CHEMICAL SYNTHESIS OF OLIGO- AND POLYNUCLEOTIDES

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INTRODUCTION

Genetics, a subject with diverse aspects, is here treated from the nucleic acid chemist's point of view, since nucleic acid chemists have contributed and still can contribute a great deal to the problem of genetic information, genetic transcription, and regulation (*Figure 1*).

Synthetic polynucleotides of known composition and known sequence in many cases allow definition of a problem more closely and more detailed questions to be answered. Polynucleotides can be synthesized by chemical synthesis, by enzymatic synthesis, or by a combination of both methods. Therefore, this paper is not confined solely to the chemical synthesis of polynucleotides.

CHEMICAL SYNTHESIS OF DEOXYPOLYNUCLEOTIDES

Homopolymers

For the condensation of mononucleotides to homopolymers picryl chloride[1] is preferable as a condensing reagent (*Figure 2*). In this way all four deoxymononucleotides can be polymerized, partly however in smaller yields, and oligomers with a chain length of about fifteen—in some cases longer—may be obtained by column separation methods (*Figure 3*). It is also possible to do a graft polymerization in elongating a given chain with a different monomer (*Figure 4*). Such short chain oligomers can serve as substrates for DNA-polymerase.

Figure 5 shows the use of such polymers as templates for DNA-polymerase as described by Kornberg and Khorana[2]. We have used such polymers in trying to determine whether DNA-dependent RNA-polymerase has a strand specificity[3]. For this purpose poly dAdT as a template was used and ATP or UTP was offered to the enzyme. As shown in *Figure 6*, the T-strand is at least ten times as well copied, i.e. polyribo A is synthesized preferentially. An analogous behaviour can be observed with poly dGdC: the transcription of the C-strand, i.e. the synthesis of poly G is much faster than the synthesis of poly C (*Figure 7*). Thus it is shown that RNA-polymerase has a strand specificity to the pyrimidine strands.

In earlier experiments with T4 DNA it had been shown by Bremer, Konrad, and Stent[4] that the transcribed messenger has A-residues at the 5′-end preferentially, and one can speculate that the beginning of the gene which is the region to be recognized by RNA-polymerase, is formed from

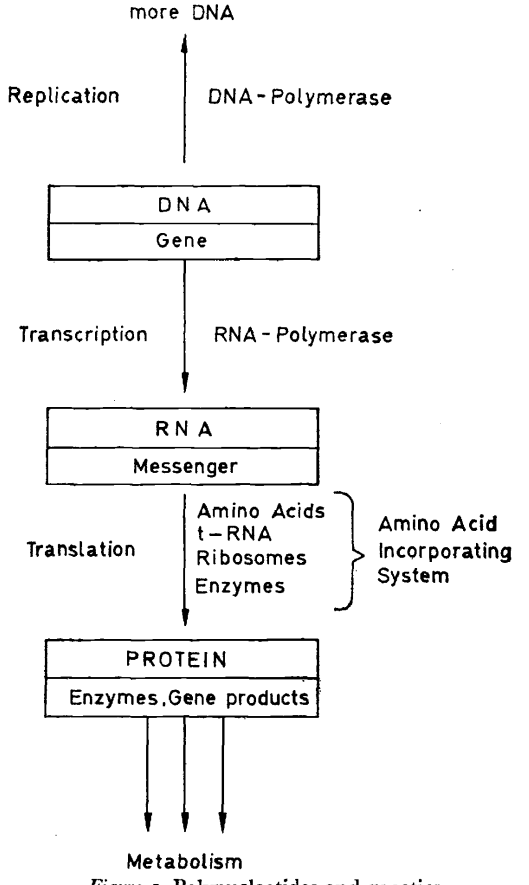


Figure 1. Polynucleotides and genetics

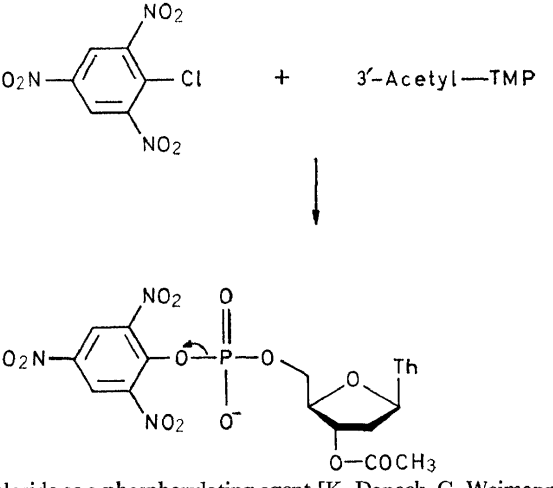


Figure 2. Picryl chloride as a phosphorylating agent [K. Daneck, G. Weimann, R. Wittmann, Darmstadt 1961–62]

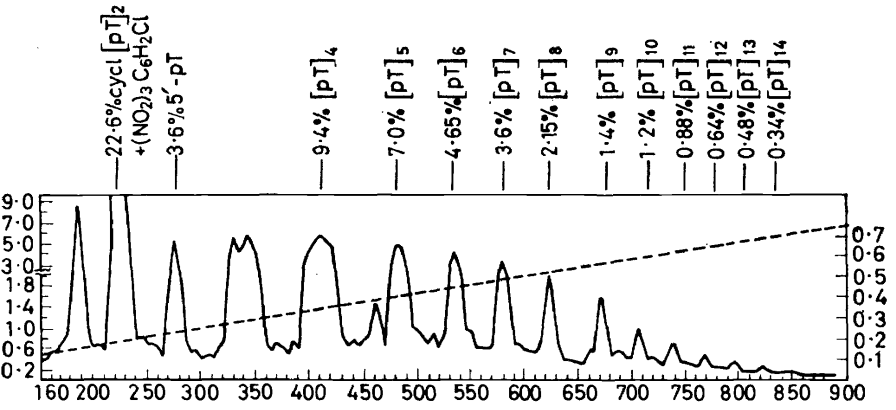


Figure 3. Column chromatographic separation of oligothymidylic acids [G. Hoffarth, Göttingen 1965]

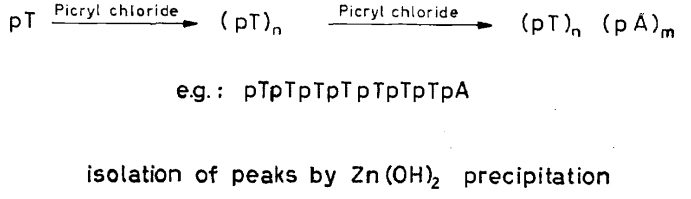


Figure 4. Graft polymerization of deoxynucleotides [K. Daneck, Göttingen 1964]

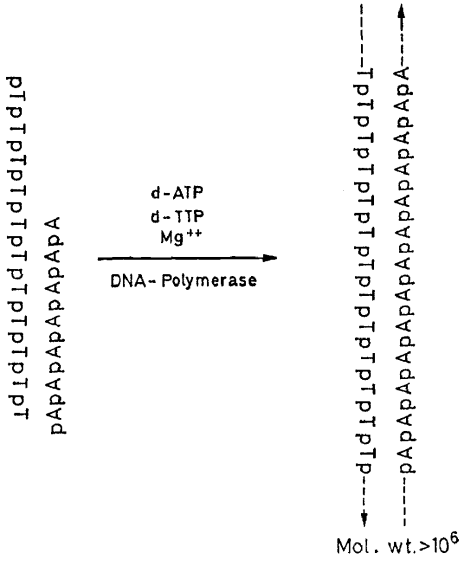


Figure 5. Biosynthesis of deoxypolynucleotides at a synthetic template [Kornberg *et al.*, 1964]

T-clusters or, more generally, from pyrimidine clusters. Another question which can be answered with this artificial, restricted DNA is whether the enzyme RNA-polymerase in the *in vitro* system can get off the strand after the synthesis has gone along the template. Bremer's explanation of the plateau in the kinetics of RNA-polymerase is that the enzyme molecules

