Chemical Synthesis of Three Deoxyribododecanucleotide Chains of Defined Sequence*

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ABSTRACT: This paper reports the chemical synthesis of three well-defined chains of deoxyribopolynucleotides bearing a 5′-phosphomonoester end group. The desired sequence of these protected oligonucleotides are related to a possible deoxyribonucleic acid sequence containing biological information for bovine insulin chain A.

The new approach described in the present synthesis is that the chain length should be approximately doubled at each condensation step. Thus products and reactants differ substantially in molecular weight and can be separated rapidly by Sephadex gel filtration. An attractive feature of this separation technique is that the product peak emerges from the column before the peaking containing starting material and thus offers great advantages over ion-exchange DEAE-cellulose chromatography. The major objective for undertaking the synthesis and the general consideration for the selection of codons sequence relate to bovine insulin chain A is also presented.

With the elucidation of the structure of the genetic code (Nirenberg *et al.*, 1966; Khorana *et al.*, 1966), it has become feasible to decode the known amino acid sequence of certain proteins into possible polynucleotide sequences. Also, with the recent discovery of polynucleotide ligase (Gilhert, 1967; Weiss and Richardson, 1967; Oliver and Lehman, 1967; Fifter *et al.*, 1967; Cozzarelli *et al.*, 1967), it should be possible to synthesize short segments of genes. Since the biosynthesis of insulin appears to follow the general pattern of protein synthesis (Humbel, 1965; Taylor and Parry, 1963), an extensive program has been initiated in this laboratory to synthesize the defined sequence of DNA containing biological information of bovine insulin chain A with the following purposes in mind. First, the DNA-directed synthesis of insulin will open up a new phase in the field of protein synthesis. Second, if the synthetic DNA could be replicated enzymatically *in vitro*, great advances can be expected in the design and synthesis of other genetic determinants and in the development of techniques for the introduction of such new determinants in the genome of living organisms.

The degenerancy of the genetic code makes it impossible to determine the exact RNA base sequence coding for a specific peptide chain. Assuming the genetic code assignment from the bacterial systems is universal, approximately two-thirds of the bases can be outlined in the sequence of the mRNA specifying the information of the polypeptide bovine insulin chain A. In most cases, synonyms in the code prohibit the determination of which of the two or more bases is the actual base in third position of a given codon. However, one of the possible sequences of mRNA for bovine insulin chain A has been outlined in Table I. The code GUA (valine at position 3) was particularly selected to ensure that if the translation process of the mRNA starts by mistake at the second base from the 5′ end (*i.e.*, out of phase reading), the fourth codon will be UAG (chain-terminating signal), and thus a tripeptide segment of an undesirable sequence will be generated. Similarly, GCU (alanine at position 8) and AGU (serine at position 9) were chosen to ensure that a reading of the mRNA beginning at the third base from the 5′ end would result in the formation of an octapeptide of another undesired sequence.

From the studies of Cozzarelli *et al.* (1967), Oliver and Lehman (1967), and Gupta *et al.* (1968a,b), it is clear that the organic synthesis of short-chain oligonucleotides followed by enzymatic joining by polynucleotide ligase provides a promising approach to the synthesis of DNA containing defined sequence. The present available methods for the chemical synthesis of oligonucleotides require the application of time-consuming and laborious ion-exchange DEAE-cellulose column chromatography at each condensation step (Jacob and Khorana, 1965; Narang and Khorana, 1965; Narang *et al.*, 1965, 1967; Kössel *et al.*, 1967; Ohtsuka and Khorana, 1967; Ohtsuka *et al.*, 1965; Jacob *et al.*, 1967). Keeping in view the magnitude of the synthetic task in the present goal, it was considered essential and realistic to explore an easier and faster separation technique for the isolation and purification of synthetic oligonucleotides. In this paper we wish to describe the use of Sephadex (superfine grade) gel filtration technique for the preparation of deoxyribopolynucleotides of defined sequence. The basic concept developed in the present study is that the chain length should be approximately doubled at each condensation step and the reactants for the condensation should be suitably protected oligonucleotides with 5′-phosphomonoester end groups. Thus products and reactants differ substantially in molecular weight and can be separated rapidly and quantitatively by gel filtration, *e.g.*, Sephadex gel with appropriate exclusion limits. An attractive feature of this separation is that the product emerges from the column before the peak containing starting material. Furthermore, the presence of a 5′-phosphomonoester group on each fragment offers more flexibility in extending the chain in either direction by the selective protection of 5′-phosphomonoester

