichloroethyl]-10)-decan-1-ol (XXIX) is abbreviated TracAt^{bz}-dOH.

c) The amount of this side product occurring is a function of the ratio of XXIX to HOdOH. Using a 10 fold quantity of HOdOH should result in a 40:1 ratio of XXIX to side product.

Synthesis of TracAt^{bz}-dOH (XXXI) was accomplished by the same method, giving 27% yield. Subsequent preparations of these two compounds gave 29% and 40% yields respectively.

Samples of these compounds were characterized by p.m.r.: both spectra contained a large multiplet at δ (relative to TMS) = 1.35 p.p.m. corresponding to the methylene protons of the decane diol group. In other respects, the spectra were similar to the parent compounds TracAt^{bz}OH, and TracUtOH. TracAt^{bz}-dOH could not be crystalized because of the presence of two diastereoisomers due to the phosphotriester chiral center but a thoroughly dried sample gave a satisfactory elemental analysis for nitrogen and phosphorus.

Phosphorylation of XXIX and XXXI proceeded rapidly as expected but both reactions remained incomplete. Again these reactions could not be forced by addition of TPS because of the possibility of sulfonylation of the terminal hydroxyl groups.

Some reaction of TPS with the terminal hydroxyl group of the diol would not be readily detectable by t.l.c., whether it occurred before or during the phosphorylation reaction, and this may explain the incomplete phosphorylation. A small proportion of sulfonylation of the decane diol itself is apparent from t.l.c. analysis of coupling reactions which have been proceeding for about one day or more.

Yields for the phosphorylation reactions were not calculated because these materials contained varying amounts of triisopropylbenzenesulfonate which rendered weights of the final product inaccurate. While the yields by t.l.c. for these compounds were lower than yields

usually expected in nucleoside coupling and phosphorylation reactions, they were considered adequate for the proposed objective.

4.2.2. Selective 5' Phosphorylation

Oligonucleotides containing a 3' terminal phosphate or no terminal phosphate are readily obtained by the stepwise method of synthesis employed in this laboratory. It would be desirable to be able to duplicate portions of natural sequences bearing a 5'-phosphate, such as tRNA, or to produce sequences requiring 5'-phosphates for increased activity, such as found with the binding of trinucleotides to tRNA in the presence of ribosomes.

Terminal phosphates can readily be introduced at the deprotected stage using the enzyme polynucleotide kinase and ATP, but on a preparative scale, it may be advantageous to do it chemically at the protected stage.

This would require additional protection of the 3'-hydroxyl group, and deprotection of the 5'-hydroxyl group of an otherwise fully protected oligonucleotide, before phosphorylation in the usual manner. This has been accomplished for several sequences as outlined in figure 19 for TracAt^{bz}-Ut-At^{bz}OH (Neilson *et al.*, 1975). The preparation and characterization of the model compounds XXIII and XXIV and the sequence pApUpA are described here.

The first step was protection of the 3' terminal hydroxyl with a tetrahydropyranyl group. The reaction was carried out under the same conditions as used for the preparation of 2'-O-tetrahydropyranyl

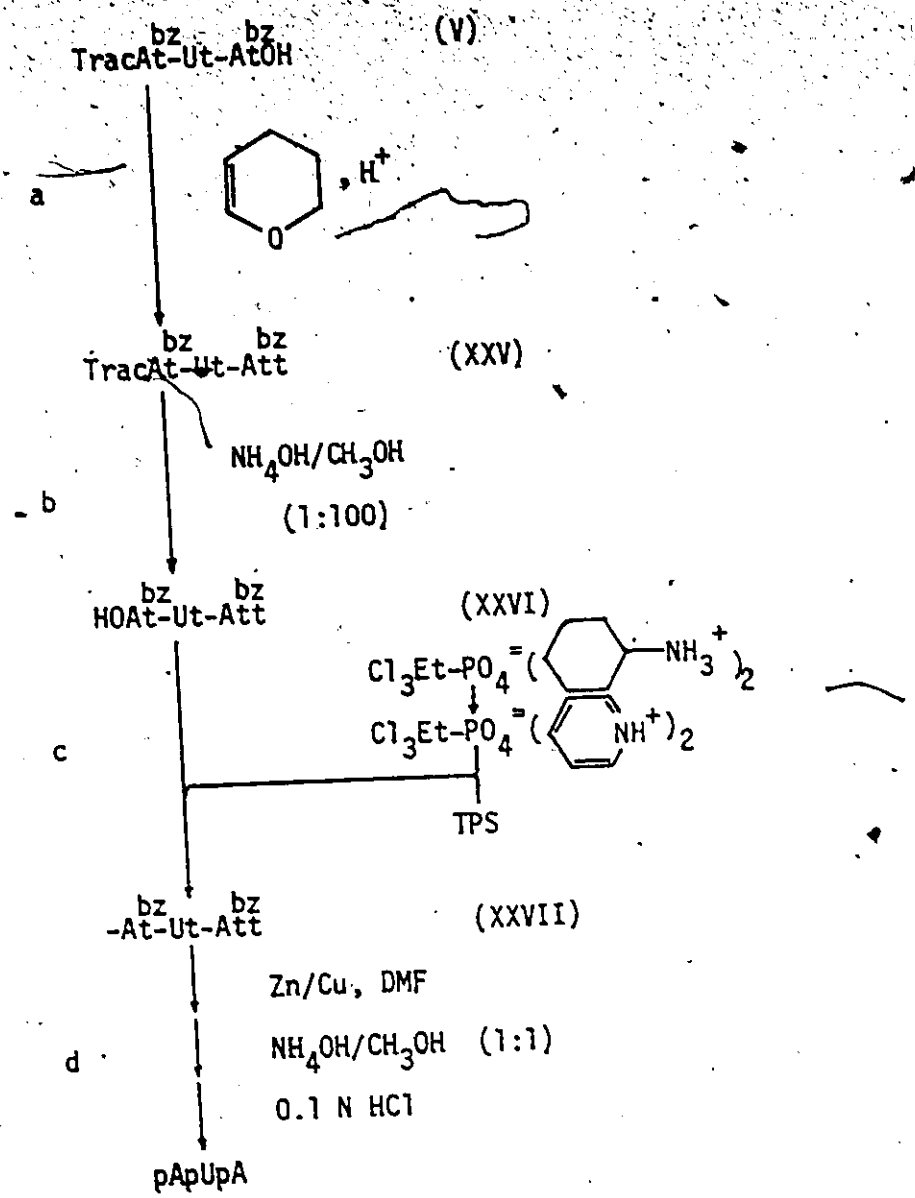


Figure 19: Synthesis of pAUpA

a) protection of 3'-hydroxyl with a tetrahydropyranyl

b) selective 5' deprotection

c) phosphorylation

d) 3 step complete deprotection.

derivatives of nucleosides (Neilson and Werstiuk, 1971a).

This reaction with TracAt^{bz}OH was readily followed by t.l.c. in 5% methanol in methylene chloride (yellow spot, R_f = 0.5 → 0.66) and appeared to be complete after two to three hours at room temperature. This product was isolated in 77% yield and its characterization by p.m.r. and elemental analysis was satisfactory.

Selective removal of the 5' triphenylmethoxyacetate group with 1% ammonium hydroxide in methanol gave 90% of XXIV (yellow spot, R_f = 0.66 → black spot, R_f = 0.29 in 5% methanol in methylene chloride). Characterization of this product by p.m.r. and elemental analysis was also satisfactory.

Phosphorylation of XXIV was not attempted, as work in this laboratory with other protected oligomers indicated no difficulty with this reaction (Neilson et al., 1975).

Formation of the 3'-O-tetrahydropyranyl derivative XXV, of TracAt^{bz}-Ut-A^{bz}OH (V), was slower requiring 5 hours at room temperature to obtain nearly complete reaction. This product was isolated in 84% yield but was not further characterized. Selective removal of the 5' triphenylmethoxyacetate group as before was accomplished in 2.5 hours giving 57% of XXVI.

Phosphorylation of XXVI by the usual procedure was rapid (2 hours) and the protected 5'-phosphorylated product XXVII was purified by silica gel chromatography.

Structural characterization of the intermediates in this synthesis was not considered practical or necessary and successful characterization

of the completely deprotected sequence is convincing evidence that these intermediates have the proposed structures.

4.2.3. Protected Oligonucleotide Affinity Sites.

The protected oligonucleotide required for the proposed affinity site is TracAt^{bz}-Ut-Ct^{bz}-At^{bz}-d- (XXXV). It was prepared in the stepwise fashion previously described.

TracAt^{bz}-Ut-Ct^{bz}OH (VIII) previously synthesized, was phosphorylated incompletely as found before with this sequence, and was purified by silica gel chromatography before coupling to the incoming adenosine nucleoside. The coupled product (XXXIII) was obtained in an average yield of 59%.

Phosphorylation of XXXIII and coupling to 1,10 decane diol, as described earlier for the model reactions, gave TracAt-Ut-Ct-At-dOH (XXXIV) in an average yield of 34%. This product, unlike the model compounds, was readily separated from the excess of decane diol by silica gel chromatography. This accounts for the lower yields reported for the model compounds. Phosphorylation of this compound was rapid but incomplete, as seen in the preparation of the model compounds, but raising the molar excess of phosphorylation reagent mixture to 5-fold, rather than two to three fold as used previously, appeared to increase the yield of XXXV as indicated by t.l.c. As for the model compounds, actual yields were not calculated due to heavy contamination of these products with residual sulfonate. The extent of this contamination (50-80% estimated) in part explains the low yields obtained on complete deprotection as described in a later section.

A second oligonucleotide sequence for an affinity site for tRNA affinity chromatography was also considered. Since a tetranucleotide binding site contains effectively two overlapping triplets, it is possible that a second species of tRNA would bind (although less efficiently) to this second codon. If this second codon, which will be the "last" or 3'-proximal three nucleotides, is a termination codon, then no tRNA with a complementary anticodon will be available for this competing binding.

The termination triplet UpApA was available in the protected form from previous work and could be extended on the 5' side to give the "first" codon of the resulting tetranucleotide. Synthesis of the tetranucleotide TracUt-Ut-At-$\overset{bz\ bz}{At}$OH (XXXVIII) by coupling TracUtOH to the selectively deprotected triplet HOUt-At-$\overset{bz\ bz}{At}$OH (XXVI) would give additonal data concerning the efficiency of coupling with respect to its dependence on the structure of the incoming nucleoside.

Using the block coupling reaction just described, a yield of 28% was obtained after allowing five days for coupling to occur. Synthesis of XXXVIII by the usual stepwise procedure gave yields of 72%, (XXXVI); 65%, (XXXVII); and 48%, (XXXVIII); at each successive addition of a nucleotide unit. (see table 10). The yield on the last coupling reaction is almost double that obtained from the block coupling reaction. The yield from the block type coupling reaction was comparable to other yields from other block coupling in this laboratory (Neilson and Werstiuk, 1974).

These data indicate that the reactivity of the incoming nucleoside is strongly influenced by the size (length, conformation, and nature) of the sequence. The 5'-hydroxyl site must be sterically hindered by the rest of the sequence attached to the 3'-hydroxyl. A similar implication

is seen in the effect of 3'-substituents on the rates of hydrolysis of the 5'-protecting group (Neilson et al., 1975) which have been discussed.

TracUt-Ut-At-At-dOH (XXXIX) was prepared as described for the model compounds XXIX and XXXI in a yield of 28%.

The low yields obtained in all of the diol coupling reactions can be understood in light of the mechanism discussed earlier for coupling reactions. If the active intermediate in the coupling reaction is the pyrophosphate, then only 50% of this material would be available for coupling without reactivation by supplemental addition of TPS. Since TPS was not added to the diol reaction because of the possibility of sulfonylation of the diol, the maximum theoretical yield can be only 50% of the molar yield. In these terms, yields obtained are comparable to yields obtained in other coupling reactions.

The solution to this problem may be to use a monosubstituted decane diol so that additional TPS could be used to drive the reaction. While some sulfonylation of the unreacted diol may occur, this problem may be overcome by use of sufficient quantities of TPS and protected diol, added by some appropriate schedule to reduce the extent of this reaction.

The protecting group used would have to be selectively removable to allow terminal phosphorylation, or perhaps a protected phosphate would be the most suitable protecting group. The synthesis and characterization of such a diol derivative was considered too time consuming at this stage of the work and it was presumed that sufficient quantities of a protected affinity site with its diol spacer and terminal phosphate could be obtained.

4.2.4. Deprotection and Characterization of Synthetic Materials

Data pertinent to the synthesis of the protected oligonucleotides with and without diol spacers have been collected into one table for convenience (see table 9). Similarly, data on the deprotection and characterization of these materials were collected for presentation and discussion in this section (see table 10).

Complete deprotection of these materials was carried out using the three step procedure described previously. The completely deprotected oligonucleotides were applied to a column of DEAE cellulose in 7M urea, 0.02M Tris pH 7.2 - 7.5, 0.001 M EDTA solution and eluted with a linear ascending gradient of NaCl. This is the method of Tomlinson and Tener (1963).

Oligonucleotides elute in order of increasing total anionic charge, which correlates with chain length: one charge for each phosphodiester linkage and two charges for each terminal phosphate. Fractions containing ultraviolet absorbing materials were identified by the use of an ultraviolet flow cell and recorder, and fractions corresponding to materials with the correct number of charges for the expected product were pooled. Urea and salts were removed by passage of the pooled fractions through gel filtration columns (Sephadex G-10 or Biogel - P2) and the resulting aqueous solutions were reduced to dryness by lyophilization after recording their volume and ultraviolet spectrum. Samples were prepared for enzymatic digestion and for characterization by thin layer and paper chromatography as before.

Table 9

Data on synthesis of protected oligonucleotides including those with a decyl spacer.

