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Synthesis of Deoxyribopolynucleotides Containing Repeating Di- and Tri-nucleotide Sequence.

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~~[illegible] P.M.'s April 17, 1964~~

Synthesis of polynucleotides has been in progress in our laboratory for the last ten years. Chemically synthesized deoxyribo-oligonucleotides of well defined size and structure have served as substrates to elucidate the mode of action of a number of enzymes which degrade nucleic acids; e.g., spleen phosphodiesterase and venom phosphodiesterase. They have also been used as models to develop chemical methods for the sequential analysis of nucleic acids; e.g., end group labelling. At present it is our conviction that the availability of synthetic deoxyribopolynucleotides would enable us/a closer study of the process to carry out of genetic information transfer. This is supported by the following recent discoveries from our own and other laboratories. 1) Short chain synthetic d-AT polymer serves as template for DNA-polymerase. 2) Several synthetic deoxyribopolynucleotides serve as templates for RNA-polymerase. 3) The ribo-polyadenylate formed using deoxyribo-polythymidylate as templates in the RNA-polymerase system brings about polylysine synthesis in the cell-free amino acid incorporation system.

Unambiguous answers are lacking as to the number of nucleotides required for the coding of an amino acid and the sequence of nucleotides in the code. It could be found by using deoxyribopolynucleotides containing specific di- and trinucleotide sequences as templates for RNA-polymerase, and utilizing the complimentary RNA thus formed, as messengers in the cell-free amino acid incorporation system. This paper reports the synthesis of five deoxyribopolynucleotides containing repeating di- and tri-nucleotide sequences.

Two main types of synthetic procedures have been used; stepwise synthesis and polymerization. Both involve formation of $C_{3'} \rightarrow C_{5'}$ internucleotide linkage after selective blocking of undesired reactive functions.

The stepwise addition method used for the synthesis of the repeating trinucleotide sequence TpTpC up to the dodecanucleotide is illustrated in Fig. 1. In this synthesis suitably protected nucleoside-5' phosphates (the 3'-hydroxyl group of both the thymidylic acid and deoxycytidylic acid protected by acetylation, and the amino group of deoxycytidylic acid protected by anisoylation) are added one at a time to the 3'-hydroxyl end of a suitably protected (5'-hydroxyl group protected by trityl or p-methoxy substituted trityl group and amino group of cytidine protected by anisoyl group) growing oligonucleotide chain, using dicyclohexylcarbodiimide (DCC) or mesitylenesulfonyl chloride as the condensing agent. The reaction is carried out at room temp., in anhydrous pyridine. At intermediate steps the protecting group at the 3'-hydroxyl function alone is removed by controlled alkali treatment.

A summary of the results obtained by using mesitylenesulfonyl chloride as the condensing agent is given in Fig. 2 and Fig. 3. Tr = Trityl. High yield of the product is maintained by using increasing amounts of the mononucleotide (3-220 fold) as the chain is elongated. The yield of the pure product isolated is around 70% at each step. The ratio of UV absorption $\frac{270\ m\mu}{302\ m\mu}$ is 1.5 when the triplet is completed. The elution pattern from a DEAE-cellulose (acetate) column for the isolation of d-$TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}TpTpC^{An}$ from the reaction mixture is given in Fig. 4. The eluant is aqueous triethylammonium acetate in 50% ethanol. The excess of mononucleotide was first removed by elution with 0.1 M buffer and is not shown in the figure. The first three peaks are by-products arising from the excess of mononucleotide. The peak marked (P) is the product and the small peak marked (S) is mainly the starting material. Other small peaks are by-products.

At the end of the synthesis, the N-anisoyl protecting group on cytidine is removed by ammonia treatment and then the protecting group at the 5'-hydroxyl function removed by mild acetic acid treatment. The compounds are tested for homogeneity at every step by paper chromatography. The ratio of UV absorption $\frac{260\ m\mu}{280\ m\mu}$ is also followed. Finally selected members of the series were degraded by spleen-diesterase and were found to give thymidine-3' phosphate, cytidine-3' phosphate and the nucleoside at the 3'-hydroxyl end. Their ratios were found as expected.

The second synthetic procedure involves the polymerization of dinucleotides using DCC. It is illustrated in Fig. 5 for the synthesis of the deoxyribopolynucleotides containing the repeating dinucleotide sequence pApG. The 5'-phosphate function of deoxyadenylic acid is protected with cyanoethyl group and the amino function with benzoyl. It is condensed with deoxyguanylic acid, protected at amino and 3'-hydroxyl functions with acetyl group, using DCC to form the fully protected dinucleotide. Controlled alkaline hydrolysis removes the cyanoethyl group at 5'-phosphate and the acetyl group at 3'-hydroxyl function to give the protected dinucleotide (I). This is polymerized using DCC. The work-up involves pyrophosphate cleavage with acetic anhydride, and then ammonia treatment to remove all the protecting groups. Polymerization gives a number of by-products together with the desired polymers. They are separated according to charge, by ion-exchange chromatography on DEAE-cellulose column using 7 M urea and salt gradient. The compounds are further purified by paper chromatography. Alkaline phosphatase is used to remove the phosphate end groups. Spleen and venom diesterases are used for degradation and characterization. The longest polymer characterized so far is the dodecanucleotide $(pApG)_6$.

Fig. 6 shows the five compounds synthesized by using the above procedures. The stepwise addition method can be extended for the preparation of deoxyribopolynucleotides of any desired sequence. The polymerization procedure gives low yields of desired products and they need elaborate purification, however it provides relatively a rapid method for the preparation of small amounts of these useful compounds. It could be extended for the synthesis of deoxyribopolynucleotides containing other repeating sequences.