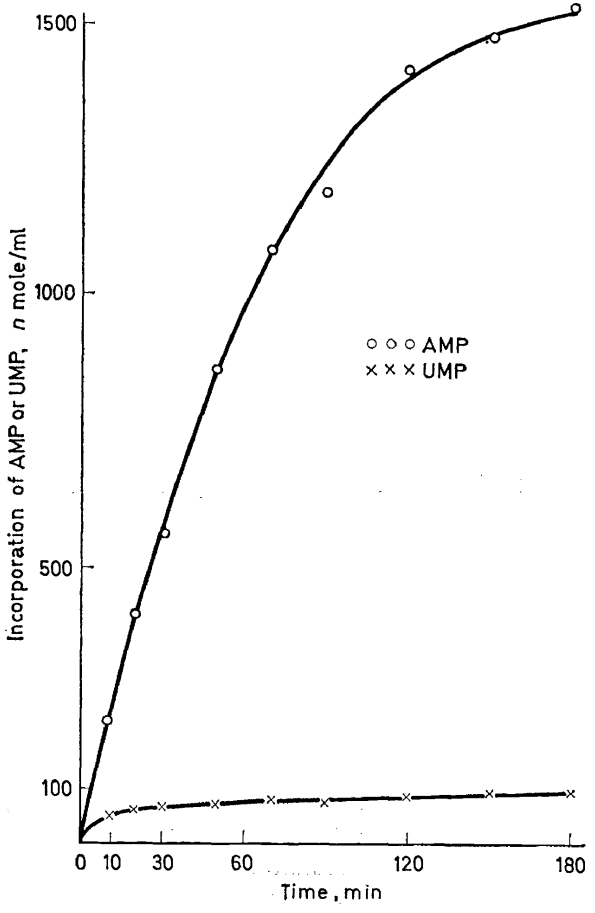


Figure 6. Incorporation of AMP or UMP in presence of only one triphosphate; template: poly dAdT [H. Matzura, Göttingen 1966]

are lined up at the end of the transcribed DNA and cannot dissociate from the template. Addition of fresh DNA according to Bremer does not cause new RNA-synthesis.

Figure 8 shows the result of an experiment in which first the polyribo A-synthesis with poly dAdT had gone to a plateau, after which poly dGdC

and GTP were added. The synthesis is reinitiated indicating that the enzyme could leave the poly dAdT strand.

Inhibition experiments can also be carried out with homopolynucleotides. *Figure 9* shows the saturation curve for the ATP-incorporation after ten minutes.

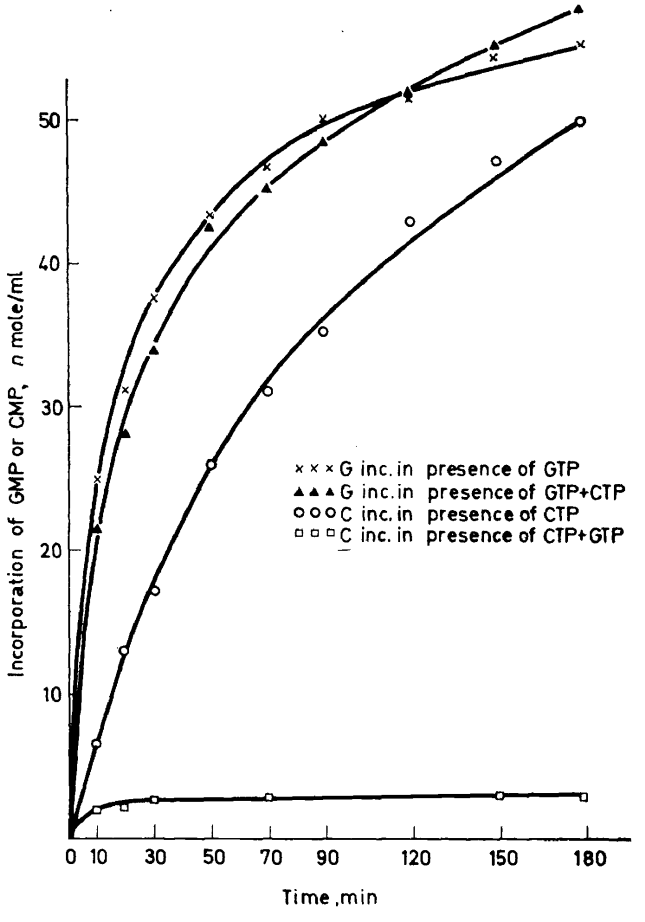


Figure 7. Incorporation of GMP and CMP; template: poly dGdC [H. Matzura, Göttingen 1967]

GTP, however, in large excess, inhibits the ATP-incorporation on a poly dAT-template. Whether there is a common binding site for all triphosphates at the polymerase molecule, cannot be decided. Apparently the enzyme exhibits a sigmoidal saturation curve, although we cannot yet indicate what factors are responsible for this phenomenon.

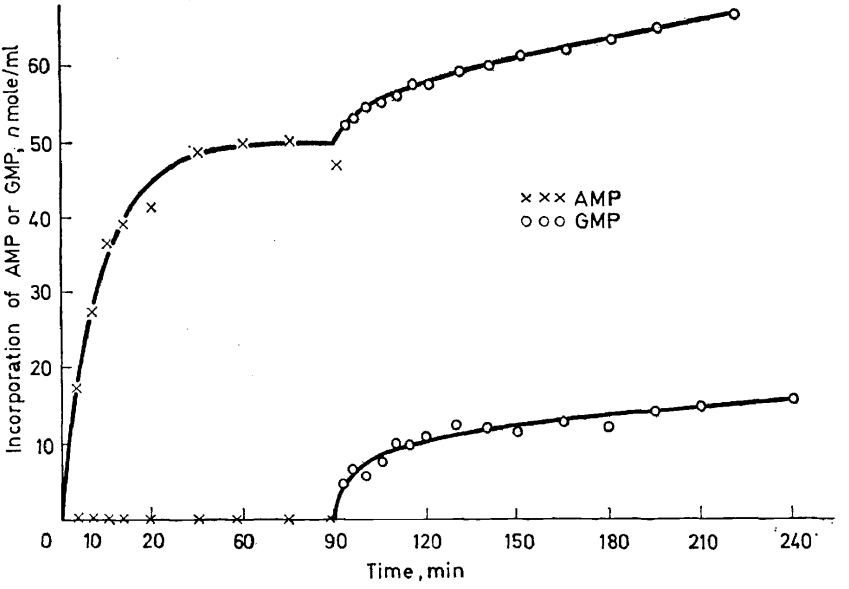


Figure 8. Incorporation of AMP and GMP at poly dAdT or poly dGdC template [H. Matzura, Göttingen 1966]

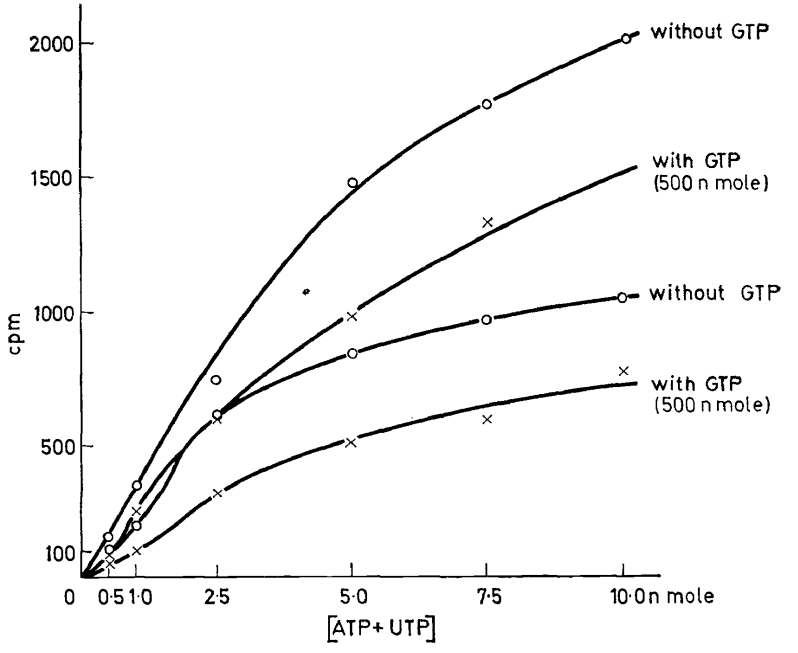


Figure 9. Saturation curve for the ATP-incorporation at two different concentrations of poly dAT template after 10 min

Polymers with defined sequences

For the synthesis of oligomers with defined sequences a complicated chemistry of protecting groups is necessary. Here only a few examples are quoted. In *Figure 10* the synthesis of a trinucleotide is given. At first a mononucleotide with a β-cyanoethyl group protection at the phosphate residue is condensed with a second mononucleotide to give a dinucleotide. The