	Sequence[a]	R_f	Yield:%	Scale: mmol	Scale: mg	Phosphorylation: t(days)	Phosphorylation: V(ml)	Coupling: t(days)	Coupling: V(ml)	Remarks
XXVI	pAUA	0.2-0.4	29[b]	0.14	59	0.1	1	-	-	b
XXIX	Ud	.72	29	0.266	167	1	5	2	5	
XXX	Udp	0.2-0.4		.05	50	1	2	-	-	
XXXI	Ad	.80	40	0.266	200	1	5	2	5	
XXXII	Adp	0.2-0.4		0.133	149	1	2	-	-	
XXXIII	AUCA	0.59	59	0.27	500	3	4	4	5	
XXXIV	AUCAd	.57	34	0.078	200	2	4	2	2	
XXXV	AUCAdp	0.2-0.4		0.032	100	2	1	-	-	
XXXVI	UU	0.75	72	1.60	1000	3	10	3	10	
XXXVII	UUA	0.68	65	1.13	1300	1	12	3	10	
XXXVIII	UUAA	0.63	27	0.08	70.5	2	3	4	0.5	d
XXXVIII	UUAA	0.63	48	0.33	600	2	5	2	4	c

(continued)

Table 9 (continued)

	Sequence[b]	R_f	Yield:%	Scale: mmol	Scale: mg	Phosphorylation: t(days)	Phosphorylation: V(ml)	Coupling: t(days)	Coupling: V(ml)	Remarks
XXXIX	UUAAd	0.58	28							

a. Abbreviations used here refer to the fully protected sequence of nucleotides

b. Yield over three steps required to insert the 5' phosphate selectively.

c. Synthesized by the stepwise addition of the 3' terminal nucleoside in the usual manner.

d. Synthesized by the block coupling style addition of HOUt-At(bz)-At(bz)OH (XVI) to TracUtOH (XXVIII).

Table 10

Data on complete deprotection and enzymatic characterization of oligonucleotides

Sequence	Yield:%	R_{Up}			Digest[a]	Products	R_{Up}[b]					
							A		B		C	
		A	B	C			Std.	Dig.	Std.	Dig.	Std.	Dig.
ApUpA[c]	93	0.62 (0.60)[c]	1.00	0.57	SVD	pA	.63	.61	1.28	1.25	0.61	0.56
						pU	.78	.81	.95	.94	.83	.79
						A	3.20	3.04	1.81	1.79	1.50	1.50
					Pan	ApUp	-	.37	-	.65	-	.46
						A	3.28	3.12	1.81	1.76	1.50	1.46
					+ Pase	ApU	1.31	1.24	-	1.19	-	.95
pApUpA	66	0.28	0.28	.20	SVD	pA	8.62	.63	1.30	1.30	.63	.59
						pU	.81	.89	.91	.94	.82	.79
					Pase	ApUpA	.63	.57	1.00	.94	.55	.56
					+ Pan	A	2.93	2.85	1.81	1.76	1.53	1.53
						ApU	1.31	1.30	-	1.07	-	0.88
Apd	77	4.1	1.89		Spleen	Ap	.81	.77	.98	.94		
Apdp	10	2.78	1.33		Spleen	Ap	.81	.78	.98	.98		
					Pase	Apd	4.1	4.1	1.89	1.89		
Upd	89	4.5	1.76		Spleen	Up	1.00	1.10	1.00	.98		
Updp	23	2.79	1.25		Spleen	Up	1.00	1.00	1.00	.92		
					Pase	Upd	4.5	4.5	1.76	1.73		
ApUpCpA	15	0.21	1.03	0.31	Pan	ApUp	-	0.32	-	0.71	-	0.45
						Cp	.85	.89	.98	.96	.95	.89
						A	3.16	3.26	2.03	2.00	1.74	1.79
					Spleen	A	3.16	2.98	1.97	1.91	1.74	1.66
						Up	1.00	1.05	1.00	.96	1.00	1.01
						Cp	.85	.83	.98	.96	.96	.92
						Ap	.79	.75	.91	.96	.67	.68

(continued)

Table 10 (continued)

Sequence	Yield:%	R_{Up} A	R_{Up} B	R_{Up} C	Digest[a]	Products	R_{Up}[b] A Std.	A Dig.	B Std.	B Dig.	C Std.	C Dig.
ApUpCpApd	27	0.45	1.21	.46	Pan	ApUp	-	.36	-	.79	-	.43
						Cp	.85	.85	.97	.94	.95	.92
						Apd	3.4	3.3	1.89	1.87	-	1.65
					Spleen	Ap	.79	.75-1.07	.94	.95	.67	.65
						Up	1.00	.75-1.07	1.00	.95	1.00	.97
						Cp	.85	.75-1.07	.97	.95	.95	.97
ApUpCpApdp	17	0.18	1.01	0.28	Pan	ApUp	-	.38	-	.76	-	0.49
						Cp	.85	.87	.97	.95	.95	1.00
						Apdp	2.20	2.45	1.33	1.36	-	1.23
					Spleen	Ap	.79	.82	.93	1.00	.67	.67
						Up	1.00	1.00	1.00	1.00	1.00	1.03
						Cp	.85	.82	.97	1.00	.95	1.03
					Pase	ApUpCpApd	0.45	0.39	1.21	1.15	0.46	0.44

a. The "Digest" column indicates the enzyme used (SVD for snake venom phosphodiesterase, Pan for pancreatic ribonuclease and Pase for alkaline phosphatase) where + indicates reaction with completed digest described in the line immediately above. The products expected from each digest are listed in the adjacent column. R_f values for each product and the corresponding standard, run concurrently, are given on the same line for three different solvent systems.

b. A = Whatman #1 paper eluted with Solvent A, B and C, cellulose t.l.c. eluted with solvents B and C respectively.

c. Standard values, Lührmann et al., 1966.

Paper chromatographic purification of these deprotected materials was less satisfactory and did not give the information regarding charge content of the products obtained. Enzymatic digestion gave the expected products in each case as indicated in table 10. This confirmation of structure prepared the way for coupling these sequences to cellulose.

4.2.5. Cellulose Coupling at the Protected Stage

Coupling of a protected oligonucleotide sequences to cellulose would appear to be analogous to coupling to another nucleoside. The protected sequence would than have to be deprotected by the three step procedure outlined previously. The reaction of Zn/Cu couple with a cellulose bound sequence in order to remove trichloroethyl protecting groups from the phosphates is likely to be less efficient than the solution phase reaction because both the cellulose substrate and the Zn/Cu couple are solids. In anticipation of this problem, two other alternatives have been considered.

If the Zn/Cu treatment is performed as the last step before coupling to cellulose, this problem may be avoided. The coupling reaction may then be performed in the same manner as before, which would now be analogous to the phosphodiester method of coupling.

The second alternative was complete deprotection of the oligonucleotide followed by purification and coupling to cellulose using the "water soluble DCC" procedure introduced by Gilham (1968). This procedure is discussed in the next section.

TracAt-$\overset{bz}{\text{U}}$t-$\overset{bz}{\text{C}}$t-$\overset{bz}{\text{A}}$t-d- (XXXV), prepared previously , was activated

with TPS in anhydrous pyridine as for coupling reactions. Dry purified cellulose was added which absorbed most the liquid. After nine days the reaction was quenched with a mixture of methanol and water and the cellulose thoroughly washed with the same solvent. The extent of reaction was estimated in two ways. T.l.c. of the residue obtained by evaporation of the combined filtrates to a small volume, and the ultraviolet spectrum of this residue compared with the spectrum of the same material before coupling at the same dilution, indicated that little material had been coupled to cellulose. Treatment of a portion of the cellulose with 1% ammonium hydroxide in methanol released the 5'-triphenylmethoxyacetate group from any material bound to the cellulose; presumably in the form of the corresponding methyl ester, (yellow spot, R_f 0.65 in methylene chloride). However only traces of this material were observed on t.l.c. analysis of the hydrolysis solution, suggesting that less than 1% of the starting material has been bound.

A sample of TracAt-d- was divided into two equal portions and one was treated with Zn/Cu couple to remove the phosphate protecting group before coupling to the cellulose. This portion will be referred to as the "diester" because the phosphate linkages present or to be formed were then unprotected, ie. diesters. Similarly, the other, untreated portion will be called the triester.

After activation of the diester and triester with TPS, these materials were added to batches of cellulose which had been dried by repeated suspension and anydrous pyridine and evaporation under reduced pressure, as done with incoming nucleosides for normal coupling reactions.

The reactions were allowed to proceed for two days. Each batch of cellulose was filtered and washed thoroughly with anhydrous pyridine. The filtrates from each batch were reduced to a small volume under reduced pressure, reactivated with TPS and recombined with their respective batches of cellulose for a further two day period.

Each batch was then filtered, washed with pyridine, methanol, and finally methylene chloride. Analysis of residues in the filtrates and of the cellulose itself as before indicated less than 1% reaction again but that the diester batch gave approximately double the amount of triphenylmethoxyacetate released from the triester batch of cellulose.

The presence of additional ultraviolet absorbing material discovered by t.l.c. analysis of the hydrolytic solutions from the cellulose suggested the presence of lignin impurities in the cellulose used. Treatment of the cellulose before coupling with pyridine, followed by ammonium hydroxide in methanol, followed by 0.1 N HCl, and drying at 100° after thorough washing with water, reduced but did not eliminate these impurities.

This cellulose, further dried by suspension in anhydrous pyridine and evaporation as before, was treated with TracUt-d- activated with TPS, in two batches. One batch was allowed to react in a larger volume of pyridine as a loose slurry, while the other was reduced in volume under reduced pressure until the reaction mixture appeared as a thoroughly damp powder. After reacting for two days, each reaction mixture was quenched and washed, as in the previous experiment and the extent of reaction estimated by the 5' deprotection method. The slurry batch gave about

1% coupling and the damp powder batch gave about 2% coupling.

Repetition of the diester and triester reactions in the damp powder form gave about 4% and 1% reaction respectively. While these methods of analysis are imprecise, the diester reaction in the damp powder form undoubtedly gave the largest yield obtained.

The uniform lack of satisfactory results obtained from these coupling reactions at the protected stage showed that coupling to cellulose was not analogous to coupling to an incoming nucleoside. The remaining alternative of coupling at the deprotected stage was then considered.

4.2.6. Cellulose Coupling at the Deprotected Stage

A suitable method of coupling of deprotected oligonucleotides to cellulose in aqueous media was proposed by Gilham (1968). Astell and Smith (1972) have modified the methods slightly and improved the yields of material bound.

Whatman 3MM chromatography paper was dampened with a solution containing the oligonucleotide, 1-cyclohexyl-3-(-2-morpholinoethyl) carbodiimide ("Water soluble DCC" or CMC) and morpholinoethane sulfonate buffer (pH 6). In the experiments of Gilham, and of Smith, the oligonucleotides carried a 5'-terminal phosphate destined to become the point of attachment. Ribonucleotide sequences bearing a 3'-phosphate do not couple adequately by this method because of the competing formation of the cyclic 2',3'-phosphate. The 3'-terminal diol-phosphate used in the current studies should resemble the phosphate of a primary 5'-hydroxyl,

and should not react intramolecularly. By the procedure of Astell and Smith, the dampened papers are allowed to dry for two days in the atmosphere, and are dampened again with a solution containing CMC and buffer as before. After two days the papers are eluted with phosphate buffer to quench the coupling reaction and to wash out the excess of coupling reagent and unbound nucleotide material.

Yields of oligonucleotide bound can then be determined by measuring the amount of nucleotide in the eluate by ultraviolet spectroscopy or by determining the amount of nucleotide bound to the cellulose by acidic or basic hydrolysis of an aliquot of the cellulose.

Yields obtained using this method for coupling simple mononucleotides were 85% ± 5% for pA and 0% ± 10% for Ap as expected. Couplings of other model compounds on the same scale as expected for the affinity sequence gave yields of 60% for Apdp, 50% for Updp and 60% for pApUpA (values ± 10%). These yields were considered adequate and if the density of bound nucleotide material should need to be increased, Astell and Smith (1972) report that repetition of the coupling procedure gives equivalent yields on subsequent couplings, indicating that the available binding sites in cellulose are not saturated by the first coupling reaction.

Yields of the deprotected sequence ApUpCpApdp coupled were uniformly about 25%. This corresponded to a ratio of 8-10 A_{260} units of synthetic oligonucleotide per gram (dry weight) of cellulose. This ratio in molar terms is comparable to that used by Gilham and that used by Smith who have used about 30 A_{260} units of oligonucleotide per gram of cellulose, and the size of the oligomers used was greater by a factor

of two or three.

Grosjean, Takada and Petre (1973) used a ratio of 4.2 A_{260} units of tRNA phenylalanine bound to 0.2 gram of polyacrylamide, giving a molar ratio approximately 10 fold less than that used here.

Thus the density of oligonucleotide bound to cellulose should be adequate for the affinity chromatography experiment and may be increased if necessary.

4.2.7. Affinity Chromatography

The proposed experiment is based on the passage of a mixture of tRNA containing isoleucine labelled species over a column of cellulose to which a synthetic tetranucleotide has been coupled. Preparation of the synthetic tetranucleotide and its coupling to cellulose by a decane diol spacer and phosphodiester have been described in previous sections. Here, remaining aspects of the affinity chromatography experiment and results obtained are discussed.

Columns containing unmodified cellulose and Apdp cellulose were prepared in order to explore the mechanics of preparing, packing and running these columns. Soft glass tubing (6 mm ID) was used to hold the cellulose after drawing one end out to a fine tip and packing a small wad of glass wool into this tip. Cellulose slurry, prepared by rapidly stirring or sonicating small chips of Whatman 3MM paper used in the coupling reactions, was packed into the column under pressure but at a very low flow rate.

Samples of tRNA and of ^{14}C isoleucine were applied to these

columns in order to obtain elution profiles for both of these species in the absence of the tetranucleotide binding site and in order to determine the recovery of ultraviolet absorbing material and radioactivity from these columns. Figure 20 is representative of the profiles obtained in these "blank" experiments. Recovery of both ultraviolet absorbing material and radioactivity were virtually quantitative. The elution conditions used were 1 M NaCl, 10 mM magnesium chloride, 0.01 M phosphate buffer at pH 7.0.

The next stage of preparation was enzymatic acylation of tRNA. Several procedures and modifications were tried (Hoskinson and Khorana, 1965; Nishimura et al., 1967), however, satisfactory results were eventually obtained using methods described by Goss and Parkhurst (1974) for preparative scale acylation of tRNA but using a magnesium ion to ATP ratio of 2.5 as suggested for optimal activity of isoleucine-tRNA synthetase (Novelli, 1967).