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TABLE I: Possible Sequence of mRNA and DNA Encoding of Bovine Insulin Chain A.[a]

		Chain initiation signal	1	2	3	4	5	6	7	8	9	10
Bovine insulin chain A		*N*-Formylmethionine -	Gly -	Ile -	Val -	Glu -	Gln -	Cys -	Cys -	Ala -	Ser -	Val
RNA coding triplets		AUG-	GG*U*-	AUU-	GUU-	GA*A*-	CA*A*-	UG*U*-	UG*U*-	GC*U*-	AG*U*-	GUU
			C	*C*	C	G	G	C	C	C	C	C
			A	A	*A*					A	UCU	*A*
			G		G					G	C	G
											A	
											G	
Possible sequence o mRNA	5′ end	AUG-	GGU-	AUC-	GUA-	GAA-	CAA-	UGU-	UGU-	GCU-	AGU-	GUA
Sequence of corresponding DNA	3′ end	TAC-	CCA-	TAG-	CAT-	CTT-	GTT-	ACA-	ACA-	CGA-	TCA-	CAT

	11	12	13	14	15	16	17	18	19	20	21	Chain-terminating signal	
	Cys -	Ser -	Leu -	Tyr -	Gln -	Leu -	Glu -	Asn -	Tyr -	Cys -	Asn -		
	UG*U*-	AG*U*-	UUA-	UA*U*-	CA*A*-	UUA-	GA*A*-	AA*U*-	UA*U*-	UG*U*-	AA*U*-	UA*A*	
	C	C	*G*	C	G	*G*	G	C	C	C	C	G	
		UCU	CUU			CUU							
		C	C			C							
		A	A			A							
		G	G			G							
Continued possible sequence of mRNA	UGU-	AGU-	UUG-	UAU-	CAA-	UUG-	GAA-	AAU-	UAU-	UGU-	AAU-	UAA	3′ end
Continued possible sequence of DNA	ACA -	TCA -	AAC -	ATA -	GTT -	AAC -	CTT -	TTA -	ATA -	ACA -	TTA -	ATT	5′ end
		III				II				I			

[a] No account has been taken of the problems of initiation and termination of transcription.

or 3′-hydroxyl group at each step. This paper deals with successful application of Sephadex gel filtration technique for the rapid chemical synthesis of three chains of dodecanucleotides (I, base 69–58), dpTpTpApApTpTpApCpApApTpA; II (base 57–46), dpApTpTpTpTpCpCpApApTpTpG; and III (base 45–34), dpApTpApCpApApApCpTpApCpA, see Table I) related to the partial DNA sequence of bovine insulin chain A from the 5′ end. A preliminary report of a part of this work has already appeared (Narang *et al.*, 1968). The general chemical method is given in Chart I.

The reactants for the condensation steps were β-cyanoethyl phosphate esters (CE)[1] of the appropriate N-protected mono-, di-, tri-, and hexanucleotides and the corresponding 3′-*O*-acetyl N-protected mono-, tri-, and hexanucleotides. MS was used as the condensing agent. The reaction mixture was concentrated shortly after the addition of the condensing agent to the anhydrous solution. At each condensation step the reaction mixture was usually given overnight aqueous pyridine-diisopropylethylamine treatment, followed by controlled alkali treatment to remove the acetyl and cyanoethyl groups. The desired product was then isolated by gel filtration on a Sephadex column at 4°. The column was eluted with 0.1 M triethylammonium bicarbonate (pH 7.5).

[1] The system of abbreviations is essentially the same as used in the *Journal of Biological Chemistry*. Thus pT-OAc is the abbreviation of 3′-*O*-acetylthymidine 5′-phosphate. CE is the abbreviation for β-cyanoethyl. MS stands for mesitylenesulfonyl chloride.