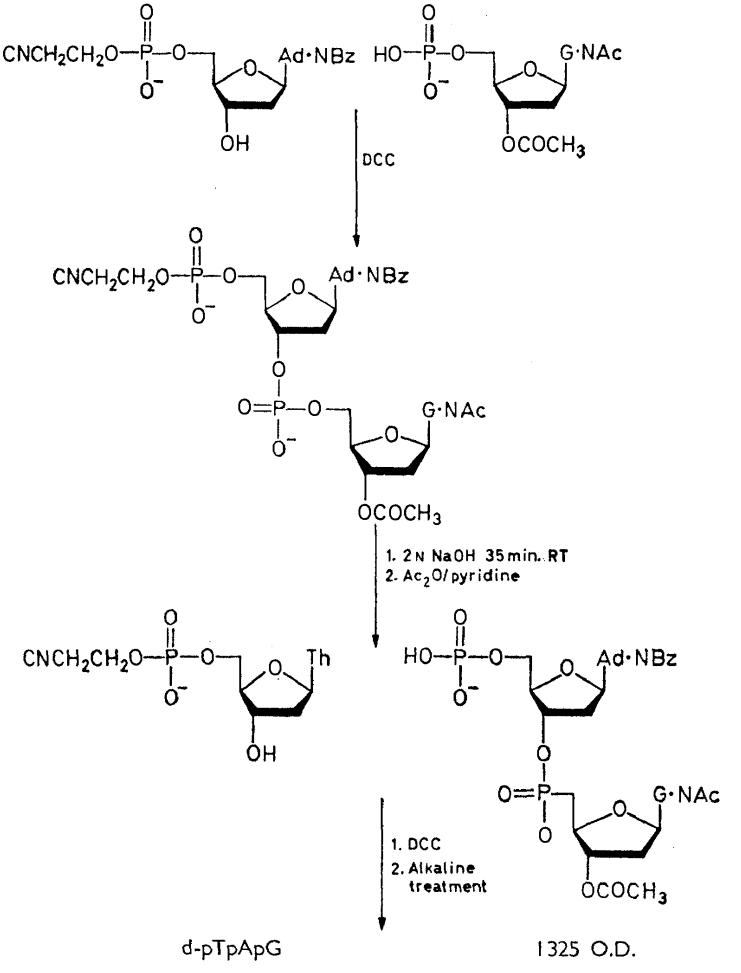


Figure 10. Synthesis of a desoxy-trinucleotide [S. Rittner, Göttingen 1964]

β-cyanoethyl grouping is alkali-labile and can be removed. Now a second β-cyanoethyl-nucleotide is condensed, whereby a trinucleotide pTpApG is obtained.

The alkali-labile β-cyanoethyl protecting group is too labile for many purposes. We therefore use in some cases a protecting group which can be removed by oxidation[5] (*Figure 11*). An acetylated deoxymononucleotide is

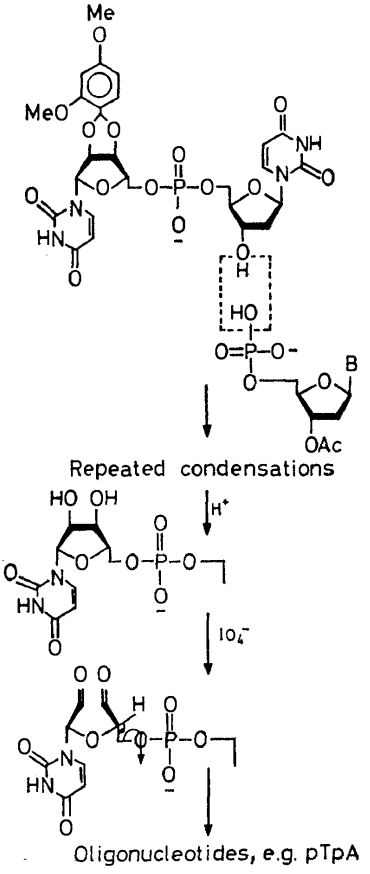


Figure 11. Removal of protecting group by oxidation [F. Kathawala and F. Cramer, Göttingen 1967]

reacted with dimethoxybenzaluridine, a reaction, which gives a high yield, since it is a 5′,5′-linkage. The protected deoxynucleotide can easily be isolated. By alkaline treatment the acetyl group is removed, and the protected nucleotide is the start of the chain to be synthesized. After the last condensation step, the benzal grouping is first removed with acetic acid and subsequently the free glycol grouping is oxidized with periodate. Thereby through a β-elimination the terminal ribonucleoside is removed, a reaction which has long been known as an end group determination method in ribonucleic acid[6, 7].

Although the sequence of reactions looks somewhat complicated, it is easily carried out.

A further protecting group which can be removed by reduction is the trichloroethyl group[8] (*Figure 12*). Trichloroethylester of nucleotides are easily obtained. The trichloroethyl grouping can be removed by reduction with zinc or copper–zinc. The easiest way to synthesize these esters is by the

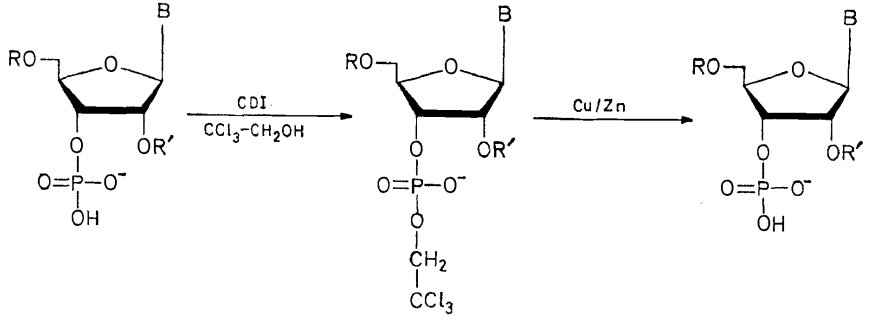


Figure 12. Trichloroethyl protecting group for phosphoric acid [F. Eckstein, Göttingen 1964]

reaction with di(trichloroethyl)-phosphochloridate (*Figure 13*). Both the solubility properties and the chromatographic properties of the products are very favourable. A scheme of such a synthesis is given in *Figure 14.*

We have used these deoxytrinucleotides for an n.m.r. study of the con-

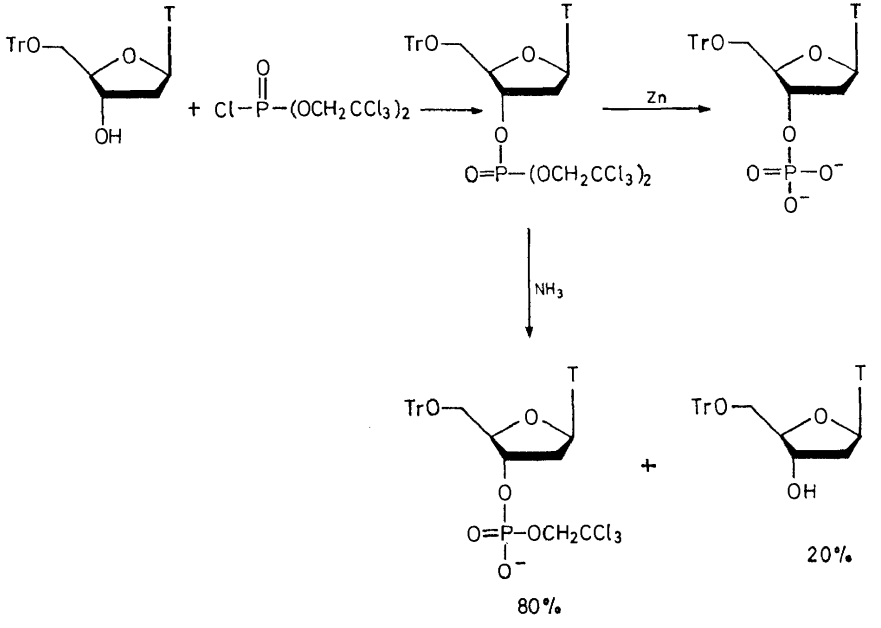


Figure 13. Synthesis of trichloroethyl esters of nucleotides

formation of these trinucleotides in aqueous solution. *Figure 15* shows the n.m.r. spectra of TTT, TGT, and TTA. These spectra cannot be fully interpreted in this paper. It can be immediately seen that the spectra of trinucleotides consisting of purine and thymine show an upfield shift of thymine proton signals as a result of stacking of the heterocyclic bases. The