A high speed, centrifugal supernatant containing the soluble components of a yeast homogenate, including the synthetase enzymes (Amino acid: tRNA Ligases, E.C. 6.1.1....), is used to attach radioactively labelled isoleucine to bulk transfer RNA, in the presence of ATP, magnesium ions, and Tris buffer (pH 7.5-7.8). The reaction is assayed by measurement of radioactivity precipitated on filter paper discs by applying an aliquot of the reaction mixture and plunging the paper into ice cold 5% trichloroacetic acid (TCA). The kinetics observed for this reaction are shown in figure 21.

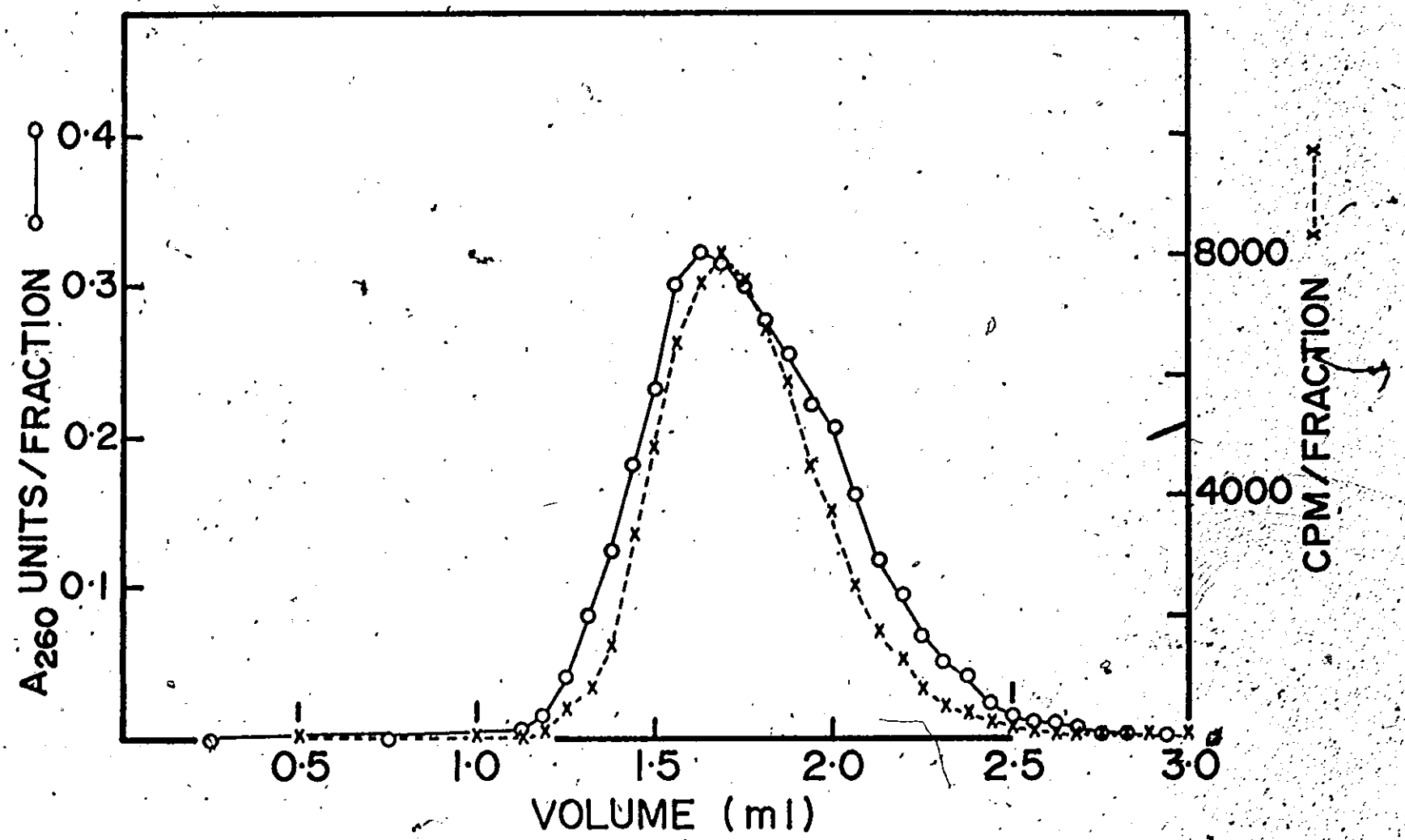


Figure 20: Elution profiles of tRNA and isoleucine from a cellulose column.

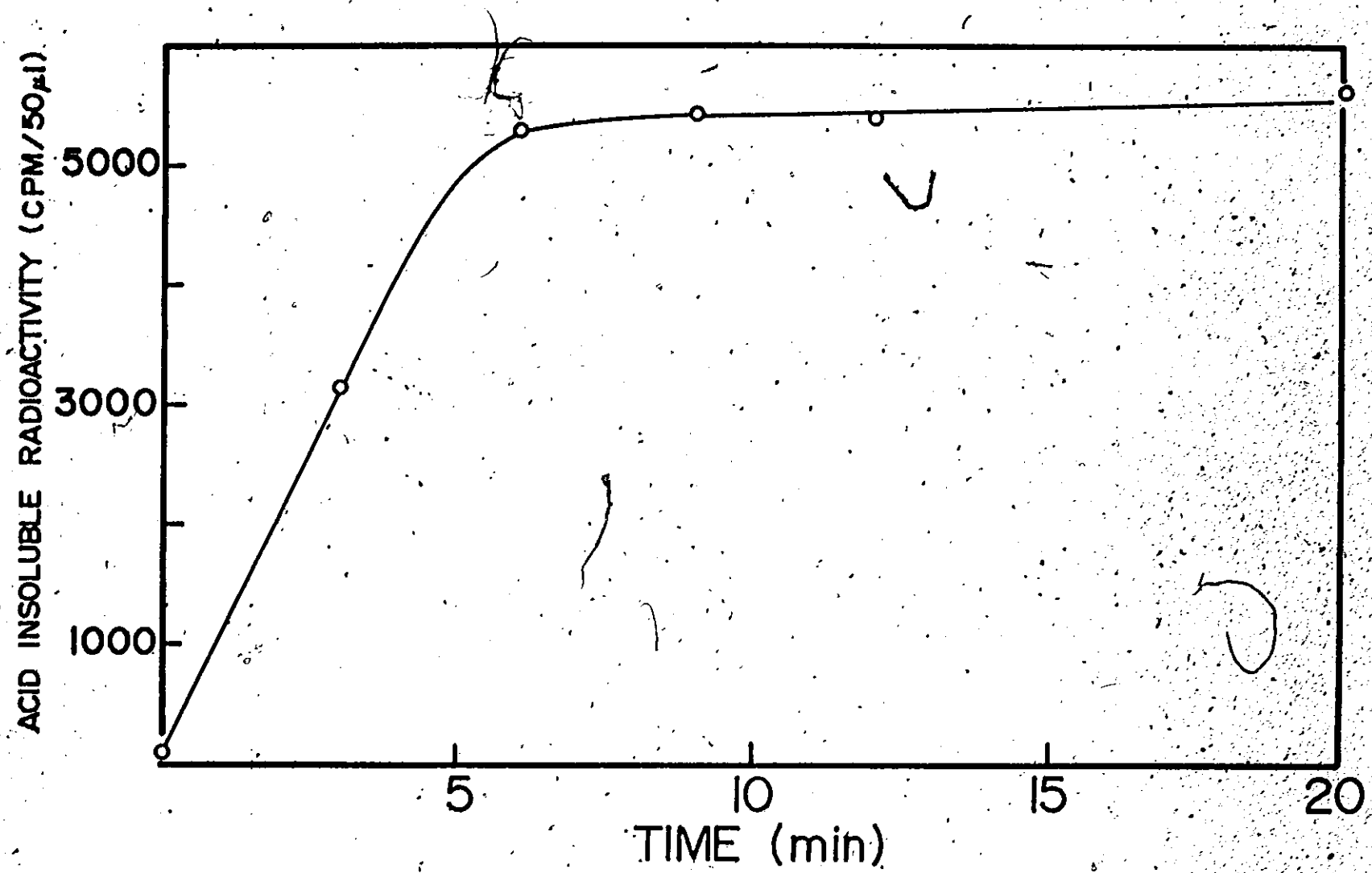


Figure 21. Kinetics of incorporation of ^{14}C isoleucine into tRNA by the method adapted from Goss and Parkhurst (1974).

On a preparative scale, the enzymatic reaction is quenched after a suitable time interval (10 - 30 min) by addition of ice cold acetate (Na^+) buffer at pH 4.5. The aminoacyl-tRNA linkage is more stable at this pH. tRNA is then precipitated with two volumes of cold ethanol and may be further purified by application to DEAE cellulose in 0.3 M NaCl, and after washing with 0.3 M NaCl to remove proteins, tRNA is eluted in a small volume by 1 M NaCl. This tRNA containing labelled amino acid may be stored in this form or precipitated with ethanol for storage. The latter method is preferable.

During the affinity chromatography experiment and during storage of the amino acylated tRNA, some spontaneous hydrolysis of the amino acyl bond may occur. This linkage is usually more stable at pH 4.5. However, for the affinity chromatography experiment this pH may not be suitable, as one of the bases in the affinity group is cytidine. The pK of the cytidine amino group is 4.15, indicating that more than 10% of these sites would likely be protonated even at pH 5. This protonation would probably eliminate effective binding at these sites.

Since the degree of stability of this amino acyl bond under conditions to be used to the affinity chromatography and at various pH values were not known, a series of experiments to determine the rate of loss of acid insoluble radioactivity with time at different pH were performed. Solutions buffered at pH 5.5, 6.0, 6.5 and 7.0 were prepared, acylated tRNA was added and these were stored at 4° for several days. Aliquots were removed, precipitated on filter paper as before and the radioactivity determined. These data are presented in figure 22.

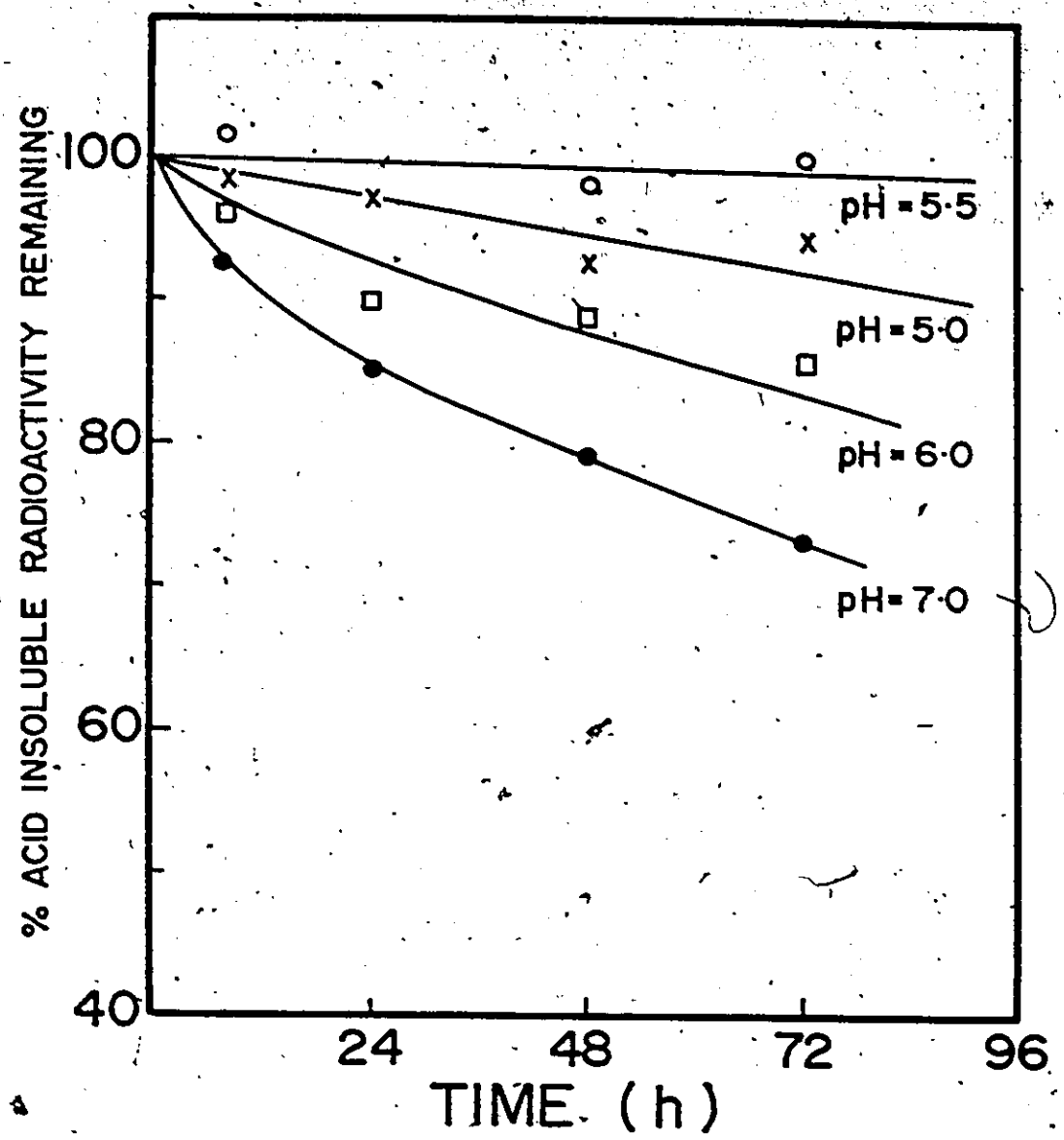


Figure 22: Rates of spontaneous deacylation of tRNA at different pH.

The results are clear. Under these conditions, radioactivity is lost from the tRNA only slowly. At lower pH values, less than 10% of the radioactivity was lost in three days. Since the anticipated time required to run affinity columns was less than this, the loss of radioactivity due to spontaneous hydrolysis should not introduce a significant error if the column were run at pH 5.5 - 6.0. At pH 6.0, slightly more than 1% of the cytidine bases would be protonated. The loss of these binding sites would not likely be significant in these experiments.