Experimental Section

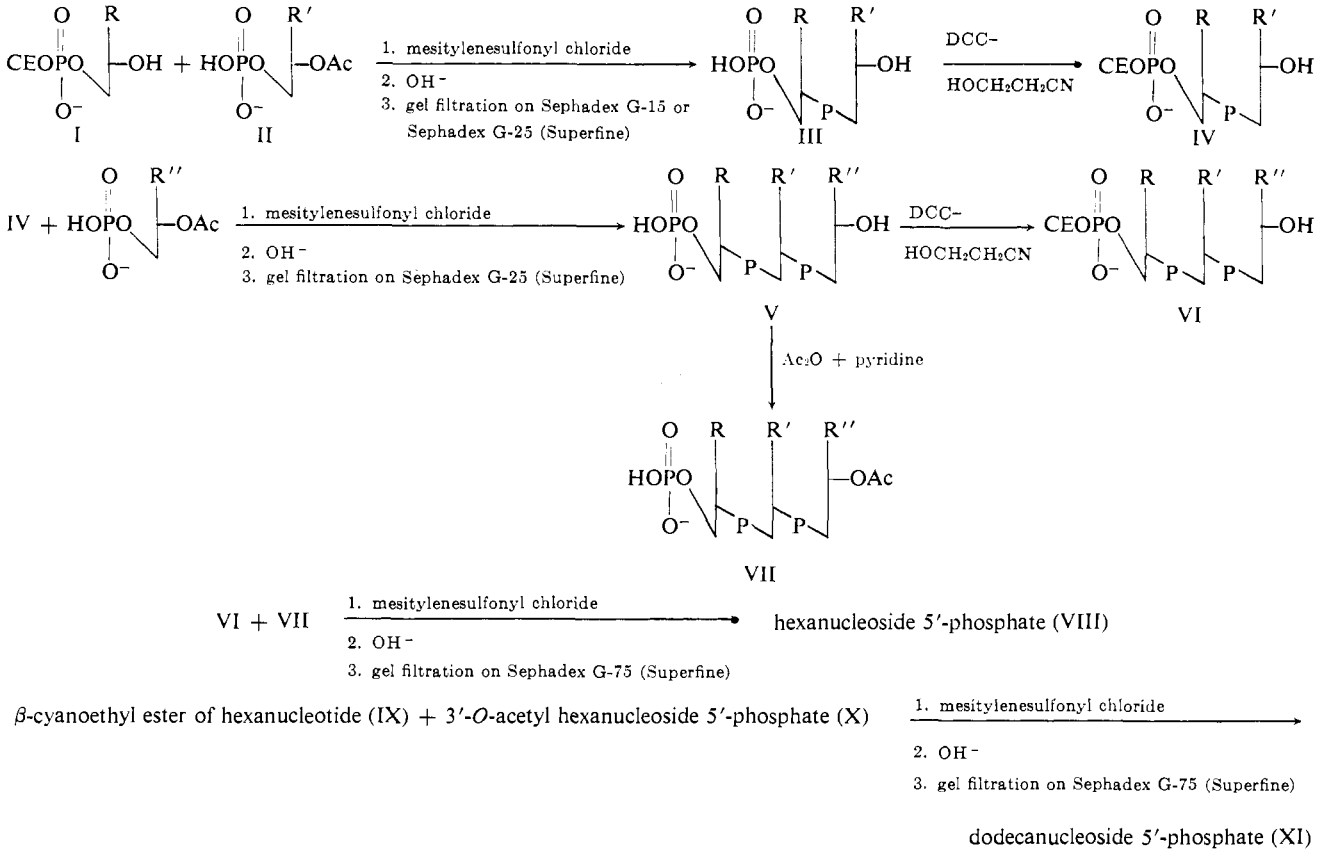
General Methods and Materials. The following commercial products were used: thymidine 5′-phosphate, deoxyadenosine 5′-phosphate, deoxycytidine 5′-phosphate, and deoxyguanosine 5′-phosphate from CalBiochem; mesitylenesulfonyl chloride from Aldrich; spleen phosphodiesterase, snake venom phosphodiesterase, and bacterial alkaline phosphatase from Worthington; and polynucleotide kinase (gift from Professors Ray Wu, Charles C. Richardson, and Jerard Hurwitz). $[\gamma\text{-}^{32}P]$ATP was prepared by the method of Glynn and Chappell (1964) as modified by Wu;[2] Sephadex G-15, G-25 (superfine), G-50 (superfine), and G-75 (superfine) were from Pharmacia, Uppsala, Sweden; columns used for gel filtration types K25–100 and K50–100 were from Pharmacia, Uppsala, Sweden.

Reagent grade pyridine was distilled and dried over Molecular Sieve (4A) from Linde Co. for several weeks.

The max values used for the protected mononucleotides are as follows: dpC^{An}, 22,450 (302 mμ) and 18,000 (280 mμ);

[2] Private communication from Professor Ray Wu.

CHART I: General Method for the Synthesis of Deoxyribopolynucleotides Bearing a 5′-Phosphomonoester End Group.