Figure 1

STEPWISE SYNTHESIS OF DEOXYRIBOPOLYNUCLEOTIDES

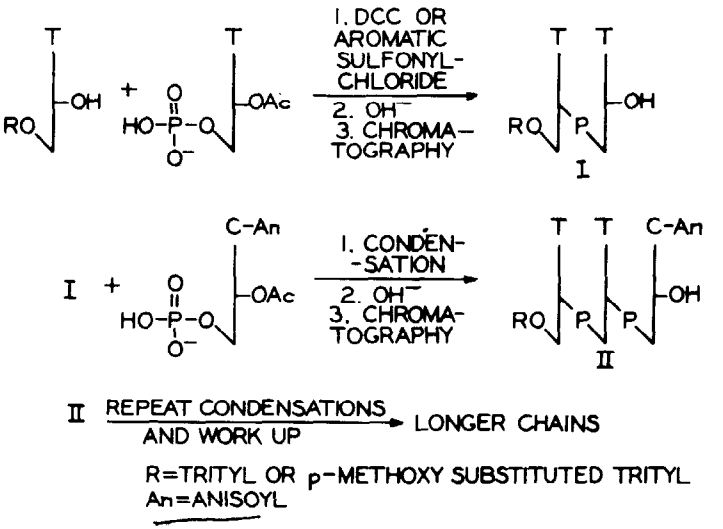


Figure 2

PRODUCT	MOLAR EQUIVA-LENT OF MONO-NUCLEOTIDE	YIELD (%)	270mμ / 302mμ
d-TrTpTpC^{An}	3	82	1·5
d-TrTpTpC^{An}pT	10	79	1·9
d-TrTpTpC^{An}pTpT	14	77	2.3
d-TrTpTpC^{An}pTpTpC^{An}	18	74	1·5
d-TrTpTpC^{An}pTpTpC^{An}pT	30	75	1·7

Figure 3

PRODUCT	MOLAR EQUIVA-LENT OF MONO-NUCLEOTIDE	YIELD (%)	270mμ / 302mμ
d-TrTpTpC^{An}pTpTpC^{An}pTpT	40	73	1·9
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	85	75	1·5
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT	120	70	1·65
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpT	185	70	1·8
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	220	56	1·5

Figure 4

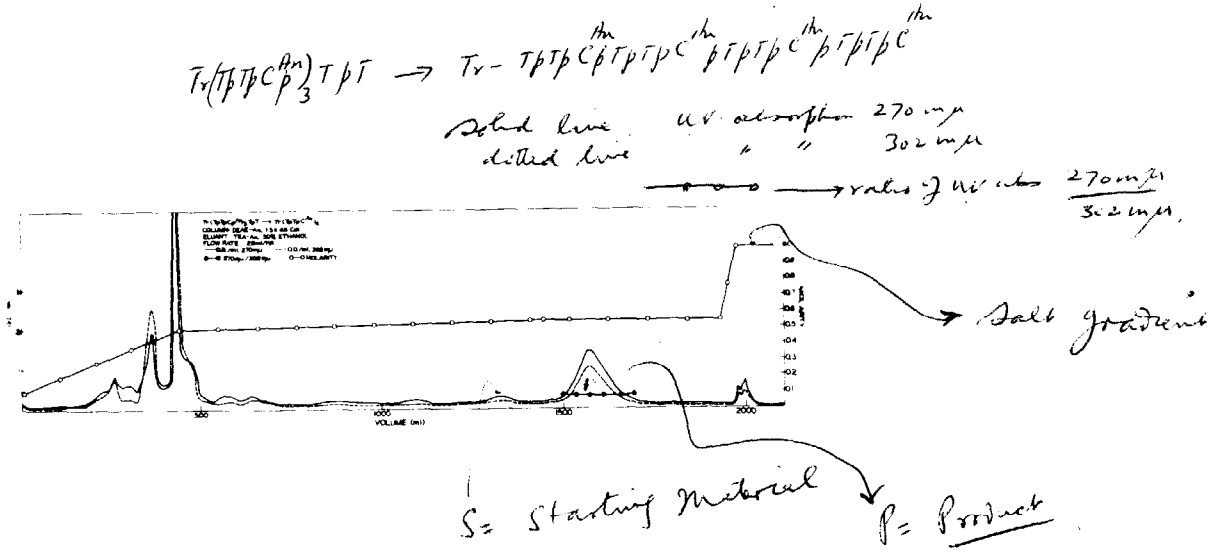
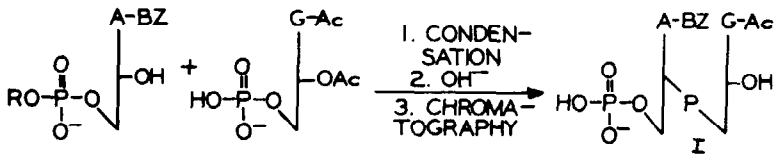


Figure 5

POLYMERIZATION OF DINUCLEOTIDES



I → (pApG)$_n$ (n = 1 TO 6)

1. DCC
2. PYROPHOSPHATE CLEAVAGE
3. NH_4OH
4. CHROMATOGRAPHY

BZ = BENZOYL
R = $-CH_2CH_2CN$

Figure 6

DEOXYRIBOPOLYNUCLEOTIDES PREPARED

BY STEPWISE SYNTHESIS

1. d-TpTpCpTpTpCpTpTpCpTpTpC
2. d-TpTpIpTpTpIpTpTpI

BY POLYMERIZATION

1. d-TpCpTpCpTpCpTpCpTpCpTpC
2. d-TpGpTpGpTpGpTpGpTpGpTpG
3. d-ApGpApGpApGpApGpApGpApG