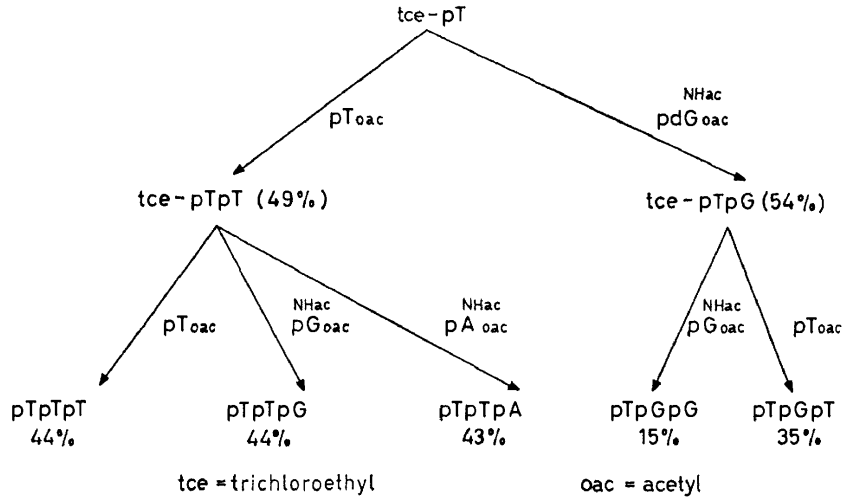


Figure 14. Stepwise synthesis of trinucleotides using trichloroethyl protecting group [A. Franke, K.-H. Scheit, F. Eckstein, Göttingen 1966]

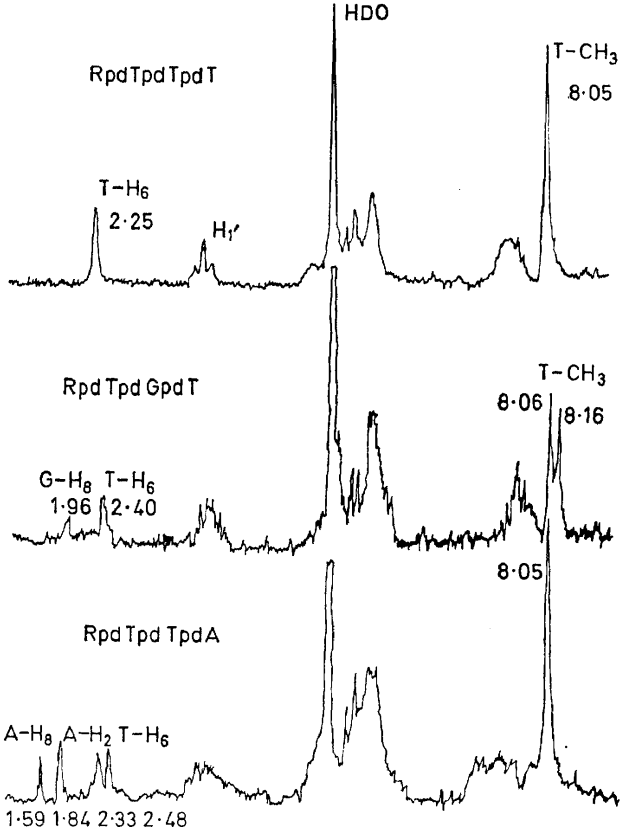


Figure 15. N.m.r. spectra of some trinucleotides [K.-H. Scheit, F. Cramer, A. Franke, 1967]

thymidine at the 3'-end of the chain has a signal at 8·16 p.p.m., the signal from the 5'-end is 8·06 p.p.m. which is identical with the monomeric thymidine. In the spectrum of TTA the thymidine signal is also unchanged. The most likely conformation of the trinucleotides which emerged from these studies is shown in *Figure 16.*

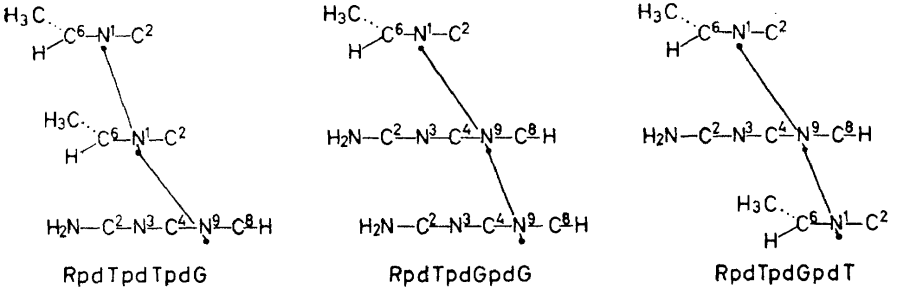


Figure 16. Conformation of trinucleotides in D_2O as determined by n.m.r. [K. H. Scheit, Göttingen 1967]

Because the stacking requires a conformation similar to a semi-Watson/Crick-helix the methyl group of thymine is situated below the ring current of the purine only in the case TGT. The triplet, therefore, has already a stacked helical conformation in aqueous solution.

BLOCK CONDENSATION

For the synthesis of higher oligomers the method of block condensation is necessary, since only by this method one can get long defined oligomers. The most simple case of a polycondensation is one of a polydinucleotide as shown in *Figure 17.* In this manner we have obtained oligomers with alternating sequence which have been similarly obtained by other workers[9]. The condensation of trinucleotides leads to polytriplets[10].

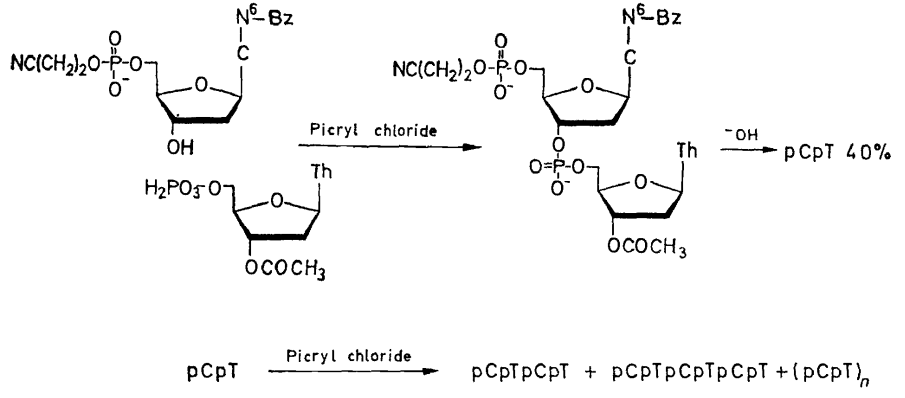


Figure 17. Synthesis of a DNA with alternating sequence [W. Grimm, K. Daneck and G. Weimann, Göttingen 1964]

In *Figure 18* the synthesis of pTpTpC, pCpTpC, and pApTpC is shown[11]. A dinucleotide was first synthesized and subsequently condensed with a protected mononucleotide; the synthesis in contrast to some previous work was carried out here in the direction $3' \rightarrow 5'$ of the growing chain. The yields in this method are around 50 per cent which must be regarded as good in polynucleotide chemistry. These trinucleotides were polycondensed.

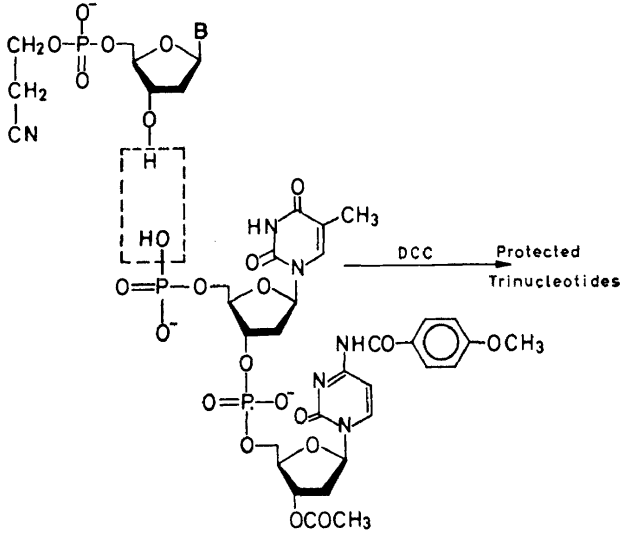


Figure 18. Synthesis of deoxy-trinucleotides pTpTpC (49·9%), pCpTpC (44·5%), pApTpC (45·2%) [W. Frölke, F. Cramer, Göttingen 1966]

Triisopropylbenzenesulphonyl chloride was used as a condensing reagent. In this reaction one obtains a mixture of oligonucleotides, the tri-, hexa-, nona-, dodeca-, and higher nucleotides (*Table 1*). The higher oligomers which are still in satisfactory yield, are the most interesting from the biochemical point of view.