I–XI, R, R′, and R″ = thymine, *N*-acetylguanine, *N*-benzoyladenine, or *N*-anisoylcytosine

TABLE II: Preparation of Protected Dinucleotides.[a]

β-CE Ester Component	Amt (mmoles)	3′-*O*-Acetyl Component	Amt (mmoles)	MS (mmoles)	Product (purity, %)	Isolated Yield (%)
dCE-pA^{Bz}	0.5	pT-OAc	0.5	5.0	dpA^{Bz}pT (81)	60
dCE-pA^{Bz}	4.0	dpC^{An}-OAc	4.0	40.0	dpA^{Bz}pC^{An} (91)	59
dCE-pC^{An}	2.0	dpA^{Bz}-OAc	2.67	26.7	dpC^{An}pA^{Bz} (98)[b]	55

[a] The desired products were isolated by gel filtration on Sephadex (G-15) columns (K50–100) at 4°. As an illustration, the elution pattern of dpA^{Bz}pC^{An} reaction mixture is given in Figure 2. [b] The reaction mixture was treated with acetic anhydride–pyridine to break the pyrophosphate.

dpA^{Bz}, 6100 (302 mμ), 18,300 (280 mμ), and 11,450 (260 mμ); dpG^{Ac}, 16,700 (260 mμ) and 10,900 (280 mμ).

Pyridinium CE-pT, dCE-pC^{An}, dCE-pA^{Bz}, pT-OAc, and pG^{Ac}-OAc were prepared as described elsewhere (Khorana and Vizolyi, 1961; Ohtsuka *et al.*, 1965; Ralph *et al.*, 1963). The general method used for the cyanoethylation, acetylation, and the synthesis of oligonucleotides has been described previously (Narang *et al.*, 1969).

Synthesis of Deoxyribopolynucleotides Bearing a 5′-Phosphomonoester End Group. Mesitylenesulfonyl chloride was added to an anhydrous pyridine solution of tri-*n*-hexylammonium salts of β-cyanoethylated oligonucleotide and 3′-*O*-acetyl oligonucleotides. The reaction was concentrated *in vacuo* with gentle shaking to a viscous solution and the sealed reaction mixture was kept at room temperature in the dark for 3 hr. Aqueous pyridine (2–4 ml) was then added with cooling, followed by excess (0.1–0.5 ml) of diisopropylethylamine. The solution was left at room temperature overnight. An equal volume of 2 N sodium hydroxide was then added and the solution was kept for 10 min at room temperature. An excess of pyridinium Dowex 50 ion-exchange resin was then added to neutralize the alkali. The resin was removed by filtration and washed thoroughly with 50% aqueous pyridine. The total filtrate was concentrated *in vacuo* to a known

TABLE III: Preparation of Protected Trinucleotides.[a]

β-CE Component	Amt (mmoles)	3′-O-Acetyl Component	Amt (mmoles)	MS (mmoles)	Product (purity, %)	Isolated Yield (%)
dCE-pTpT	0.78	dpA^{Bz}-OAc	1.2	10.5	dpTpTpA^{Bz} (92)	74
dCE-pA^{Bz}	2.5	pTpT-OAc	2.0	7.0	dpA^{Bz}pTpT (92)	40
dCE-pA^{Bz}pT	1.62	dpA^{Bz}-OAc	2.4	24.0	dpA^{Bz}pTpA^{Bz} (91)	37
dCE-pA^{Bz}pC^{An}	0.55	pT-OAc	2.75	27.5	dpA^{Bz}pC^{An}pT (97)	40
dCE-pA^{Bz}pC^{An}	1.37	dpA^{Bz}-OAc	4.0	30.0	dpA^{Bz}pC^{An}pA^{Bz} (95)	50
dCE-pTpT	0.55	dpC^{An}-OAc	2.0	20.0	dpTpTpC^{An} (95)	75
dCE-pC^{An}pA^{Bz}	0.33	dpA^{Bz}-OAc	1.0	10.0	dpC^{An}pA^{Bz}pA^{Bz} (96)	40
dCE-pTpT	0.25	dpG^{Ac}-OAc	1.0	10.0	dpTpTpG^{Ac} (95)	50

[a] The desired products were isolated by gel filtration on Sephadex G-25 (superfine) columns (K50–100) at 4°. As an illustration, the elution pattern of dpTpTpA^{Bz} reaction mixture is given in Figure 3.