A column was prepared from the ApUpCpApdp cellulose and tRNA labelled with ^{14}C isoleucine was applied in 1 M NaCl. The column was eluted with the same solution as used previously but buffered at pH 6.0. Fractions were collected and diluted with water before recording the ultraviolet absorbance at 260 nm and counting the radioactivity by liquid scintillation counting.

The shape and distribution of the absorbance and radioactivity profiles did not appear to differ significantly from the blank profiles shown in figure 20, and since recovery of absorbance units and radioactivity were virtually quantitative, no residual tightly bound material was suspected.

Since sample size and eluted volume were large compared to total bed volume of the column, a small retardation of part of the radioactivity relative to the optical density may not be readily apparent. In order to try to improve the resolution, the experiment was repeated using a cooling bath to reduce the temperature of the column to about 0° rather than 4°.

In a third experiment, the column loading was reduced to 1/20 of the original loading, and the column was again run at the lower temperature. The low level of radioactivity and ultraviolet absorbance rendered low accuracy of measurement but neither of these variations altered the elution profiles obtained previously.

Another variation tried was the use of an annealing period of 18 hours, after loading the column. After elution of the main peak from the column, elution with a solution of 10 MM EDTA with no salt and at 23° gave a very small peak containing less than 1% of the radioactivity and less than 5% of the absorbance units.

Alkaline hydrolysis of the cellulose gave little ultraviolet absorbing material, indicating that the oligonucleotide sites had been lost.

The tRNA used in these experiments had been isolated only by precipitation with ethanol from the enzymatic reaction mixture and resuspension in a high salt solution. Thus it may have contained significant contamination with nucleases. This fact may explain the low or nonexistent binding and the loss of oligonucleotide material.

Another factor which was considered is that the tRNA sample applied contained no magnesium. As the tRNA elutes with very little nonspecific retardation, the magnesium concentration present with the tRNA during its passage through the column may be very low.

A new experiment, incorporating these modifications and several other improvements was performed. Transfer RNA was acylated with leucine (^{3}H labelled) to serve as a control, and with ^{3}H isoleucine (giving approximately 200 fold greater specific activity) and proteins were

removed by DEAE cellulose chromatography as described earlier. These samples were made up in a more concentrated form in 1.5 M NaCl and 10 mM magnesium chloride. Thus the density of the sample would stabilize it during its application to the column, and the sample volume would be smaller. The column was prepared from a new batch of oligonucleotide cellulose of similar site density and jacketed for circulation of a salt ice mixture to maintain the operating temperature of the column in the range -2 - 0°. Samples applied were about 0.5 A_{260} units of tRNA and the column was eluted very slowly after annealing the applied sample for about 2 hours.

After elution of the main peak and the level of ultraviolet absorbance in the eluate had dropped to background levels, the column was brought to 30° and eluted with 10 mM EDTA and 0.5 NaCl. The elution profile obtained for the experiment using isoleucine labelled tRNA is shown in figure 23. The release of approximately 5% of the radioactivity with this treatment indicates some binding. The amount of ultraviolet absorbing material released by this treatment at first appeared to be large but a scan of wavelengths indicated that much of this material was not nucleotide, but was probably residual lignins present in the cellulose. A column run with the leucine labelled tRNA indicated virtually indistinguishable elution profiles.

Using half the amount of tRNA showed that the amount of material bound, rather than the ~~fraction~~ of total material applied, was constant. A ratio of radioactivity to ultraviolet absorbance was not meaningful in these cases where the level of absorbance was low and the

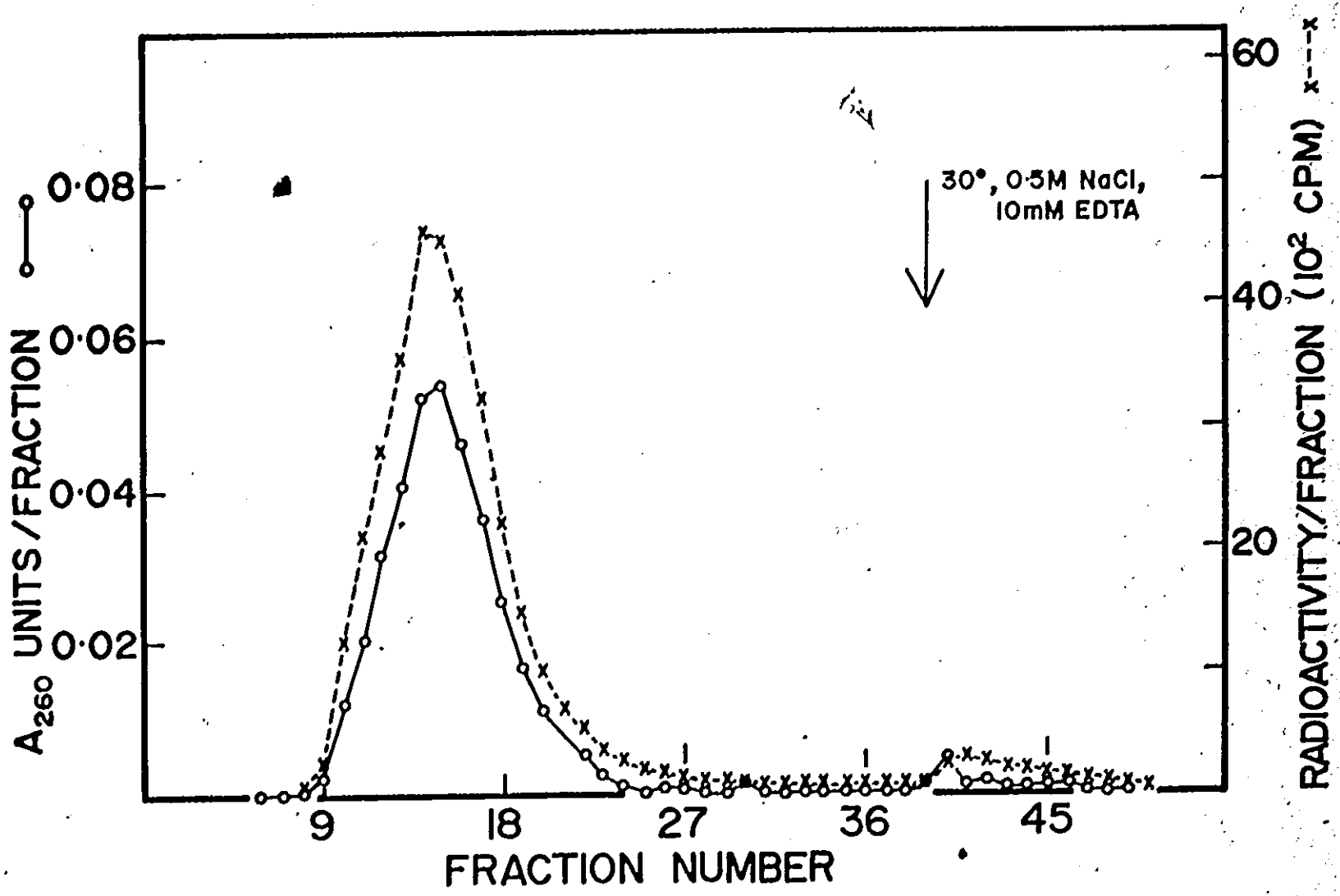


Figure 23: Elution of tRNA from ApUpCpApdp - cellulose affinity column. These profiles were obtained using ^{3}H isoleucine labelled tRNA. Profiles obtained using ^{3}H leucine labelled tRNA were indistinguishable.

background was variable. Alkaline hydrolysis of an aliquot of the cellulose used indicated the presence of approximately 80-90% of the original nucleotide material still bound to the cellulose. It could not be concluded that the isoleucine tRNA was binding specifically to the synthetic oligonucleotide binding sites.

On the basis of evidence presented to introduce this study, binding or significant retardation of 50 - 90% of the radioactivity was expected. Granting that a new system might not behave ideally, a clear increase in the amount of radioactivity bound or retarded was still expected. Since this is not the case, there must be some particular cause for the lack of significant retardation or binding.

4.2.8. Analysis

It should be possible to estimate the stability of binding to the column in a more quantitative manner, given the experimental conditions and the equilibrium constants determined for tRNA - tRNA interactions and tRNA - oligonucleotide interactions.

First let us examine the simple case of tRNA binding to the affinity site, assuming that the tRNA is spread throughout the whole column bed and given time to equilibrate.

Let K be the association constant for tRNA - tetranucleotide interaction in units of litres/mole.

Let C be the concentration of tRNA - tetranucleotide complex in moles/litre.

Let S be the concentration of tetranucleotide affinity sites in the column bed in moles/litre.

Let F be the concentration of free tRNA complementary to but not bound to the site in moles/litre.

Let T be the total concentration of complementary tRNA in moles/litre.

Then: the fraction of $tRNA_{ile}$ bound to the column will be

$$\frac{C}{T} = \frac{(T + S + \frac{1}{K}) \pm \sqrt{(T + S + \frac{1}{K})^2 - 4 * TS}}{2 * T}$$

(See appendix I for derivation)

Substituting the values:

$K = 1.2 * 10^5$ litres/mole (Schimmel et al., 1972)

$S = 2.9 * 10^{-5}$ moles/litre (Calculated from the observed site density as in appendix II)

and varying the loading T (see appendix III), it can be seen that the loading affects the fraction of $tRNA_{ile}$ bound very little until T is greater than 10^{-5} moles/litre (table 11A).

Now holding the value of T at 10^{-7} moles/litre and varying S, it can be seen that binding is much more sensitive to the affinity site density (table 11B). The last entry in table 11 indicates that if the site density is very low, that decreased loading can improve the fraction bound.

Table 11

Calculated fraction of $tRNA_{ile}$ bound to the affinity column:

A) at different $tRNA_{ile}$ concentrations ($S = 2.9 \times 10^{-5}$ M):

Concentration (T)	Fraction bound (C/T)
10^{-8}M	0.7767
10^{-7}	.7763
10^{-6}	.7732
10^{-5}	.7715
$2*10^{-5}$	.6557
$5*10^{-5}$	.4450
10^{-4}	.2596

B) at different binding site concentrations ($T = 10^{-7}$ M):

Concentration (S)	Fraction bound (C/T)
10^{-4}M	0.9230
$2.9*10^{-5}$	.7763
10^{-5}	.544
$5*10^{-6}$	.373
$2*10^{-6}$	.096
$2*10^{-6}$	.193 ($T = 10^{-8}$)

Fraction of $tRNA_{ile}$ bound to the affinity column is given by the formula $\frac{C}{T} = \frac{(T + S + {}^{1}/K) \pm \sqrt{(T + S + {}^{1}/K)^2 - 4*TS}}{2 * T}$ where C is the concentration of $tRNA_{ile}$ bound, T is the total $tRNA_{ile}$ concentration, S is the concentration of binding sites, and K is the association constant.

Various aspects of the real experimental system which could alter these parameters have been considered.

The salt and magnesium concentrations and the temperature used are the same as those used in the other studies quoted and should not contribute to the low binding levels.

The effective site density of the column may be lower than that calculated above due to two factors. The first is the conformation of the decane-diol spacer. If its conformation in aqueous solution is a tightly coiled, rigid structure, then the binding face of the oligonucleotide may not be as readily available as if the spacer were flexible and extended. No convenient method to investigate or estimate the extent of this effect was available. However use of a hydrophilic spacer for affinity chromatography has been found effective, and could be tried in this system.

The second factor is the nature of the cellulose matrix. If the oligonucleotide is small enough to bind to the cellulose in locations inaccessible to the tRNA molecule, then these sites will be wasted. Astell and Smith (1972) found that the maximum effective capacity of their columns was only 35% of the site density. This may suggest that as many as 65% of the affinity sites are not fully available for binding. As the affinity sites used here are smaller, and the target molecule is larger, this effect may be greater still in this study.

The availability of the oligonucleotide sites were tested by comparing the release of ultraviolet absorbing material from aliquots of modified cellulose with several hydrolytic enzymes and sodium hydroxide. These results are shown in Table 12. It was found that the blanks used

Table 12

Accessibility of cellulose-bound oligonucleotides to ribonuclease digestion.

A. ApUpCpApdp - cellulose

Digested with:	Material Released: (vs digest solution) in A_{260} units/g cellulose	(less blank)	(% Theoretical)
1. Spleen diesterase	3.0	2.4	28.5%
2. Pancreatic RNase	4.4	3.8	58%*
3. Water	0.6	-	-
4. 0.2 M NaOH	-	8.7	100%

B. cellulose - pApUpA

Digested with:	Material Released (vs digest solution) in A_{260} units/g cellulose	(less blank)	(% Theoretical)
1. SVD	2.0	1.7	13%
2. Pancreatic RNase	4.5	4.2	140%*
3. Water	0.3	-	-
4. 0.2 M NaOH	-	9.0	100%

* High background absorbance in these samples prevented accurate measurement, and released additional non-nucleotide materials increased these values greatly.

for each reaction had a significant absorbance due to excess coupling reagent (CMC) which was not completely removed after the coupling procedure. As a result, values obtained from these experiments had to be corrected for this background as well as well as the background due to the enzyme solution. In experiments with pancreatic RNase, background levels due to the presence of phenol prevented accurate determination of the amount of nucleotide released. (Background levels had previously been compensated for in the sodium hydroxide hydrolysis, thus these values required no further correction.)

While the accuracy of this method is not good due to these low levels of absorbance and irregular background levels, it is immediately apparent that only a fraction of the ribonucleotide material can be digested with these enzymes. These estimates of 10 - 40% of the nucleotide sequences available should be considered maximum estimates.

Better results may be obtained using Sepharose instead of cellulose as has previously been found for other affinity chromatographic studies, or a longer hydrophilic spacer group may be required.

The possibility that formation of dimers between complementary tRNA molecules may reduce the available tRNA concentration was also considered. Binding of two tRNA molecules in the absence of the affinity column should follow the same relationship described for the interaction of tRNA with the column, where S now becomes the concentration of tRNA complementary to the desired species. If the concentrations of the two tRNA species are assumed to be equal and set at $2 * 10^{-7}$ moles/litre, (see appendix III) and $K = 2 * 10^{5}$ litres/mole, then substituting these values in equation (4) returns the value 3.5% of the tRNA bound as dimer.