Table 1. Block-condensation of d-trinucleotides
[0·05 mmole in pyridine, 3-fold excess of TIPS, 12 h, room temperature, W. Frölke, Göttingen 1966]

Starting material	*Reaction products*				
	cyclo-	tri-	hexa-	nona-	dodeca and higher
An pTpTpC	24·4	22·2	17·9	16·3	19·7
An An pCpTpC	23·2	24·6	22·2	12·4	17·5
Bz An pApTpC	23·5	18·7	18·2	13·2	26·4

FULLY ESTERIFIED OLIGONUCLEOTIDES

The internucleotidic bond is not completely inert to the condensing reagents, be it dicyclohexylcarbodiimide or a sulphonyl chloride. Therefore, with longer oligonucleotides chain breakages and subsequent rearrangements occur which upset the defined sequences. We have, therefore, started on a programme to use fully esterified oligonucleotides[12]. Dr Eckstein has used the following approach. A nucleotide trichloroethylester is reacted with the 5'-position of a nucleoside or nucleotide (*Figure 19*). The protected

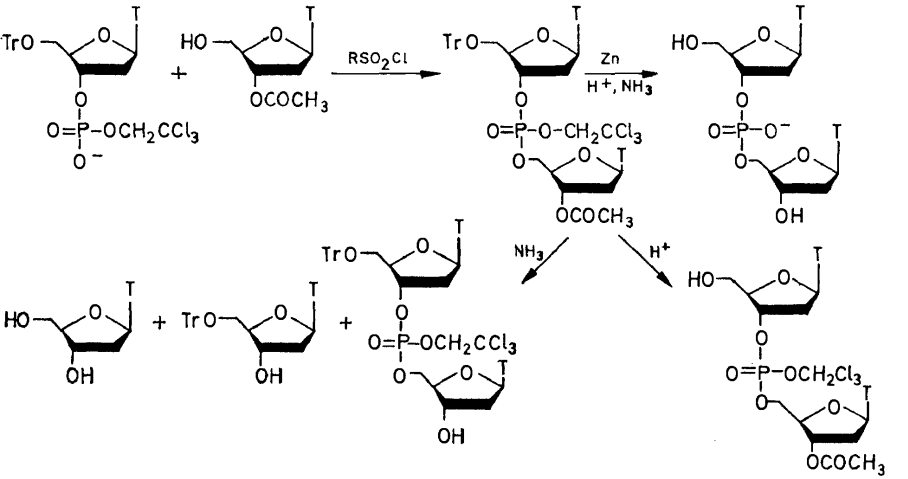


Figure 19. Fully esterified oligonucleotides

derivative is thereby obtained from which the terminal trityl group can be removed with acid and the synthesis continued. The triisopropylsulphonyl chloride (TIPS) is suitable to effect this condensation. It is also possible to fully esterify a trinucleotide with phenyldiazomethane[13] as shown in *Figure 20.*

These oligonucleotides are completely soluble in non-aqueous solvents and very easy to handle chromatographically. A scheme which combines all the possible features for a standard deoxytriplet-synthesis is shown in *Figure 21.* It operates from the 3'- to the 5'-direction. A benzyltrichloroethyl nucleotide was condensed with a trityltrichloroethyl nucleotide after the first trityl group had been removed. The terminal trityl group at the Y-nucleotide can be removed with acid since it is the only acid labile group. Thus the reaction can be continued. The fully protected trinucleotide may be made reactive either at the 5'-end with acid or at the 3'-phosphate with sodium iodide depending on which direction the further synthesis should go. It may be noticed that this is a suitable oligomer for block polymerization in both directions.

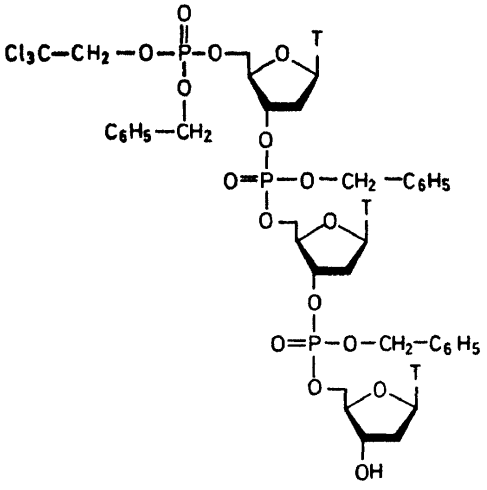


Figure 20. Oligonucleotide triester by reaction of oligonucleotide with phenyldiazomethane [K.-H. Scheit, Göttingen 1967]

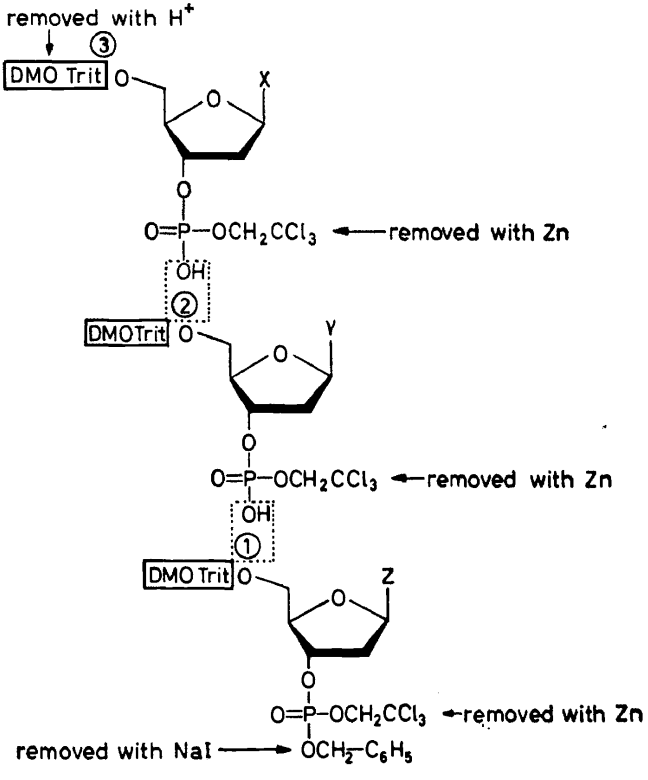


Figure 21. Scheme for deoxytriplet synthesis [F. Eckstein, A. Franke, W. Frölke, K.-H. Scheit, W. Siehr, F. Cramer, 1967]

SYNTHESIS AT A MACROMOLECULAR CARRIER

According to Merrifield[14], the method of peptide synthesis on a polymer support allows because of the simplification of the working-up procedures synthesis of polypeptides in a much improved fashion. We have tried an analogous method in the nucleic acid chemistry and have obtained the first results in this field[15].