TABLE IV: Synthesis of Protected Hexanucleotides.[a,b]

β-CE Component	Amt (mmoles)	3′-O-Acetyl Component	Amt (mmoles)	MS (mmoles)	Products (purity, %)	Isolated Yield (%)
dCE-pTpTpA^{Bz}	0.24	dpA^{Bz}pTpT-OAc	0.25	2.5	dpTpTpA^{Bz}pA^{Bz}pTpT (92)	31
dCE-pA^{Bz}pC^{An}pA^{Bz}	0.29	dpA^{Bz}pTpA^{Bz}-OAc	0.31	3.0	dpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpA^{Bz} (90)	33
dCE-pA^{Bz}pTpT	0.17	dpTpTpC^{An}-OAc	0.2	2.0	dpA^{Bz}pTpTpTpTpC^{An} (92)	30
dCE-pC^{An}pA^{Bz}pA^{Bz}	0.11	dpTpTpG^{Ac}-OAc	0.12	2.0	dpC^{An}pA^{Bz}pA^{Bz}pTpTpG^{Ac} (94)	29
dCE-pA^{Bz}pTpA^{Bz}	0.18	dpC^{An}pA^{Bz}pA^{Bz}-OAc	0.19	2.0	dpA^{Bz}pTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz} (98)	29
dCE-pA^{Bz}pC^{An}pT	0.075	dpA^{Bz}pC^{An}pA^{Bz}-OAc	0.08	0.8	dpA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz} (97)	25

[a] The reaction mixture was given acetic anhydride–pyridine treatment in order to cleave the pyrophosphates. [b] The desired products were isolated by gel filtration on Sephadex G-75 (superfine) columns (K25-100) at 4°. As an illustration, the elution pattern of dpA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz} reaction mixture is given in Figure 4.

TABLE V: Synthesis of Protected Dodecanucleotides.[a,b]

β-CE Components	Amt (mmoles)	3′-O-Acetyl Components	Amt (mmoles)	MS (mmoles)	Products (purity, %)	Isolated Yield (%)
dCE-pTpTpA^{Bz}pA^{Bz}pTpT	0.03	dpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpA^{Bz}-OAc	0.031	0.3	dpTpTpA^{Bz}pA^{Bz}pTpTpA^{Bz}pC^{An}-pA^{Bz}pA^{Bz}pTpA^{Bz} (89)	18
dCE-pA^{Bz}pTpTpTpTpC^{An}	0.0081	dpC^{An}pA^{Bz}pA^{Bz}pTpTpG^{Ac}-OAc	0.0091	0.2	dpA^{Bz}pTpTpTpTpC^{An}pC^{An}pA^{Bz}-pA^{Bz}pTpTpG^{Ac} (80)	17
dCE-pA^{Bz}pTpA^{Bz}pC^{An}-pA^{Bz}pA^{Bz}	0.015	dpA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz}-OAc	0.017	0.2	dpA^{Bz}pTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}-pA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz} (82)	15

[a] The reaction mixture was given acetic anhydride–pyridine treatment in order to cleave the pyrophosphates. [b] The desired products were isolated by gel filtration on Sephadex G-75 (superfine) columns (K25–100) at 4°. As an illustration, the elution pattern of dpTpTpA^{Bz}pA^{Bz}pTpTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpT reaction mixture is given in Figure 5.

TABLE VI: Paper Chromatography of Deoxyribopolynucleotides.[a]

Compd	R_F with 5′-Phosphate End Group in Solvents			R_F without 5′-Phosphate End Group in Solvents		
	A	B	C	A	B	C
Dinucleotides						
dpA^{Bz}pT	1.02					
dpApT	0.44	1.14		1.1		
dpA^{Bz}pC^{An}	1.08					
dpApC	0.40	1.27	0.81	1.08		
dpC^{An}pA^{Bz}	1.0					
dpCpA	0.32	1.24		1.21		
Trinucleotides						
dpTpTpA^{Bz}	0.82					
dpTpTpA	0.32	0.80	0.77	1.0		
dpA^{Bz}pTpT	0.81					
dpApTpT	0.31	0.70	0.68	0.93		
dpA^{Bz}pTpA^{Bz}	0.89					
dpApTpA	0.21	1.21		0.70		
dpA^{Bz}pC^{An}pT	0.70					
dpApCpT	0.25	0.95	0.57	0.60		
dpA^{Bz}pC^{An}pA^{Bz}	0.70					
dpApCpA	0.22	1.33	0.56	0.55		
dpTpTpC^{An}	0.83					
dpTpTpC	0.40	0.61		0.85		
dpC^{An}pA^{Bz}pA^{Bz}	0.69					
dpCpApA	0.27	1.34		0.56		
dpTpTpG^{Ac}	0.54					
dpTpTpG	0.35	0.60		0.90	1.30	
Hexanucleotides						
dpTpTpA^{Bz}pA^{Bz}pTpT	0.39					
dpTpTpApApTpT	0.04	0.32	0.35			0.71
dpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpA^{Bz}	0.32					
dpApCpApApTpA		1.03	0.25			0.48
dpA^{Bz}pTpTpTpTpC^{An}	0.30					
dpApTpTpTpTpC	0.08		0.25	0.29		0.60
dpC^{An}pA^{Bz}pA^{Bz}pTpTpG^{Ac}	0.25					
dpCpApApTpTpG		0.15	0.21		0.32	0.45
dpA^{Bz}pTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}	0.30					
dpApTpApCpApA	0.18	1.01	0.27		1.59	0.49
dpA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz}	0.31					
dpApCpTpApCpA	0.19	0.97	0.29			0.43
Dodecanucleotides						
dpTpTpApApTpTpApCpApApTpA		0.19			0.34	0.025
dpApTpTpTpTpCpCpApApTpTpG			0.05			0.09
dpApTpApCpApApApCpTpApCpA			0.06			0.10