While this value may be sensitive to these parameters, especially the total concentration of tRNA, this would still not likely be a major contribution to the low level of binding to the column observed.

While it was recognized from the beginning of this study that a high site density would be advantageous, the increases in site density obtainable by repeated coupling reactions with the available material may not raise the available site density by more than a factor or two or three. This would certainly not be adequate to make the tRNA binding nearly quantitative and difficulties encountered in synthesis of this affinity site, its deprotection, and coupling to cellulose have limited the quantity of this material available for further coupling reactions.

The alternatives which have been discussed with respect to the supportive matrix, methods of attachment and type of spacer group would undoubtedly be worthwhile investigating. The geometry and mechanics of running the affinity column may also be improved in further experimentation. Unfortunately, the limited time available for this research have prevented further studies along these lines.

4.3. Summary

A proposal for the design of an affinity chromatographic system to show the selective retardation of isoleucine tRNA by association with a matrix bound synthetic tetranucleotide was made on the basis of available data on the stability of similar interactions and the availability of synthetic oligoribonucleotides of defined sequence and length. In spite of the very promising theoretical analysis of this proposal, in fact, no

positive evidence for the selective binding of this tRNA species was found.

Analysis of the results obtained suggested that the principal reason for the lack of binding was a low density of available binding sites. This resulted from low yields on several consecutive chemical reactions including the last steps in synthesis of the affinity sequence, and spacer group, deprotection and attachment of the affinity site to the cellulose matrix. A second reason for the possible lack of available sites was that conformation of the decane diol spacer or the structure of cellulose itself may prevent association with tRNA molecules.

Several alternative approaches were offered which would be worthwhile investigating in order to overcome these difficulties. The use of a different matrix and spacer group and new methods of attachment to the matrix were advocated with the specific aim of increasing density of binding sites and increasing their availability.

The negative results obtained in this study cannot be interpreted as evidence against the proposal of tRNA affinity chromatography. The alternative explanations are ample to account for the results obtained. Lack of time has prevented exploration of these alternatives. Thus the principal value of this work has not been the actual results obtained, but instead, has been in the direction of attention to the problems which exist; their nature and extent, and in the offering of some potential solutions to them.

5. CONCLUSION

Two applications of the chemical synthesis of oligoribonucleotides were proposed. One sequence proposed was designed to serve as a messenger RNA, coding for N-formylmethionylangiotensin. This would provide a basic structure to which future additions and modifications could be made in order to study their effects on initiation and termination of protein biosynthesis. The second was to act as an affinity site for affinity chromatography of isoleucine tRNA. This would be the first step in developing a general method for the selective isolation of tRNA molecules responding to any particular codon. Rapid and effective purification would be a definite asset in the study of structure and function of tRNA molecules.

The successful synthesis and characterization of TracAt^{bz}OH (II) led to the synthesis of protected oligonucleotides. A variety of di-, tri-, and tetranucleotide blocks required for block coupling were synthesized by the two-step coupling procedure and successfully deprotected and characterized. Consistent yields of each sequence were obtained but yields varied for different sequences: for example, yields of TracAt-Ut-At-Ct$^{bz\,bz}$OH (IX) and TracUt-At-At$^{bz\,bz}$OH (XV) were high (~ 70%) while yields of TracAt-Gt$^{bz\,bz}$OH (XIII) and TracAt-Ut-Ct-Ct$^{bz\,bz}$OH (XIX) were low (~ 30%).

Data obtained on selective deprotection of these sequences confirmed both the unpredictable rates of hydrolysis of the N-benzoyl and triphenylmethoxyacetyl groups in different sequences and the difficulty in obtaining selective 5' hydrolysis in sequences containing

adjacent adenosine and cytidine or cytidine and cytidine residues. Optimization of reaction conditions for each individual sequence was recommended.

Two block coupling reactions were attempted but no evidence for the block coupled products after deprotection was obtained. Binding of these sequences to the zinc/copper couple used in the first step of the deprotection reaction and possible degradation at this step were proposed as possible reasons for this and the low yields obtained of other deprotected sequences. These hypotheses could be tested by characterizing the products of this reaction via DEAE-cellulose chromatography as employed in the purification of oligonucleotides synthesized by the diester method. The problem may, alternatively, be solved by experimentally determining reaction conditions which do not result in degradation or loss of binding. Time was not available, though, to pursue these ideas here.

Model studies on preparation of decane diol coupled sequences and 5'-phosphorylated sequences were successful although the yields of the former were low due to the mechanism proposed to be oeprating in these coupling reactions. The proposed mechanism (a tetrasubstituted pyrophosphate as the active intermediate) or the alternative mixed phosphoric-sulfonic anhydride mechanism also indicated the sterically crowded nature of these active intermediates. Proposals of less sterically hindered activating agents and possible use of a "mixed" diester-triester approach to the synthesis of the internucleotide bond were offered as potential improvements, particularly in the block coupling reaction.

Coupling of protected sequences to cellulose by the diester and

triester methods were not successful, but the proposed mechanism also readily explained the lack of reaction in the triester case. Coupling of deprotected nucleotides with a decyl spacer group to cellulose was carried out with modest success.

Failure to demonstrate selective retardation of isoleucine tRNA in the affinity chromatography experiments could only be understood in terms of extremely low availability of the oligonucleotide binding sites present, and this hypothesis was qualitatively supported by the low level of nucleotides released by treatment with three ribonucleases. Use of other chromatographic supports, spacer groups and coupling methods were proposed with the aim of increasing the available site density, as the theoretical validity of the proposal was demonstrated.

That the objectives of this research were not reached is not grounds for concluding that the objectives cannot be reached. The data and observations collected in the course of this work have directed attention to the particular problems encountered with the use of these methods. In response, several potential solutions were discussed, though time to investigate alternatives was not available. However, it is anticipated that these will provide a firmer ground from which future work may begin.

APPENDIX I

This appendix shows the derivation of a formula for estimating the amount of tRNA bound to an affinity column from the association constants, and concentrations of species involved, assuming that tRNA is spread through the total bed volume and given time to reach equilibrium.

Let K be the association constant for tRNA-tetranucleotide interaction in units of litres/mole.

Let C be the concentration of tRNA-tetranucleotide complex in moles/litre.

Let S be the concentration of tetranucleotide affinity sites in the column bed in moles/litre.

Let F be the concentration of free tRNA complementary, but not bound, to the affinity site in moles/litre.

Let T be the total concentration of complementary tRNA in moles/litre.

Then:

$$K = \frac{C}{F * (S-C)} \quad (1),$$

$$T = F + C \quad \text{or} \quad F = T-C \quad (2).$$

Substituting 2 in 1:

$$K = \frac{C}{(T-C) * (S-C)}.$$

and solving for C:

$$K * (TS - TC - SC + C^2) = C \qquad (3)$$

$$TS - (T + S) * C + C^2 = \frac{C}{K}$$

$$TS - (T + S + {}^1/K) * C + C^2 = 0$$

$$C = \frac{T + S + {}^1/K \pm \sqrt{(T + S + {}^1/K)^2 - 4 * TS}}{2}$$

Therefore, the fraction of tRNA bound to the column will be

$$\frac{C}{T} = \frac{(T + S + {}^1/K) \pm \sqrt{(T + S + {}^1/K)^2 - 4 * TS}}{2 * T} \qquad (4)$$

APPENDIX II

This appendix shows the calculation of concentration of binding sites (S) from the density of oligonucleotide binding sites measured.

1. Column volume (V):

$r = 0.25$ cm
$h = 15.9$ cm

$V = \pi r^2 h$, 1 cm^3 = 1 ml $\qquad$ V = 3.09 ml.

2. Binding site density (M):

8.7 A_{260} units/g cellulose (dry)
0.5 g cellulose/column
ε ApUpCpA = 48.6×10^3 l/mol

Since 1 A_{260} unit = $\dfrac{1}{\varepsilon * 1000}$ moles

the column contains

$$\frac{4.35}{48.6 * 10^6} \text{ moles} \qquad = 0.897 \times 10^{-7} \text{ moles}$$

3. Concentration of binding sites (S):

$$S = \frac{M}{V} \qquad S = 2.9 \times 10^{-5} \text{ moles/litres}$$

APPENDIX III

This appendix shows the estimation of concentrations of isoleucine tRNA (T) used in calculating the fraction of tRNA bound to the affinity column.

0.5 A_{260} unit of tRNA (bulk) was used.

If the molar extinction coefficient of tRNA is estimated as 6×10^5, and the fraction of total tRNA which is isoleucine is estimated at 3% then the concentration of isoleucine tRNA (assuming it is spread throughout the column volume of 3.09 ml) will be

$$0.03 * 0.5\ A_{260} * \frac{1}{6 * 10^5 * 10^3}\ \text{moles}/A_{260}\ \text{unit} * 0.00309\ \text{litres.}$$

$$= 0.8\ 3 \times 10^{-8}\ \text{moles/litre}$$

The tRNA applied to this column will always be more concentrated than this.

6. BIBLIOGRAPHY

Adesnik, M. and Darnell, J.E. (1972); J. Mol. Biol. 67, 397. "Biogenesis and Characterization of Histone Messenger RNA in HeLa Cells".

Adler, A.J. and Rich, A. (1962); J. Amer. Chem. Soc. 84, 3977. "Cellulose Columns Containing Polyribonucleotides and Ribonucleic Acids".

Agarwal, K.L. and Khorana, H.G. (1972); J. Amer. Chem. Soc. 94, 3578. "Studies on Polynucleotides, CII. The Use of Aromatic Isocyanates for Selective Blocking of the Terminal 3'-Hydroxyl Group in Protected Deoxyribooligonucleotides".

Alberts, B.M., Amodio, F.J., Jenkins, M., Gutman, E.D. and Ferris, F.J. (1968); Cold Spring Harbour Symp. Quant. Biol. 33, 289. "Studies with DNA-Cellulose Chromatography. I. DNA-binding Proteins from Escherichia coli".

Alberts, B. and Herrick, G. (1971); Methods in Enzymology. 21, 198. "DNA-Cellulose Chromatography".

Anderson, L.E. and McClure, W.O. (1973); Anal. Biochem. 51, 173. "An Improved Scintillation Cocktail of High-Solubilizing Power".

Anon. (1970); Chem. and Eng. News Feb. 9, page 11.

Arnott, S., Hukins, D.W.L., Dover, S.D., Fuller, W. and Hodgson, A.R. (1973); J. Mol. Biol. 81, 107. "Structures of Synthetic Polynucleotides in the A-RNA and A'-RNA Conformation: X-ray Diffraction Analysis of the Molecular Conformations of Polyadenylic acid: Polyuridylic acid and Polyinosinic acid: Polycytidylic acid.

Astell, C.R. and Smith, M. (1971); J. Biol. Chem. 246, 1944. "Thermal Elution of Complementary Sequences of Nucleic Acids from Cellulose Columns with Covalently Attached Oligonucleotides of Known Sequence and Length".

Astell, C.R. and Smith, M. (1972); Biochem. 11, 4114. "Synthesis and Properties of Oligonucleotide-Cellulose Columns".

Astell, C.R., Doel, M.T., Jahnke, P.A. and Smith, M. (1973); Biochem. 12, 5068. "Further Studies on the Properties of Oligonucleotide Cellulose Columns".

Atherton, F.R., Openshaw, H.T. and Todd, A.R. (1945); J. Chem. Soc. 382. "Studies on Phosphorylation. Part I. Dibenzyl Chlorphosphonate as a Phosphorylating Agent".

Avery, O.T., McLeod, C.M. and McCarty, M. (1944); J. Exp. Med. 79, 137. "Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types: Induction of Transformation by a Deoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III".

Bautz, E.K.F. and Hall, B.D. (1962); Proc. Nat. Acad. Sci. U.S.A. 48, 400. "The Isolation of T4-Specific RNA on a DNA-Cellulose Column".

Bautz, E.K.F. and Reilly, E. (1966); Science 151, 328. "Gene-Specific Messenger RNA: Isolation by the Deletion Method".

Beadle, G.W. and Tatum, E.L. (1941); Proc. Nat. Acad. Sci. U.S.A. 27, 499. "Genetic Control of Biochemical Reactions in Neurospora".

Beadle, G.W. (1945); Chem. Rev. 37, 15. "Biochemical Genetics".

Berlin, Y.A., Chakhmakhcheva, O.G., Efimov, V.A., Kolosov, M.N. and Korobko, V.G. (1973); Tet. Lett. 1353. "Arenesulfonyl Imidazolides, New Reagents for Polynucleotide Synthesis".

Bishop, J., Leahey, J. and Schweet, R. (1960); Proc. Nat. Acad. Sci. U.S.A. 46, 1030. "Formation of the Peptide Chain of Hemoglobin".

Blackburn, G.M., Brown, M.J. and Harris, M.R. (1967); J. Chem. Soc. 2438. "Synthetic Studies of Nucleic Acids on Polymer Supports I. Oligodeoxynucleotide Synthesis on an Insoluble Support".

Bonnavida, B., Fuchs, S. and Sela, M. (1970); Biochem. Biophys. Res. Comm. 41, 1335. "Antibodies to Transfer RNA Obtained with Covalently Linked tRNA Conjugates".

Boulton, E.T. and McCarthy, B.J. (1962); Proc. Nat. Acad. Sci. U.S.A. 48, 1390. "A General Method for the Isolation of RNA Complementary to DNA".

Brawerman, G. (1974); Ann. Rev. Biochem. 43, 621. "Eukaryotic Messenger RNA".

Brown, D.M. and Todd, A.R. (1952); J. Chem. Soc. 52, 52. "Nucleotides. Part X. Some Observations on the Structural Chemical Behaviour of the Nucleic Acids.

Butzow, J.J. and Eichhorn, G.L. (1965); Biopolymers 3, 95. "Interactions of Metal Ions with Polynucleotides and Related Compounds. IV. Degradation of Polyribonucleotides by Zinc and Other Divalent Metal Ions".

Catlin, J.C. and Cramer, F. (1973); J. Org. Chem. 38, 245. "Deoxy Oligonucleotide Synthesis via the Triester Method".

Chang, M.M., Leeman, S.E., and Nial, H.D. (1971); Nature N. Biol. 232, 86. "Amino Acid Sequence of Substance P".