Polystyrene carrying a trityl chloride grouping is reacted with a nucleoside which is the beginning of the growing chain (*Figure 22*). After completion of the polynucleotide synthesis the product is removed from the support

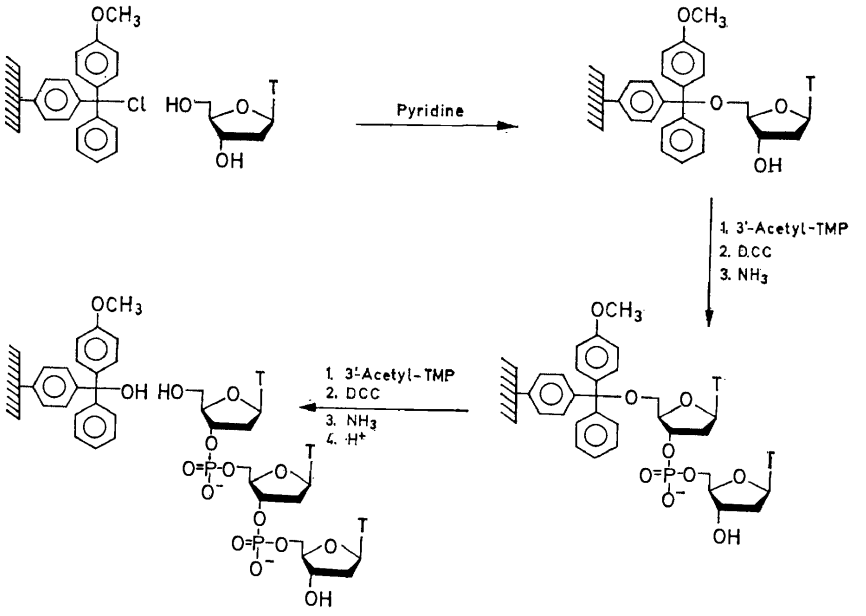


Figure 22. Synthesis of oligonucleotides on a polymer support [H. Helbig, H. Seliger, Göttingen 1966]

with acid. In this way we have synthesized heptanucleotides in yields which in some cases correspond to a 90 per cent reaction in the single step. This, however, is not yet sufficient for a practicable synthesis. In order to render the reaction workable one should have around 99 per cent yield in the single step, in order to avoid side products in a stepwise synthesis. We hope to continue in the desired direction.

A different but less promising method is to fix the first monomer on an aminopolystyrene by a phosphamide bond and to remove the chain after the reaction by acid[16] (*Figure 23*). In this case we had some difficulty in removing the molecule from the support at the end of the reaction.

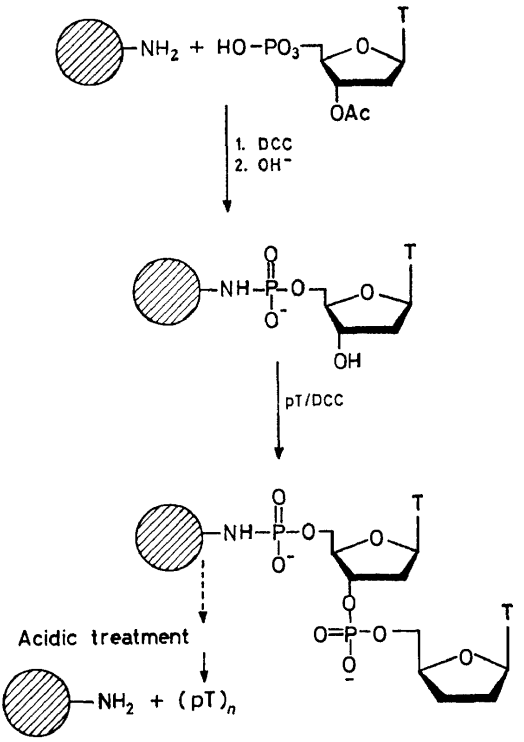


Figure 23. Oligonucleotide synthesis on Merrifield-carrier [H. Seliger, Göttingen 1965]

TEMPLATE PROPERTIES OF SYNTHETIC DEOXYPOLYMERS

When single-stranded oligodeoxynucleotides are used as templates for RNA-polymerase and the corresponding triphosphates are offered in excess the incorporation described in *Table 2* is obtained[11].

With the template pCpTpC and a chain length 12 and higher G and A are incorporated in the ratio 2·04:1. Correspondingly with ATC as a template, UAG is incorporated in the ratio 1·02:1:0·95, or with TTC as a template A

Table 2. Transcription of oligonucleotides with RNA-polymerase
[10^{-3} M triphosphates, 1·0 O.D. template, 100 µg enzyme (200 e.u./mg) in 0·25 ml, H. Matzura, W. Frölke, Göttingen 1967]

	Incorporation in nmole after 90 min			
Template	A	U	G	
$(pCpTpC)_{n \geq 4}$	4·65	—	9·50	G:A = 2·04:1·00
$(pApTpC)_{n \geq 4}$	4·20	4·10	3·90	U:A:G = 1·02:1·00:0·95
$(pTpTpC)_{n \geq 4}$	24·30	—	12·20	A:G = 1·99:1·00

and G are incorporated in the ratio 1·99:1·0. The transcription is—as can be seen from these experiments—highly specific even with single strands. From comparatively short deoxypolymers, messengers can be transcribed.

Khorana in his work during the last years has transcribed a number of deoxyoligo- and polynucleotides and has translated the transcription products into proteins. In this he has made a great contribution to the decoding problem, and I may quote some of his work here.

The transcription of an alternating polymer[17] leads to four different messengers—depending on which strand is transcribed (*Figure 24*) which

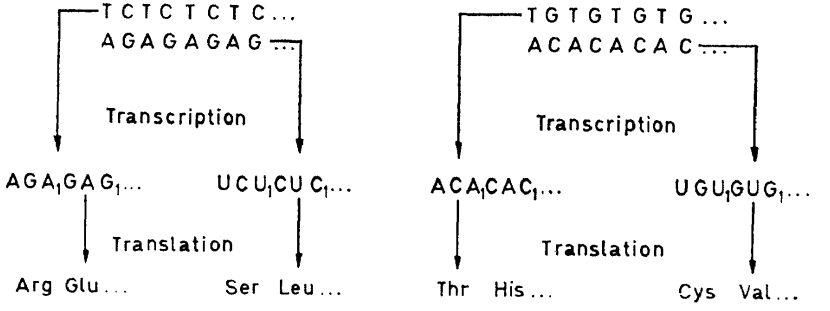


Figure 24. Transcription and translation of polynucleotides with repeating doubletts [H. G. Khorana, S. Nishimura, M. W. Moon, E. Ohtsuka, Madison 1965–66]

can be regulated by the offer of the triphosphates. When these synthetic messengers are used in polypeptide synthesis the following polypeptides are obtained: in the case AGAG, arginine and glutamic acid, in case UCUC, serine and leucine, in the case ACAC, threonine and histidine, and in the case UGUG, cysteine and valine[18]. A remarkable experiment gives independent evidence of the direction of the reading of the messenger given by an oligonucleotide made from alternating quadruplets[19] (*Figure 25*).

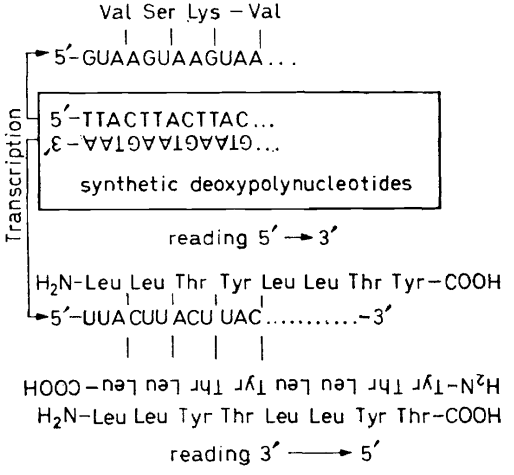
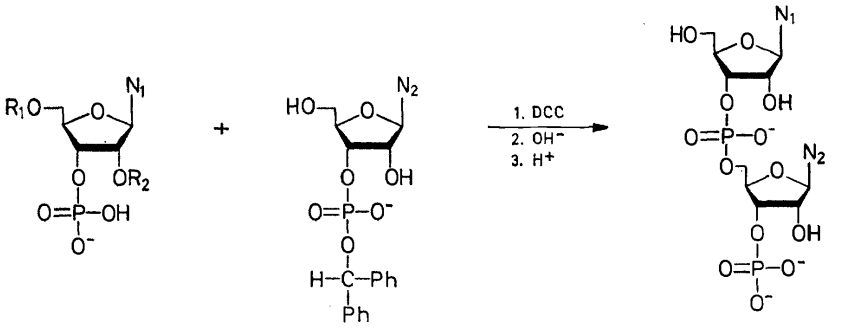


Figure 25. Direction of message reading from repeating tetranucleotide sequences [H. Kössel, H. G. Khorana, A. R. Morgan, Madison 1966]

The double-strand TTAC on the one side and GTAA on the other side was transcribed. Thereby in one case the messenger GUAAGUAA etc. is obtained, an oligonucleotide which has a repeating unit of four triplets which would give a polypeptide, that is repeated after four amino acids. This messenger did not yield a polypeptide, since the messenger contains the terminator-codon UAA. This codon will terminate the chain at its place, making impossible the biosynthesis of longer oligopeptides. More interesting is the transcription of the other strand, GTAA. This will result in a messenger which has the sequence read from the 5′-end: UUACUUAC. With this messenger the question of the direction of reading can be answered unambiguously. When reading from the 5′-end the peptide formed should have the sequence (read from the amino end) leucine, leucine, threonine, tyrosine. When the reading occurs from the 3′-end the sequence would be leucine, leucine, tyrosine, threonine. The analysis of the polypeptide, carried out by Khorana, showed the sequence in the way that the reading occurs from the 5′- to the 3′-end of the messenger. The polypeptide was split with acid at the threonine bond.