[a] R_F with respect to pT.

volume. The summary of the pertinent reaction conditions and yields in the preparation of various di-, tri-, hexa-, and dodecanucleotides are given in Tables II, III, IV, and V.

Gel Filtration on Sephadex Column. Sephadex columns types K25–100 and K50–100 equipped with flow adaptors were packed with Sephadex gel of the appropriate particle size grades. The adaptor was connected to graduated syringe (sample container) and an elution reservoir by means of a three-way valve (Tomac). The column was preequilibrated with 0.1 M triethylammonium bicarbonate (pH 7.5) at 4°. The reaction mixture was applied as a narrow band through the flow adaptor as this operation eliminated the manual sample application.

Isolation of Oligonucleotides. Following chromatography

TABLE VII: Characterization of Products by Snake Venom Phosphodiesterase Degradation.

Compound	OD_{260} Degraded	Nucleoside:Nucleotides	Molar Ratio of Products	
			Found	Theoretical
dApT	9.2	dA:pT	1:0.98	1:1
dApC	4.37	dA:dpC	1:1.14	1:1
dCpA	7.21	dC:dpA	1:1.12	1:1
dTpTpA	7.5	T:pT:dpA	1:0.92:0.85	1:1:1
dApTpT	5.1	dA:pT	1:2.08	1:2
dApTpA	9.2	dA:pT:dpA	1:1.0:1.04	1:1:1
dApCpT	5.6	dA:dpC:dpA	1.1:1.2:1	1:1:1
dApCpA	8.2	dA:dpC:dpA	1:0.99:0.95	1:1:1
dTpTpC	7.4	T:pT:dpC	1:1.05:0.98	1:1:1
dCpApA	8.1	dC:dpA	1.17:2.0	1:2
dTpTpG	6.3	T:pT:dpG	1:0.97:0.99	1:1:1
dTpTpApApTpT	8.8	T:dpA:pT	1.1:2.0:3.2	1:2:3
dApCpApApTpA	11.2	dA:dpC:pT:dpA	0.86:1.10:1.0:3.0	1:1:1:3
dApTpTpTpTpC	9.2	dA:pT:dpC	1.1:4.01:1.0	1:4:1
dCpApApTpTpG	8.1	dC:dpA:pT:dpG	1.1:2.0:1.8:1.17	1:2:2:1
dApTpApCpApA	9.1	dA:pT:dpC:dpA	0.9:1.0:0.8:3.0	1:1:1:3
dApCpTpApCpA	8.5	dA:dpC:pT:dpA	1.0:1.95:0.91:2.05	1:2:1:2
dTpTpApApTpTpApCpApApTpA	12.3	T:dpC:pT:dpA	0.9:0.89:3.96:6.0	1:1:4:6
dApTpTpTpTpCpCpApApTpTpG	10.9	dA:pT:dpC:dpA:dpG	0.9:5.8:1.8:1.82:0.8	1:6:2:2:1
dApTpApCpApApApCpTpApCpA	11.4	dA:dpA:pT:dpC	0.9:5.95:1.89:2.86	1:6:2:3

on Sephadex columns, the appropriate fractions were pooled and evaporated under reduced pressure at temperatures below 20° in the presence of added pyridine. The syrupy residues obtained finally were rendered anhydrous by evaporation of added anhydrous pyridine. The resulting solutions were added dropwise to anhydrous ether (25–50-fold excess in volume). The nucleotidic materials separated as fine white precipitates.