Chargaff, E. (1950); Experientia 6, 201. "Chemical Specificity of Nucleic Acids and Mechanism of their Enzymatic Degradation".

Chargaff, E. (1951a); Fed. Proc. 10, 654. "Structure and Function of Nucleic Acids as Cell Constituents".

Chargaff, E. (1951b); J. Cell and Comp. Physiol. 38, (supp. 1), 41. "Some Recent Studies on the Composition and Structure of Nucleic Acids".

Chladek, S. and Smrt, J. (1964); Chem. and Ind. 1719. "2'-O-(α-Ethoxyethyl) -5-O-acetyluridine 3'-Phosphate, a New Key Intermediate for Oligonucleotide Synthesis".

Cohen, L.A. and Steele, J.A. (1966); J. Org. Chem. 1, 2333. "Protection of the Hydroxyl Groups with Vinyl Thioethers".

Cook, A.F., Holman, M.J. and Nussbaum, A.L. (1969a); J. Amer. Chem. Soc. 91, 1522. "Nucleoside 5-Alkyl Phosphorothioates. II. Preparation and Enzymatic Properties".

Cook, A.F., Holman, M.J. and Nussbaum, A.L. (1969b); J. Amer. Chem. Soc. 91, 6479. "Nucleoside 5-Alkyl Phosphorthioates. III. Application to Oligonucleotide Synthesis".

Cook, A.F. and Maichuk, D.T. (1970); J. Org. Chem. 35, 1940. "Use of Chloroacetic Anhydride for the Protection of Nucleoside Hydroxyl Groups".

Cozzarelli, N.R., Melachen, N.E., Jovin, T.M. and Kornberg, A. (1967); Biochem. Biophys. Res. Comm. 28, 578. "Polynucleotide Cellulose as a Substrate for a Polynucleotide Ligase Induced by Phage T4".

Cramer, F., Baldhauf, H.J. and Kuntzel, H. (1962a); Angew. Chem. 1, 54. "Oligonucleotide Synthesis with Trichloracetonitrile".

Cramer, F., Neunhoeffer, F., Scheit, K.H., Schneider, G. and Tennigkeit, J. (1962b); Angew. Chem. 1, 331. "New Phosphorylation Reactions and Protecting Groups for Nucleotides".

Cramer, F. and Scheit, K.H. (1962); Angew. Chem. 1, 510. "A New Method for the Synthesis of Ribo-oligonucleotides".

Cramer, F., Saenger, W., Scheit, K.H. and Tennigkeit (1964); Ann. Chem. "Uber Substituierte 2.3-benzylidenribonucleoside".

Cramer, F., Helbig, R., Hetler, H., Scheit, K.H. and Seliger, H. (1966); Angew. Chem. 5, 601. "Oligonucleotide Synthesis with a Soluble Polymer as Carrier".

Crick, F.H.C. (1958); Symp. Soc. Exp. Biol. London 12, 138. "On Protein Synthesis".

Crick, F.H.C. (1966); J. Mol. Biol. 19, 548. "Codon-Anticodon Pairing: the Wobble Hypothesis".

Cuatrecasas, P. and Anfinsen, C.B. (1971); Ann. Rev. Biochem. 40, 259. "Affinity Chromatography".

Cusack, N.J., Reese, C.B. and van Boom, J.H. (1973); Tetrahedron Letters, 2209, "Block Synthesis of Oligonucleotides by the Phosphotriester Approach"

Dayhoff, M.O. (1969); Atlas of Protein Sequence and Structure. National Biomedical Research Foundation.

Dintzis, H.M. (1961); Proc. Nat. Acad. Sci. U.S.A. 47, 247. "Assembly of the Peptide Chains of Hemoglobin".

Dodd, G.H., Golding, B.T. and Ioannou, P.V. (1975); Chem. Comm. 249. "Limitations of t-Butyl Dimethylsilyl as a Protecting Group for Hydroxy Functions".

Doel, M.T. and Smith, M. (1973); FEBS Lett. 34, 99. "The Chemical Synthesis of Deoxyribo-oligonucleotides Complementary to a Portion of the Lysozyme Gene of Phage T4 and Their Hybridization to a Phage Specific RNA and Phage DNA".

Dounce, A.L. (1952); Emzymologia 15, 151.

Dube, S.K. (1973); Nature N. Biol. 243, 103. "Evidence for 'Three Point' Attachment of tRNA to Methionyl tRNA Synthetase".

Eckstein, F. (1967a); Chem. Ber. 100, 2228. "βββ-Trichlor-äthanol als Phosphorsaureschutzgruppe fur Nucleotide".

Eckstein, F. (1967b); Chem. Ber. 100, 2236. "Oligodesoxynucleotide mit Endstandiger 5'-Phosphatgruppe".

Eckstein, F. and Rizk, I. (1969); Chem. Ber. 102, 2362. "Synthese von Oligodesoxynucleotiden uber Phosphorsauretriester".

Edmonds, M., Vaughn, M.H. and Nakazoto. (1971); Proc. Nat. Acad. Sci. 68, 1336. "Polyadenylic Acid Sequences in Heterogeneous Nuclear RNA and Rapidly Labelled Polyribosomal RNA of HeLa Cells. Possible Evidence for a Precursor Relationship".

Eisinger, J., Feuer, B. and Yamane, T. (1970); Proc. Nat. Acad. Sci. 65, 638. "Luminescence and Binding Studies on tRNA phe".

Eisinger, J. (1971a); Biochem. Biophys. Res. Comm. 43, 854. "Complex Formation Between Transfer RNAs with Complementary Anticodons".

Eisinger, J. (1971b); Biochem. Biophys. Res. Comm. 44, 1135. "Visible Gel Electrophoresis and Determination of Association Constants".

Eisinger, J., Fuer, B. and Yamane, T. (1971); Nature New Biol. 231, 126. "Codon-Anticodon Binding in Transfer RNA phe".

England, T. (1975); Personal Communication.

Erhan, S., Northrup, L.G. and Leach, F.R. (1965); Proc. Nat. Acad. Sci. U.S.A. 53, 646. "A Method Potentially Useful for Establishing Base Sequences in Codewords".

Felsenfeld, G. and Miles, H.T. (1967); Ann. Rev. Bioch. 36, 407. "Physical and Chemical Properties of Nucleic Acids".

Franklin, R.E. and Gosling, R.G. (1953); Nature 171, 740. "Molecular Configuration in Sodium Thymonucleate".

Freist, W., Helbig, R. and Cramer, F. (1970); Chem. Ber. 103, 1032. "2-[α-Pyridyl]-athanol als Phosphorschutzgruppe".

Fromageot, H.D.M., Griffin, B.E., Reese, C.B. and Sulston, J.C. (1967); Tetrahedron 23, 2315. "Synthesis of Oligoribonucleotides. III. Monoacylation of Ribonucleosides and Derivatives via Orthoester Exchange".

Fuller, W. and Hodgson, A. (1967); Nature 215, 817. "Confoimation of the Anticodon Loop in tRNA".

Ganoza, C.M. (1966); Cold Spring Harbour Symp. Quant. Biol. 31, 273. "Polypeptide Chain Termination in Cell Free Extracts of E. coli".

Gauss, D.H., von des Haar, F., Maelicke, A. and Cramer, F. (1971); Ann. Rev. Biochem. 40, 1045. "Recent Results of tRNA Research".

Ghosh, K. and Ghosh, H.P. (1970); Biochem. Biophys. Res. Comm. 41, 519. "Role of Modified Nucleoside Adjacent to 3' End of Anticodon in Codon-Anticodon Interaction".

Gilham, P.T. and Khorana, H.G. (1958); J. Amer. Chem. Soc. 80, 6212. Studies on Polynucleotides. I. A New and General Method for the Chemical Synthesis of the $C_{5'}$-$C_{3'}$ Internucleotidic Linkage. Synthesis of Deoxyribo-dinucleotides".

Gilham, P.T. and Khorana, H.G. (1959); J. Amer. Chem. Soc. 87, 4647. "Studies on Polynucleotides. V. Stepwise Synthesis of Oligonucleotide. Synthesis of Thymidylyl-(5'-3')-thymidylyl-(5'-3')-Thymidine and Deoxycytidylyl-(5'-3')-deoxyadenylyl-(5'-3')-thymidine".

Gilham, P.T. (1962); J. Amer. Chem. Soc. 84, 1311. "Complex Formation in Oligonucleotides and Its Application to the Separation of Polynucleotides".

Gilham, P.T. (1964); J. Amer. Chem. Soc. 84, 4982. "The Synthesis of Polynucleotide-Celluloses and Their Use in the Fractionation of Polynucleotides".

Gilham, P.T. and Robinson, W.E. (1964); J. Amer. Chem. Soc. 86, 4985. "The Use of Polynucleotide-Cellulose in Sequence Studies of Nucleic Acids".

Gilham, P.T. (1968); Biochem. 7, 2809. "The Synthesis of Celluloses Containing Covalently Bound Nucleotides, Polynucleotides, and Nucleic Acids".

Gillam, S., Waterman, K. and Smith, M. (1975); Nucleic Acids Research 2, 625. "The Base Pairing Specificity of Cellulose-pdT9".

Goldstein, A. and Brown, B.J. (1961); Biochim. Biophys. Acta. 53, 438. "Addition of Amino Acids to C-Terminal Ends of Growing Protein in Escherichia coli".

Goss, D.J. and Parkhurst, L.J. (1974); Biochem. Biophys. Res. Comm. 59, 181. "Ultra-rapid Quantitative Isolation of Specific Transfer Ribonucleic Acids. A Solid Phase Method".

Greenberg, J.R. and Perry, R.P. (1972); J. Mol. Biol. 72, 91. "Relative Occurrence of Polyadenylic Acid Sequences in Messenger and Heterogeneous Nuclear RNA of L Cells as Determined by Poly(U)-Hydroxyapatite Chromatography".

Griffin, B.E., Reese, C.B., Stephenson, G.F. and Trentham, D.R. (1966); Tetrahedron Letters 4349. "Oligonucleotide Synthesis from Nucleoside 2'-O-benzyl ethers".

Griffin, B.E., Jarman, M., Reese, C.B. and Sulston, J.E. (1967); Tetrahedron 23, 2301. "Synthesis of Oligoribonucleotides.2. Methoxymethylidene derivatives of Ribonucleosides and 5' Ribonucleotides".

Griffin, B.E., Jarman, M. and Reese, C.B. (1968); Tetrahedron 639, "The Synthesis of Oligoribonucleotides. IV. Preparation of Dinucleoside Phosphates from 2',5' Protected Ribonucleoside Derivatives".

Grosjean, H., Takada, C. and Petre, J. (1973); Biochem. Biophys. Res. Comm. 53, 882. "Complex Formation Between Transfer RNAs with Complementary Anticodons - Use of Matrix Bound Transfer RNA".

Handbook of Biochemistry. (1970); The Chemical Rubber Co., 2nd Ed'n; H. Sober, Ed.

Haselkorn, R. and Rothman-Denes, L.B. (1973); Ann. Rev. Biochem. 42, 397. "Protein Synthesis".

Hawley, D.A., Miller, M.J., Slobin, L.I. and Wahba, A.J. (1974); Biochem. Biophys. Res. Comm. 61, 329. "The Mechanism of Initiation Factor 3 in Protein Synthesis I. Studies on Ribosomes Crosslinked with Dimethylsuberimidate".

Hershey, A.D., Dixon, J. and Chase, M. (1953); J. Gen. Physiol. 36, 777. "Nucleic Acid Economy in Bacteria Infected with Bacteriophage T2".

Hoagland, M.B., Stephenson, M.L., Scott, J.F., Hecht, L.I. and Zamecnik, P.C. (1958); J. Biol. Chem. 231, 241. "A Soluble Ribonucleic Acid Intermediate in Protein Synthesis".

Hogenaur, G. (1967); Z. Physiol. Chem. 348, 227. "Nachweiss eine Wechselwirkung zwischen Trinucleotiden and Loslichen Ribonucleinsaure".

Hogenaur, G. (1970); Eur. J. Biochem. 12, 527. "The Stability of a Codon Transfer RNA Complex".

Hoskinson, R.M. and Khorana, H.G. (1965); J. Biol. Chem. 240, 2129. "Studies on Polynucleotides.XLI. Purification of Phenylalanine-Specific Transfer Ribonucleic Acids from Yeast by Countercurrent Distribution".

Hung, P.P. (1965); Science 149, 639. "Enrichment of Serine Acceptor Soluble RNA by Nucleic Acid Gels".

Ikehara, M. (1974); Acc. Chem. Res. 7, 92. "Synthesis of Riboologonucleotides Having Sequences of Transfer Ribonucleic Acids".

Jacob, F. and Monod, J. (1961); J. Biol. Chem. 3, 318. "Genetic Regulatory Mechanisms in the Synthesis of Proteins".

Jacob, T.M. and Khorana, H.G. (1964); J. Amer. Chem. Soc. 86, 1630. "Studies on Polynucleotides. XXX. A Comparative Study of Reagents for the Synthesis of the $C_{3'}$-$C_{5'}$ Internucleotide Linkage".

Jacob, T.M. and Khorana, H.G. (1965); J. Amer. Chem. Soc. 87, 368. "Studies on Polynucleotides. XXXVII. The Synthesis of Specific Deoxyribopolynucleotides. Further Examination of the Approach Involving Stepwise Synthesis".

Jovin, T.M. and Kornberg, A. (1968); J. Biol. Chem. 243, 250. "Polynucleotide Celluloses as Solid State Primers for Polymerases".

Jukes, T.H. and Gatlin, L. (1971); Prog. in Nucl. Ac. Res. and Mol. Biol. 11, 303. "Recent Studies Concerning the Coding Mechanism".

Katagiri, N., Itakura, K. and Narang, S.A. (1974). J.C.S. Chem. Comm. 325. "Novel Condensing Reagents for Polynucleotide Synthesis".

Kennard, O., Motherwell, W.D., Coppola, J.C., Griffin, B.E., Reese, C.B. and Larson, A.C. (1971); J. Chem. Soc. (B) 1940. "X-Ray Crystal-Structure Determination of More Levorotatory Diastereomer of 2'-O-Tetrahydropyranyl-Adenosine. Correlation of Physical Properties of Tetrahydropyranyl Ethers with Absolute Configuration".