Dinucleotide	R_1	R_2	N_1	N_2	*Yield* (%)
UpUp	Acetyl	Tetrahydropyranyl	Uracil	Uracil	29
	Acetyl	Acetyl	Uracil	Uracil	22
UpAp	Acetyl	Tetrahydropyranyl	Uracil	Adenine	20
	Acetyl	Acetyl	Uracil	Adenine	20
ApUp	Benzoyl	Tetrahydropyranyl	N^6-Benzoyladenine	Uracil	14
ApAp	Benzoyl	Tetrahydropyranyl	N^6-Benzoyladenine	Adenine	11
CpAp	Acetyl	Acetyl	N^6-Acetylcytosine	Adenine	24
CpUp	Acetyl	Acetyl	N^6-Acetylcytosine	Uracil	21

Figure 26. Synthesis of dinucleotides with 3′-phosphate [H. J. Rhaese, K.-H. Scheit, G. Schneider, Darmstadt/Göttingen 1963–64]

RNA-SYNTHESIS

Chemically

The first synthesis of a ribodinucleotide was carried out by Scheit and Cramer[20] according to the benzhydryl ester method (*Figure 26*). The benzhydryl protecting group can be removed with acid. Another possibility is to use the cyclophosphates as protecting units[21] (*Figure 27*).

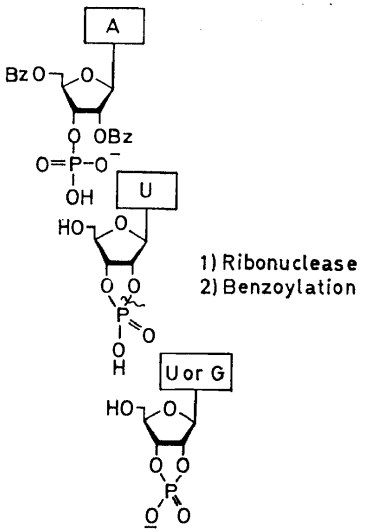


Figure 27. Synthesis of a ribotrinucleotide [W. Siehr, F. Cramer, Göttingen 1966]

This method gives trinucleotides with 3′-terminal phosphate in good yield. All 64 trinucleoside diphosphates were synthesized by Khorana[22] in the following manner. He began with three different building blocks which are condensed in the sequel 1, 2, 3 (*Figure 28*). With these trinucleotides and with trinucleotides which were synthesized partly biochemically by Nirenberg[23], the known and famous code table of Nirenberg and Khorana was obtained.

Enzymatic synthesis of ribopolymers

The enzymatic synthesis of polyribonucleotides can also be carried out with the enzyme polynucleotide phosphorylase, besides the above described transcription of known DNA sequences with RNA-polymerase. Polynucleotide phosphorylase is neither template-dependent nor base-specific. Thus the enzyme accepts all kinds of modified nucleotides, e.g. 4-thio-UDP.

Figure 29 shows a somewhat different experiment with thio-UTP and RNA-polymerase; as can be seen the thio-UTP is incorporated at about half the rate as UTP is at a poly-dAT template. This reaction leads to a very interesting polymer. The most interesting feature of this is probably that it has a UV-absorption at 335 far outside the other nucleotide and

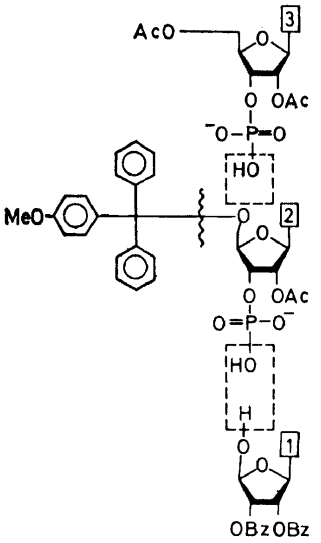


Figure 28. Synthesis of the 64 tripletts [D. Soell, H. G. Khorana, Madison 1965]

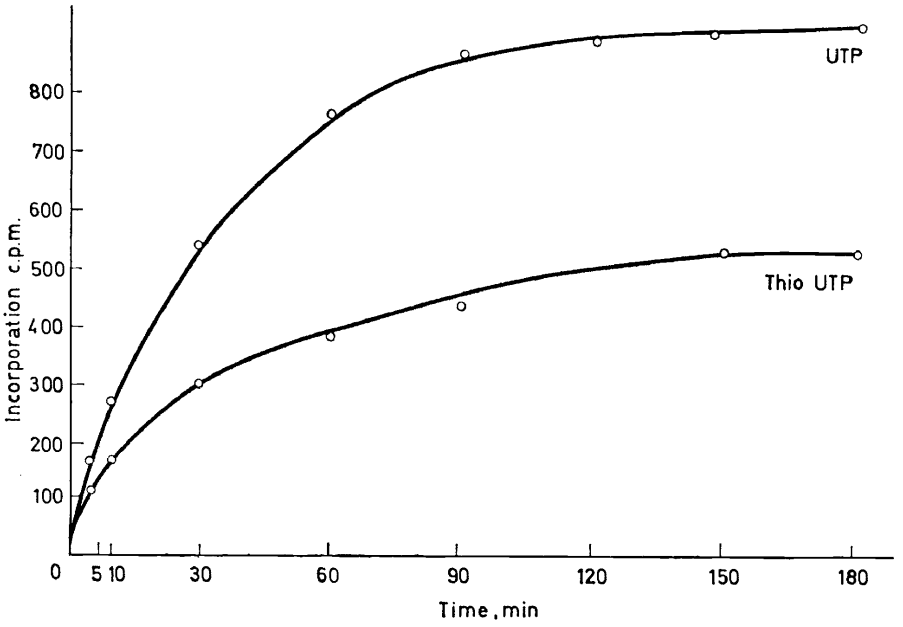


Figure 29. Incorporation of thio UTP with RNA-polymerase (50 nmole ATP-^{14}C, 50 nmole thio UTP, 0·1 O.D. poly dAT) [K.-H. Scheit, H. Matzura, F. Cramer, Göttingen 1967]

protein absorption. Thus this material might become interesting for enzymatic and physico-chemical studies.

For the following studies with polynucleotide phosphorylase the enzyme was prepared in a method similar to that of Singer[24]. The primer dependence of polynucleotide phosphorylase is a very interesting question in itself. Primer dependency is strongly dependent on the salt content of the solution[25]. The results are given in *Figures 30* and *31*.

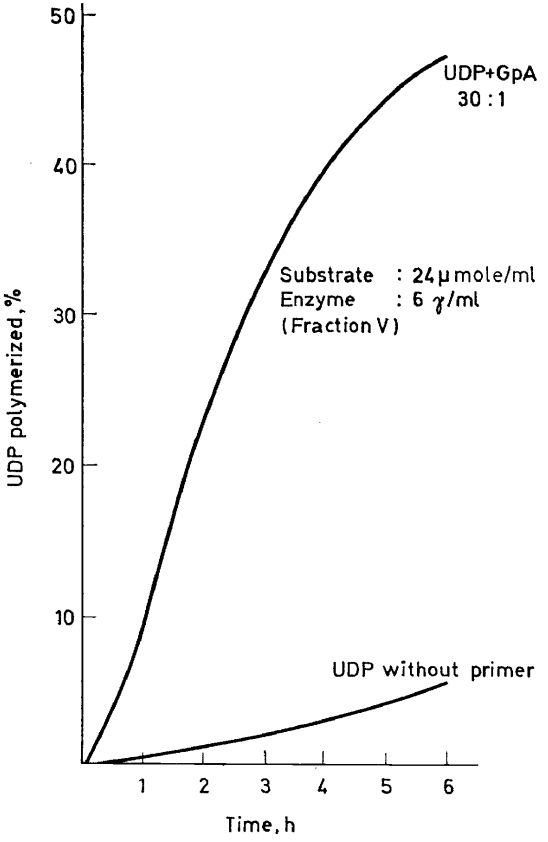


Figure 30. Primer requirement of PNPase from *M. lysodeicticus* [E. Gaertner, F. Cramer, Göttingen 1967]

With increase in the sodium chloride concentration the enzyme becomes more and more dependent on the primer. Under the described conditions the enzyme has an absolute primer dependence. When dinucleoside phosphate is used as a primer in such a reaction, all synthesized chains begin with this dinucleoside phosphate. According to this principle we have prepared a number of polymers with a defined triplet at the 5'-end[1]. The various polymers synthesized are shown in *Table 3*[26].