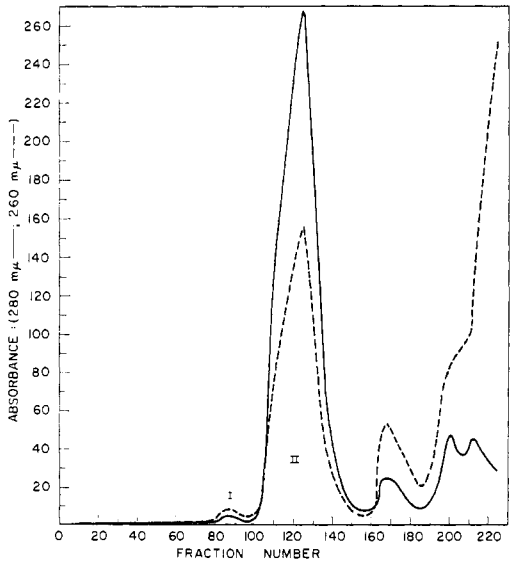


FIGURE 1: Chromatography of the reaction mixture on a Sephadex G-15, K50–100 column in the isolation of dpA^{Bz}. Fractions of 6 ml were collected every 6 min. Peak II contained the desired product.

These were collected by centrifugation and washed with fresh ether by resuspension in the centrifuge tube.

Characterization of Deoxyribopolynucleotides. The homogeneity of the isolated products was checked by paper chromatography under the following conditions.

PAPER CHROMATOGRAPHY. Paper chromatography was carried out by the descending technique using mostly Whatman No. 40 or No. 1 paper. The solvents used were solvent A, ethyl alcohol–1 M ammonium acetate (pH 7.5, 7:3, v/v); solvent B, isobutyric acid–concentrated ammonia–water (pH 3.7, 66:1:33, v/v, solvent C, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v); and solvent D, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); The R_F values are given in Table VI.

ENZYMATIC DEGRADATIONS. The structure and purity of all the compounds were checked by the analysis of the products formed on degradation with snake venom phosphodiesterase after the removal of the 5′-phosphomonoester end group. The results are given in Table VII. The homogeneity of the dodecanucleotides was further checked by the selective labeling of the 5′-OH terminal with polynucleotidic kinase followed by chromatography on DEAE-cellulose paper with 0.3 M triethylammonium bicarbonate as eluent. The radioactive product on degradation with snake venom phosphodiesterase was further analyzed on paper chromatography. All of the radioactivity was found in an area with mobility corresponding to the expected mononucleotide region.

Preparation of N-Protected Mononucleotides. dpA^{Bz}. Pyridinium deoxyadenosine 5′-phosphate (CalBiochem lot no. 50217) (2 mmoles) was benzoylated according to the earlier method (Ralph and Khorana, 1961; Khorana *et al.*, 1961). After the usual work-up, the aqueous pyridine solution was

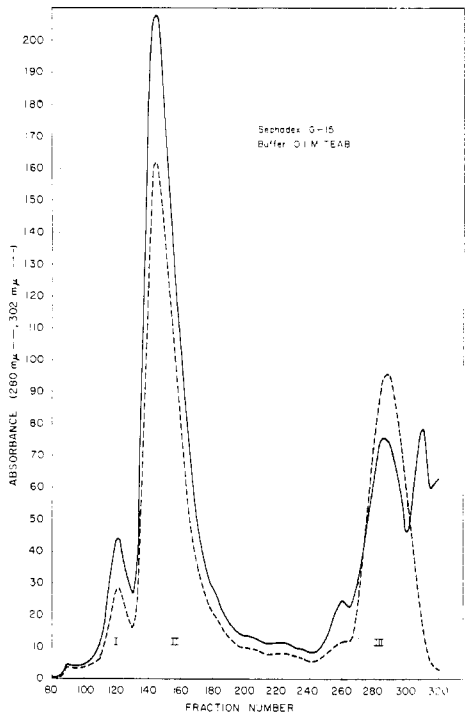


FIGURE 2: Chromatography of the reaction mixture (one-fourth portion) on a Sephadex G-15, K50–100 column in the preparation of $dpA^{Bz}pC^{An}$. Fractions of 4 ml were collected every 6 min. Peak II contained the desired product.