Khorana, H.G. (1961); Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest. New York, Wiley.

Khorana, H.G., Turner, A.F. and Vizsolyi, J.P. (1961); J. Amer. Chem. Soc. 83, 686. "Studies on Polynucleotides. IX. Experiments on the Polymerization of Mononucleotides. Certain Protected Derivatives of Deoxycytidine-5' Phosphate and Synthesis of Deoxycytidine Polynucleotides".

Khorana, H.G., Buchi, H., Ghosh, H.P., Gupta, N., Jacob, T.M., Kossel, H., Morgan, R., Narang, S.A., Ohtsuka, E. and Wells, R.D. (1966); Cold Spring Harbour Symp. Quant. Biol. 31, 39. "Polynucleotide Synthesis and the Genetic Code".

Knorre, D.G. and Zarytova, V.F. (1974); Recent Developments in Oligonucleotide Synthesis and Chemistry of Minor Bases of tRNA. Ed. Z. Paryzek. Universytet Im. Adama Mickiewicza Institut Chemii Kiekrz-Poznan Poland. 89. "The Mechanism of the Chemical Synthesis of Oligonucleotides".

Koster, H. (1972a); Tetrahedron Letters 1527. "Polymer Support Oligonucleotide Synthesis VI. Use of Inorganic Carriers".

Koster, H. (1972b); Tetrahderon Letters 1535. "Polymer Support Oligonucleotide Synthesis VIII. Use of Polyethylene Glycol".

Koster, H. and Heyns, K. (1972); Tetrahedron Letters 1531. "Polymer Support Oligonucleotide Synthesis VII. Use of Sephadex LH 20".

Koster, H. (1974); Recent Developments in Oligonucleotide Synthesis and Chemistry of Minor Bases of tRNA. 125. "Some Recent Improvements in the Synthesis of Deoxyoligonucleotides".

Kumar, A. and Khorana, H.G. (1969); J. Amer. Chem. Soc. 91, 2743. "Studies on Polynucleotides. XCII. The Synthesis of a Deoxyribododecanucleotide Containing Specific Amino Acid Codons".

Kuruhashi, K. (1974); Ann. Rev. Biochem. 43, 445. "Biosynthesis of Small Peptides".

LeGoff, E. (1964). J. Org. Chem. 29, 2408. "Cyclopropanes from Easily Prepared, Highly Active Zinc-Copper Couple, Dibromomethane and Olefins".

Lengyel, P. and Soll, D. (1969); Bact. Rev. 33, 264. "Mechanism of Protein Synthesis".

Letsinger, R.L., Fontaine, J., Mahadevan, V.L., Schexnayder, D.A. and Leone, R.E. (1964); J. Org. Chem. 29, 2615. "2,4-Dinitrobenzenesulfenyl as a Blocking Group for Hydroxyl Functions in Nucleosides".

Letsinger, R.L. and Mahadevan, V.L. (1965); J. Amer. Chem. Soc. 87, 3526. "Oligonucleotide Synthesis on a Polymer Support".

Letsinger, R.L. and Mahadevan, V.L. (1966); J. Amer. Chem. Soc. 88, 5319. "Stepwise Synthesis of Oligodeoxynucleotides on an Insoluble Polymer Support".

Letsinger, R.L., Caruthers, M.H. and Jerina, D.M. (1967); Biochem. 6, 1379. "Reactions of Nucleosides on Polymer Supports. Synthesis of Thymidylthmidylthymidine".

Letsinger, R.L., Caruthers, M.H., Miller, P.S. and Ogilvie, K.K. (1967); J. Amer. Chem. Soc. 89, 7146. "Oligonucleotide Synthesis Using β-Benzoylpropionyl. A Blocking Group with a Trigger for Selective Cleavage".

Letsinger, R.L. and Ogilvie, K.K. (1967); J. Org. Chem. 32, 296. "Use of p-Nitrophenylchloroformate in Blocking Hydroxyl Groups in Nucleosides".

Letsinger, R.L. and Ogilvie, K.K. (1969a); J. Amer. Chem. Soc. 91, 3350. "Synthesis of Oligothymidylates via Phosphotriester Intermediates".

Letsinger, R.L. and Ogilvie, K.K. (1969b); J. Amer. Chem. Soc. 91, 3356. "Protecting Groups for Nucleosides Used in Synthesizing Oligonucleotides".

Letsinger, R.L. (1975); "Synthetic Challenges in Nucleotide Chemistry", Presented at Sixteenth Annual Medicinal Chemistry Symposium: Nucleotides and Peptides. State University of New York, Buffalo.

Lewis, J.B. and Doty, P. (1970); Nature 225, 510. "Derivation of the Secondary Structure of 5S RNA from its Binding of Complementary Oligonucleotides".

Lipsett, M.N. (1964); J. Biol. Chem. 239, 1256. "Complex Formation Between Polycytidylic Acid and Guanine Oligoribonucleotides".

Lipsett, M.N., Heppel, L.A. and Bradly, D.F. (1961); J. Biol. Chem. 236, 857. "Complex Formation Between Oligonucleotides and Polymers".

Lohrmann, R. and Khorana, H.J. (1966); J. Amer. Chem. Soc. 88, 829. "Studies on Polynucleotides. LII. The Use of 2,4,6-Triisopropylbenzenesfulfonyl Chloride for the Synthesis of Internucleotide Bonds".

Lohrmann, R., Soll, D., Hayatsu, H., Ohtsuka, E. and Khorana, H.G. (1966); J. Amer. Chem. Soc. 88, 819. "Studies on Polynucleotides. CI. Synthesis of 64 Possible Ribotrinucleotides Derived from the Four Major Ribomononucleotides".

Lucas-Lenard, J. and Lipmann, F. (1971); Ann. Rev. Biochem. 40, 409. "Protein Biosynthesis".

Matsuo, H., Abba, Y., Nair, R.M.G., Arimura, A. and Schally, A.V. (1971); Biochem. Biophys. Res. Comm. 43, 1334. "Structure of Procine LH-Releasing and FSH-Releasing Hormone.I. Proposed Amino Acid Sequence".

Matthei, J.H., Voigt, H.P., Heller, G., Neth, P., Schoch, G., Kubler, H. Arnelunxen, F., Sander, G. and Parmeggiani, A. (1966); Cold Spring Harbour Symp. Quant. Biol. 31, 25. "Specific Interaction of Ribosomes in Decoding".

McCarthy, B.J. and Boulton, E.T. (1963); Proc. Nat. Acad. Sci. U.S.A. 50, 156. "An Approach to the Measurement of Genetic Relatedness Among Organisms".

Melby, L.R. and Strobach, D.R. (1967); J. Amer. Chem. Soc. 89, 450. "Oligonucleotide Synthesis on Insoluble Polymer Supports. I. Stepwise Synthesis of Trithymidine Diphosphate".

Melby, L.R. and Strobach, D.R. (1969a); J. Org. Chem. 34, 421. "Oligonucleotide Synthesis on Insoluble Polymer Supports. II. Pentathymidine Tetraphosphate".

Melby, L.R. and Strobach, D.R. (1969b); J. Org. Chem. 34, 427. "Oligonucleotide Synthesis on Insoluble Polymer Supports. III. 15 Di-(Deoxyribonucleoside) phosphates and several Trinucleoside Diphosphates".

Meselson, M. and Stahl, F.W. (1958); Proc. Nat. Acad. Sci. U.S.A. 44, 671. "The Replication of DNA in Escherichia coli".

Michelson, A.M., Szabo, L. and Todd, A.R. (1956); J. Chem. Soc. 1546. "Nucleotides. Part XXXVI. Adenosine-2' Uridine-5' Phosphate".

Michelson, A.M. (1959); J. Chem. Soc. 3655. "Polynucleotides. Part II. Homopolymers of Cytidylic and Pseudouridylic Acid, Copolymers with Repeating Subunits, and the Stepwise Synthesis of Polynucleotides".

Michelson, A.M. and Monny, C. (1967); Biochem. Biophys. Acta 149, 107. "Polynucleotides X. Oligonucleotides and Their Association with Polynucleotides".

Miller, P.S., Fang, K.N., Kondo, N.S. and Ts'O, P.O.P. (1971); J. Amer. Chem. Soc. 93, 6657. "Conformation and Interaction of Dinucleoside Monophosphates and Diphosphates.5. Syntheses and Properties of Adenine and Thymine Nucleoside Alkyl Phosphotriesters, Neutral Analogs of Dinucleoside Monophosphates".

Min Jou, W., Haegeman, G., Ysebaert, M. and Fiers, W. (1972); Nature 237, 82. "Nucleotide Sequence of the Gene Coding for the Bacteriophage MS2 Coat Protein".

Molloy, G.R., Sporn, M.B., Kelley, D.E. and Perry, R.P. (1972); Biochem. 11, 3256. "Localization of Polyadenylic Acid Sequences in Messenger Ribonucleic-Acid of Mammalian Cells".

Mungall, W.S., Greene, G.L., Miller, P.S. and Letsinger, R.L. (1974); Nucleic Acids Research 1, 615. "Use of Phosphorous Oxychloride in Synthesizing Nucleotides and Oligonucleotides".

Nair, R.M.G., Kastin, A.J. and Schally, A.V. (1971); Biochem. Biophys. Res. Comm. 43, 1376. "Isolation and Structure of Hypothalamic MSH Release Inhibiting Hormone".

Narang, S.A., Bhanot, O.S., Goodchild, J., Michniewicz, J., Wightman, R. H. and Dheer, S.K. (1970a); Chem. Comm. 516. "Use of New Protecting Groups in the Synthesis of Deoxyribo-oligonucleotides of Defined Sequence".

Narang, S.A., Bhanot, O.S., Goodchild, J. and Wightman, R.H. (1970b); J. Chem. Soc.(D) 91, 70. "Use of Substituted Phenol as a Phosphate Protecting Group in Synthesis of Deoxyribo-Oligonucleotides Bearing 5'-Phospho-monoester End Group".

Narang, S.A., Bhanot, O.S., Goodchild, J., Wightman, R.H. and Dheer, S.K. (1972); J. Amer. Chem. Soc. 94, 6183. A New General Method for the Synthesis of Phosphate-Protected-Deoxyribooligonucleotides. IV".

Narang, S.A. and Wightman, R.H. (1973); "The Total Synthesis of Nucleic Acids" page 279 in The Total Synthesis of Natural Products. New York, Wiley-Interscience, ApSimon, J., Ed.

Neilson, T. (1969); Chem. Comm. 1139. "A Novel Synthesis for Oligoribonucleotides".

Neilson, T. and Werstiuk, E.S. (1971a); Can. J. Chem. 49, 493. "Oligoribonucleotide Synthesis. II. Preparation of 2'-O-Tetrahydropyranyl Derivatives of Adenosine and Cytidine Necessary for Insertion in Stepwise Synthesis".

Neilson, T. and Werstiuk, E.S. (1971b); Can. J. Chem. 49, 3004. "Oligoribonucleotide Synthesis III. Synthesis of Ribnucleotides Using a Stepwise Phosphortriester Method".

Neilson, T., Wastrodowski, E.V. and Werstiuk, E.S. (1973); Can. J. Chem. 51, 1068. "Oligoribonucleotide Synthesis. V. Preparation of 2'-O-Tetrahydropyranyl Derivatives of Guanosine and Their Insertion into a General Stepwise Synthesis".

Neilson, T. and Werstiuk, E.S. (1974); J. Amer. Chem. Soc. 96, 2295. "Synthesis of the Anticodon Loop of Escherichia coli Methionine Transfer Ribonucleic Acid".

Neilson, T., Deugau, K.V., England, T.E. and Werstiuk, E.S. (1975); Can. J. Chem. 53, 1039. "Oligoribonucleotide Synthesis VIII. Insertion of Terminal 5'-Phosphate Groupings.

Nelidova, O.D. and Kiselev, L.L. (1968); Mol. Biol. 2, 47. "Enzyme-Substrate Complexes Between Aminoacyl-RNA-Synthetases and Transfer RNAs Fixed on a Column".

Nirenberg, M.W. and Matthei, J. (1961); Proc. Nat. Acad. Sci. U.S.A. 47, 1588. "The Dependence of Cell-free Protein Synthesis in E. coli Upon Naturally Occuring or Synthetic Polyribonucleotides".

Nirenberg, M.W., Jones, O.W., Leder, P., Clark, B.F.C., Sly, W.S. and Petska, S. (1963); Cold Spring Harbour Symp. Quant. Biol. 28, 549. "On the Coding of Genetic Information".

Nirenberg, M.W. and Leder, P. (1964); Science 145, 1399. "RNA Codewords and Protein Synthesis".

Nirenberg, M.W., Caskey, T., Marshal, R., Birmacombe, R., Kellogg, D., Doctor, P., Hatfield, D., Levin, J., Rottman, F., Petska, S., Wilcox, M. and Anderson, F. (1966); Cold Spring Harbour Symp. Quant. Biol. 31, 11. "The RNA Code and Protein Synthesis".

Nishimura, S., Harada, F., Narushima, U. and Seno, T. (1967); Biochem. Biophys. Acta. 142, 133. Purification of Methionine-, Valine-, Phenylalaine-, and Tyrosine-Specific tRNA from Escherichia coli".

Novelli, G.D. (1967); Ann. Rev. Biochem. 36 449. "Amino Acid Activation for Protein Synthesis".

Nussbaum, A.L. and Tiberi, R. (1965); J. Amer. Chem. Soc. 87, 2513. "A New Phosphorylation Procedure".

Nygaard, A.P. and Hall, B.D. (1963); Biochem. Biophys. Res. Comm. 12, 98. "A Method for the Detection of RNA:DNA Complexes".

Ogilvie, K.K. and Letsinger, R.L. (1967); J. Org. Chem. 32, 2365. "Use of Isobutyloxycarbonyl as a Blocking Group in Preparation of 3'-O-p-Monomethoxytritylthymidine".

Ogilvie, K.K. (1973); Can. J. Chem. 51, 3799. "The tert-Butyldimethylsilyl Group as a Protecting Group in Deoxynucleosides".

Ogilvie, K.K., Sadana, K.L., Thompson, E.A., Quilliam, M.A. and Westmore, J.B. (1974); Tetrahedron Lett. 2861. "Use of Silyl Groups in Protecting Hydroxyl Functions of Ribonucleosides".