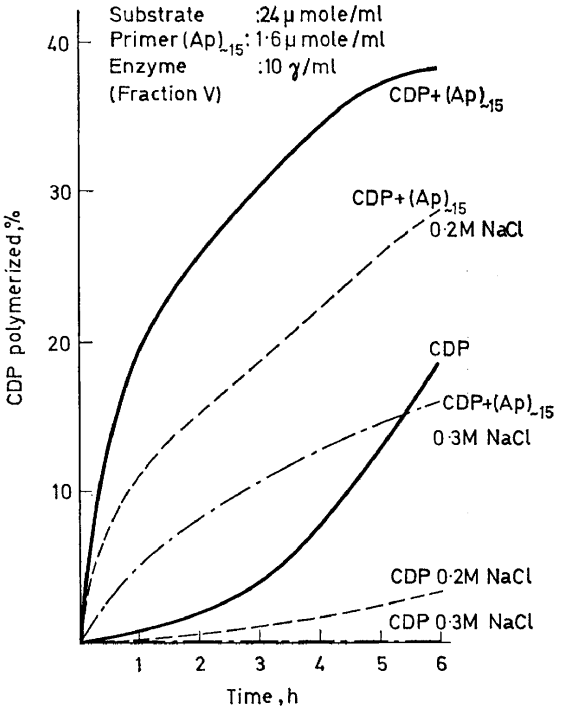


Figure 31. Primer requirement of PNPase at increasing NaCl concentration [Erika Gaertner, F. Cramer, Göttingen 1967]

Table 3. Polynucleotides with defined 5′-terminal triplett
[H. Küntzel, Göttingen 1965]

ApG + ADP	→ AGAAAA .....
UpG + UDP	→ UGUUUU .....
GpA + ADP	→ GAAAAA .....
GpA + UDP	→ GAUUUU .....
ApU + ADP	→ AUAAAA .....
ApU + UDP	→ AUUUUU .....
ApA + UDP	→ AAUUUU .....
UpC + ADP	→ UCAAAA .....
GpC + ADP	→ GCAAAA .....
GpC + UDP	→ GCUUUU .....
UpG + ADP	→ UGAAAA .....
CpU + ADP	→ CUAAAA .....
GpU + ADP	→ GUAAAA .....
GpU + UDP	→ GUUUUU .....

With these polymers several codons were confirmed[27]. Other questions of the direction of reading polymers with a 3′-terminal end may be important. These can be prepared in the following manner[1]:

A copolymer from 30 parts of ADP and one part of UDP was prepared (*Figure 32*). In the polymer in average every thirtieth place in the chain is a

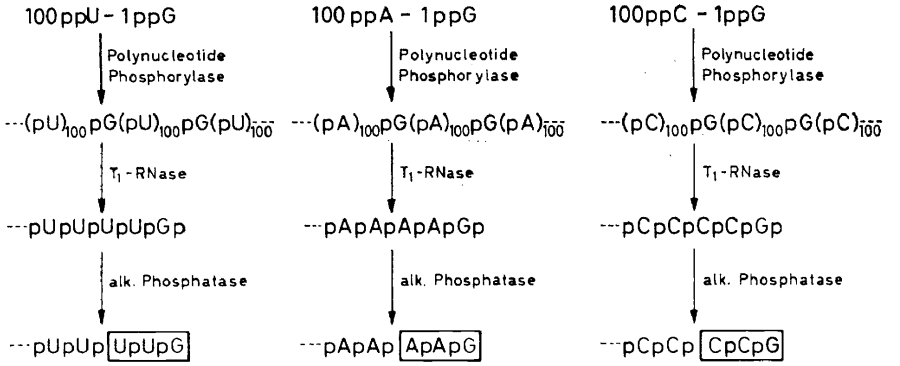


Figure 32. Synthesis of ribopolynucleotides with defined 3′-terminal triplet by cleavage with T_1-ribonuclease [H. Küntzel, Göttingen 1964]

U. At this link the chain can be split with ribonuclease. By the action of RNase one obtains defined short chain polymers which all have a U-terminus. The molecular weight of these fractions varies around the chain length of thirty. At the same time this seems to be the most easy way to obtain oligomers of fairly defined chain length. A further purification according to molecular weight can be achieved on Sephadex G 100 columns. Thus fractions are obtained which have, for example, a chain length of 30 ± 3 nucleotides. The determination of the chain length is done by *labelling* the terminal nucleotide. Thus the ratio of radioactivity versus UV-absorption gives the average chain length. Such a separation is shown in *Figure 33.*

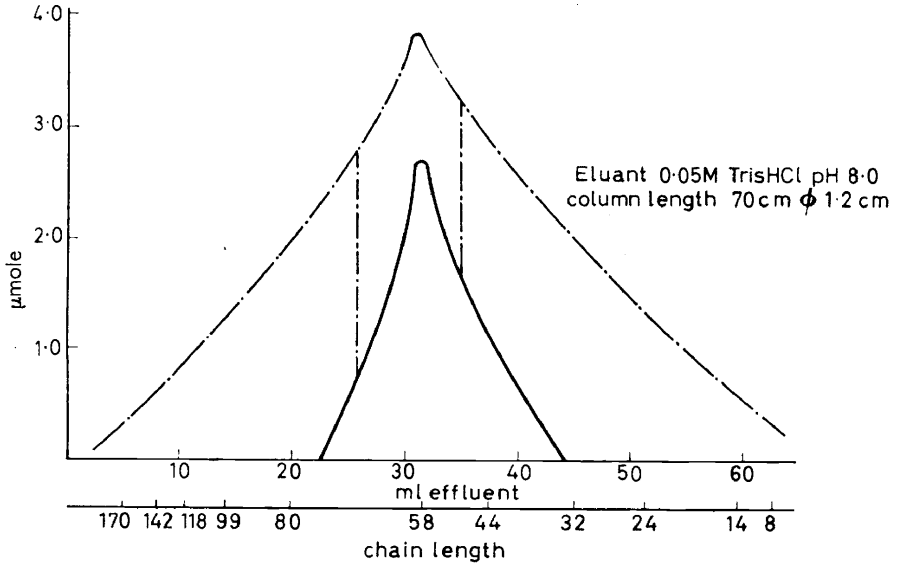


Figure 33. Separation of 15 µmole poly ApAp . . . U on Sephadex G-100 [Erika Gaertner, Göttingen 1967]

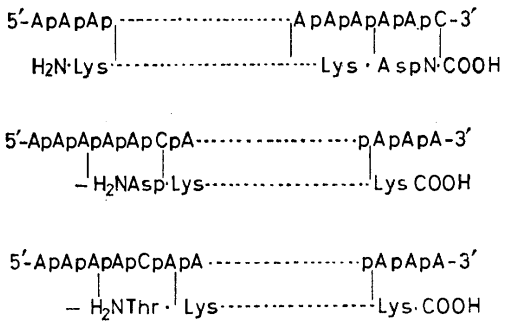


Figure 34. Experiment of Ochoa *et al.* (reading 5′ → 3′)

All these polymers have found numerous applications in coding and translating studies. Here just one example is given: the determination of the direction of the reading of the genetic message[28] (*Figure 34*).

The polymer AAAA . . . C directs the synthesis of an oligopeptide which at its carboxyterminal end has an aspartic acid besides the various lysine residues in the middle of the chain.

CONCLUSION

It is difficult to deal with all aspects of this rich and interesting field and this paper has been restricted to the more chemical and synthetic aspect. The numerous facets of this work—from protecting groups, sugar chemistry, polymer chemistry, physical chemistry, physico-organic chemistry, biochemistry, and enzymology—have been demonstrated.

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