Ohtsuka, E., Murao, K., Ubasawa, M. and Ikehara, M. (1970); J. Amer. Chem. Soc. 92, 3441. "Studies on Transfer RNA and Related Compounds.I. Synthesis of Ribooligonucleotides Using Aromatic Phosphoramidates as a Protecting Group".

Ohtsuka, E., Morioka, S. and Ikehara, M. (1972); J. Amer. Chem. Soc. 94, 3229. "Studies on Transfer Ribonucleic Acids and Related Compounds.V. Synthesis of Ribonucleotides with Phosphomonoester and Groups on a Polymer Support".

Ohtsuka, E., Morioka, S. and Ikehara, M. (1973); J. Amer. Chem. Soc. 95, 8437. "Polynucleotides. XIX. Synthesis of Oligonucleotides by the Use of the N-Trityl-p-aminophenyl Group, A New Protecting Group for the Terminal Phosphate Residues".

Poonian, M.S., Schlabach, A.J. and Weissbach, A. (1971); Biochemistry 10, 424. "Covalent Attachment of Nucleic Acids to Agarose for Affinity Chromatography".

Ralph, R.K. and Khorana, H.G. (1961); J. Amer. Chem. Soc. 83, 2926. "Studies on Polynucleotides. XI. Chemical Polymerization of Monoucleotides. The Synthesis and Characterization of Deoxyadenosine Polynucleotides".

Ralph, R.K., Connors, W.J., Schaller, H. and Khorana, H.G. (1963); J. Amer. Chem. Soc. 85, 1983. "Studies on Polynucleotides. XVIII. Experiments on the Polymerization of Mononucleotides. The Synthesis and Characterization of Deoxyguanosine Oligonucleotides".

Ramirez, F. and Ugi, I. (1974); Recent Developments in Oligonucleotide Synthesis and Chemistry of Minor Bases of Transfer RNA, 53. "Phosphorylating Reagents for Oligonucleotide Synthesis".

Rammler, D.H. and Khorana, H.G. (1962); J. Amer. Chem. Soc. 84, 3114. "Studies on Polynucleotides. XVI. Specific Synthesis of the $C_{3'}$-$C_{5'}$ Interribonucleotidic Linkage. Examination of Routes Involving Protected Ribonucleosides and Ribonucleoside 3'-Phosphates. Synthesis of Uridylyl-(3'→5')-Cytidine, Adenylyl-(3'→ 5')-Adenosine and Related Compounds".

Reese, C.B. and Saffhill, R. (1968); Chem. Comm. 767. "Oligonucleotide Synthesis via Phosphotriester Intermediates, Phenyl-Protecting Group".

Reese, C.B. and Stewart, J.C.M. (1968); Tetrahedron Lett. 4273. "Methoxyacetyl as a Protecting Group in Ribonucleoside Chemistry".

Reese, C.B., Saffhill, R. and Sulston, J.E. (1970); Tetrahedron 26, 1073. "4-Methoxytetrahydropyran-4-yl. A Symmetrical Alternative to the Tetrahydropyranyl Protecting Group".

Rich, A. (1974); Biochimie. 56, 1441. "Transfer RNA and Protein Synthesis".

Robberson, D.L. and Davidson, N. (1972); Biochem. 11, 533. "Covalent Coupling of Ribonucleic Acid to Agarose".

Sachdev, H.S. and Starkovsky, N.A. (1969); Tetrahedron Lett. 733. "Enzymatic Removal of Acyl Protecting Groups. Use of Dehydrocinnamoyl Group in Oligonucleotide Synthesis and its Cleavage by Alpha Chymotrypsin".

Sander, E.G., McCormick, D.B. and Wright, L.D. (1966); J. Chromatogr. 21, 419. "Column Chromatography of Nucleotides over Thymidylate Cellulose".

Saxinger, W.C., Ponnamperuma, C. and Gillespie (1972); Proc. Nat. Acad. Sci. U.S.A. 69, 2975. "Nucleic Acid Hybridization with RNA Immobilized on Filter Paper".

Scheit, K.H. (1967); Tetrahedron Lett. 3243. "Die Benzyl-ester von Desoxynucleosidphosphaten und 5'-O-(β,β,β) Trichloro-athylphosphorylthymidylyl-(3'-5')-Thymidylyl-(3'-5')-Thymidin".

Schimmel, P.R., Uhlenbeck, O.C., Lewis, J.B., Dickson, L.A., Eldred, E.W. and Schrier, A.A. (1972); Biochem. 11, 642. "Binding of Complementary Oligonucleotides to Free and Aminoacyl Transfer Ribonucleic Acid Synthetase Bound Transfer Ribonucleic Acid".

Schott, H. (1974a); FEBS Lett. 41, 203. "The Base Pairing Specificity in Chromatographic Systems".

Schott, H. (1974b); J. Chromatogr. 96, 79. "Affinatatschromatographie an Stationar Gebundenen Oligonucleotiden I. Synthese und Anwendung von Desoxyoligothymidin-5'-Phosphat-DEAE Cellulose".

Schott, H., Eckstein, H. and Bayer, E. (1974); J. Chromatog. 99, 31. "Template Chromatography. Application of Principles of Molecular Biology for Selective Separation of Nucleotides and Peptides. Progress Report".

Sen, G.C. (1974); Ph.D. Thesis, McMaster University. "Role of Modified Nucleosides in tRNA".

Sheldon, R., Kates, J., Kelley, D.E. and Perry, R.P. (1972); Biochem. 11, 3829. "Polyadenylic Acid Sequences on 3' Termini of Vaccinia Messenger Ribonucleic Acid and Mammalian Nuclear and Messenger Ribonucleic Acid".

Shih, T.Y. and Martin, M.A. (1974); Biochem. 13, 3411. "Chemical Linkage of Nucleic Acids to Neutral and Phopshorylated Cellulose Powders and Isolation of Specific Sequences by Affinity Chromatography".

Slor, H. (1975); Nucl. Acids Res. 2, 587. "Purification of S1 Nuclease from Takadiastase by Affinity Chromatography on Single Stranded DNA-acrylamide Columns".

Smith, M., Moffat, J.G. and Khorana, H.G. (1958); J. Amer. Chem. Soc. 80, 6204. "Carbodiimides. VIII. Observations on the Reactions of Carbodiimides with Acids and Some New Applications in the Synthesis of Phopshoric Acid Esters".

Smith, M. and Khorana, H.G. (1959); J. Amer. Chem. Soc. 81, 2911. "Specific Synthesis of the $C_{5'}$-$C_{3'}$, Interribonucleotide Linkage" The Synthesis of Uridyl-(5'→3')Uridine".

Smith, M., Rammler, D.H., Goldberg, I.H. and Khorana, H.G. (1962); J. Amer. Chem. Soc. 84, 430. "Studies on Polynucleotides. XIV. Specific Synthesis of the $C_{3'}$-$C_{5'}$ Interribonucleotide Linkage. Synthesis of Uridylyl-(3'→5')Uridine and Urdiylyl-(3'→5')-Adenosine".

Smrt, J. (1972a); Coll. Czech. Chem. Comm. 37, 1870. "Oligonucleotidic Compounds. 40. Aspects of Triester Synthesis of Oligonucleotides in the Ribo Series".

Smrt, J. (1972b); Tetrahedron Lett. 3437. "Protection of Internucleotidic Bond After Its Synthesis. Approach to Synthesis of Oligonucleotidic Chains".

Soll, D. and Khorana, H.B. (1965); J. Amer. Chem. Soc. 87, 360. "Studies on Polynucleotides XXXVI. The Specific Synthesis of C_3,-C_5,-Linked Ribo-oligonucleotides Bearing a 3'-Phosphomonoester Group".

Speyer, J.F., Lengyel, P., Basilio, C., Wahba, A.J., Gardiner, R.S. and Ochoa, S. (1963); Cold Spring Harbour Symp. Quant. Biol. 28, 559. "Synthetic Polynucleotides and the Amino Acid Code".

Steers, E., Cuatercasas, P. and Pollard, H. (1971); J. Biol. Chem. 246, 196. "The Purification of β-galactosidase from Escherichia coli by Affinity Chromatography".

Stothart, P.H., Brown, I.D. and Neilson, T. (1973); Acta Cryst. (B) 29, 2237. "The Crystal Structure of Dextrorotatory 2'-O-tetrahydropyranyluridine".

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E. and Inouye, M. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 77. "Frameshift Mutations and the Genetic Code".

Tager, H.S. and Steiner, D.F. (1974); Ann. Rev. Biochem. 43, 509. "Peptide Hormones".

Tener, G.M. (1961); J. Amer. Chem. Soc. 83, 159. "2-Cyanoethyl Phosphate and its use in Synthesis of Phosphate Esters".

Thatch, R.E., Sundararajan, T.A., Dewey, K.F., Brown, J.C. and Doty, P. (1966); Cold Spring Harbour Symp. Quant. Biol. 31, 85. "Translation of Synthetic Messenger RNA".

Tinoco, I., Uhlenbeck, O.C. and Levine, M.D. (1971); Nature 230, 362. "Estimation of Secondary Structure in Ribonucleic Acids".

Tinoco, I., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C. Crothers, D.M. and Gralla, J. (1973); Nature New Biology 246, 40. "Improved Estimation of Secondary Structure in Ribonucleic Acids".

Tomlinson, R.V. and Tener, G.M. (1963); Biochem. 2, 697. "The Effect of Urea, Formamide and Glycols on the Secondary Binding Forces in the Ion Exchange Chromatography of Polynucleotides on DEAE Cellulose".

Uhlenbeck, O.C., Baller, J. and Doty, P. (1970); Nature 225, 508. "Complementary Oligonucleotide Binding to the Anticodon Loop of f-met-transfer RNA".

Uhlenbeck, O.C. (1972); J. Mol. Biol. 65, 25. "Complementary Oligonucleotide Binding to Transfer RNA".

Ungar, G., Desiderio, D.M. and Parr, W. (1972); Nature 238, 198. "Isolation, Identification and Synthesis of a Specific-behavior-inducing Brain Peptide".

van Boom, J.H., Owen, G.R., Preston, J., Ravindranathan, T. and Reese, C.B. (1971); J. Chem. Soc. 3230. "The Synthesis of Oligoribonucleotides. Part IX. Preparation of Ribonucleoside 2'-Acetal 5' Esters".

van Boom, J.H., van Deursen, P., Meeuwse, J. and Reese, C.B. (1972); Chem. Comm. 766. "Two Sulfur Containing Protecting Groups for Alcoholic Hydroxyl Functions".

Volkin, L. and Astrachan, L. (1956) Virol. 2, 149. "Phosphorus Incorporation in Escherichia coli Ribonucleic Acid After Infection with Bacteriophage T2".

Wagner, A.F., Bugianesi, R.L. and Shen, T.Y. (1971); Biochem. Biophys. Res. Comm. 45, 184. "Preparation of Separose-bound Poly(rI:rC)".

Wahba, A.J., Salas, M. and Stanley, W.M. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 103. "Studies on the Translation of the Genetic Message.II. Translation of Oligonucleotide Messenger of Specified Base Sequence".

Waller, J.P. (1963); J. Mol. Biol. 7, 483. "The NH_2 Terminal Residues of the Proteins from Cell-free Extracts of E. coli".

Wastrodowski, E. (1972); Master's Thesis, McMaster University. "Chemical Synthesis of Guanosine Oligoribonucleotides: The Synthesis of N^2-Benzoyl-2'-O-Tetrahydropyranylguanosine and N^2-benzoyl, 2'-O-Tetrahydropyranyl-5'-O-Triphenylmethoxyacetylguanosine".

Watson, J.D. and Crick, F.H.C. (1953a); Nature 171, 737. "A Structure for Deoxyribose Nucleic Acid".

Watson, J.D. and Crick, F.H.C. (1953b); Nature 171, 964. "Genetical Implications of the Structure of Deoxyribonucleic Acid".

Watson, J.D. (1963); Science 140, 17. "Involvement of RNA in the Synthesis of Proteins".

Weimann, G. and Khorana, H.G. (1962a); J. Amer. Chem. Soc. 84, 419. "Studies on Polynucleotides. XIII. Stepwise Synthesis of Deoxyribo-oligonucleotides. An Alternative General Approach and the Synthesis of Thymidine Di-, Tri- and Tetranucleotides Bearing 3'-Phosphomonoester End Groups".

Weimann, G. and Khorana, H.G. (1962b); J. Amer. Chem. Soc. 84, 4329. "Studies on Polynucleotides. XVII. On the Mechanism of Internucleotide Bond Synthesis by the Carbodiimide Method".

Werstiuk, E.S. and Neilson, T. (1972); Can. J. Chem. 50, 1283. "Oligoribonucleotide Synthesis, IV. Approach to Block Synthesis".

Werstiuk, E.S. and Neilson, T. (1973); Can. J. Chem. 51, 1889. "Oligoribonucleotide Synthesis. VI. Selective Deblocking of the 5'-O-Triphenylmethoxyacetyl Grouping in Protected Dinucleotides"

Wilkins, M.H.F., Stokes, A.R. and Wilson, H.R. (1953); Nature 171, 738. "Molecular Structure of Deoxypentose Nucleic Acids".

Wittmann, H.G. and Wittmann-Liebold, B. (1963); Cold Spring Harbour Symp. Quant. Biol. 28, 589. "Tobacco Mosaic Virus Mutants and the Genetic Coding Problem".

Yanofsky, C. (1963); Cold Spring Harbour Symp. Quant. Biol. 28, 581. "Amino Acid Replacements Associated with Mutation and Recombination in the A Gene and the Relationship to In Vitro Coding Data".

Yip, K.F. and Tsou, K.C. (1971); J. Amer. Chem. Soc. 93, 3272. "A New Polymer Support Method for the Synthesis of Ribooligonucleotides".

Zemlicka, J., Chladek, S., Holy, A. and Smrt, J. (1966); Coll. Czech. Chem. Comm. 31, 3198. "Oligonucleotidic Compounds. XIV. Synthesis of Some Diribonucleoside Phosphates Using the N-Dimethylaminomethylene Derivative of 2', 3'-O-ethoxymethylene Ribonucleosides".