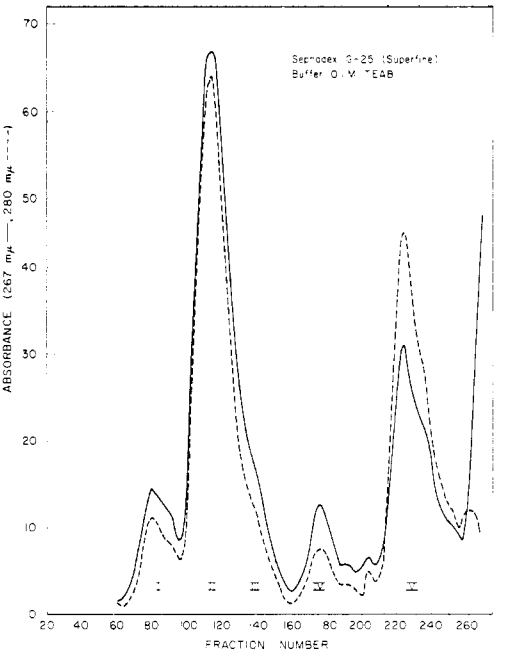


FIGURE 3: Chromatography of the reaction mixture (one-third portion) on a Sephadex G-25 (superfine), K50–100 column in the preparation of $dpTpTpA^{Bz}$. Fractions of 4 ml were collected every 6 min. Peak II contained the desired product.

concentrated *in vacuo* to 30 ml when most of the benzoic acid crystallized out. It was removed by filtration and the filtrate was chromatographed on Sephadex (G-15) column[3] K50–100 at 4°. Elution was carried out by using 0.1 M triethylammonium bicarbonate (pH 7.5) and the elution pattern is shown in Figure 1. Peak II (fraction 106–136) contained dpA^{Bz} (97%), dpA (0.6%), and $d(pA^{Bz})_2O$ (2.4%). The yield of the desired product was 31,000 OD_{280} units (83%).

The compound from peak I moved to R_F 1.26 (with respect to pT) in solvent A. It was next treated with concentrated ammonia for 48 hr then it moved to R_F 0.25 (with respect to pT) on rechromatography in the same solvent. The unprotected compound, after bacterial alkaline phosphatase treatment and on rechromatography moved to R_F 0.49 (with respect to pT) in solvent A. Finally 10.5 OD_{280} was degraded with snake venom phosphodiesterase. The products on separation by paper chromatography in solvent A were found to be deoxycytidine and deoxycytidine 5′-phosphate the relative concentration of 1:0.92 and the structure assigned was dCpC with one phosphate group either at 5′ or 3′ end. This impurity was also detected in the commercial sample of deoxyadenosine 5′-phosphate disodium salt. The yield of this impurity in peak I was 240 OD_{280} units (0.76%).

d-pC^{An}. Pyridinium deoxycytidine 5′-phosphate (1 mmole) was anisoylated according to the earlier method (Ralph and Khorana, 1961; Khorana *et al.*, 1961). After the usual workup, the aqueous pyridine solution was concentrated *in vacuo* to 20 ml when most of the anisic acid crystallized out. It was removed by filtration and the filtrate was chromatographed on Sephadex (G-15) column K50–100 at 4°. The desired product (19,500 OD_{302} units) was isolated in 87% yield.

[3] The column was found to break at the region of concentration of benzoic acid but patched up with time and the separation was not effected.

Results and Discussion

In the present synthetic work, the N-protected mononucleotides 5′-phosphates *i.e.*, dpA^{Bz} and dpC^{An}, were readily isolated from the reaction mixture by Sephadex G-15 gel filtration technique. The elution pattern of dpA^{Bz} is given in Figure 1. By this technique, an additional impurity of oligonucleotide (dCpC containing one terminal phosphate, either at 5′ or 3′ end) (0.78%) present in the commercial sample of dpA was also removed. The fractionation of the dinucleotide reaction mixtures was satisfactorily carried out on Sephadex G-15 column. As an illustration, the elution patterns for the preparation of $dpA^{Bz}pC^{An}$ is given in Figure 2. The purity of the isolated dinucleotide products was from 81 to 98%. Similarly, the isolation of trinucleotides was next carried out on Sephadex G-25 (superfine). Figure 3 of $pTpTpA^{Bz}$ fractionation represents a typical elution pattern for trinucleotide reaction mixtures. When isolated, the desired trinucleotide products were from 85 to 96% pure. In the case of hexanucleotides synthesis, it was considered essential to treat the crude reaction mixture with acetic anhydride–pyridine to degrade the pyrophosphate side products as far as possible (Narang *et al.*, 1969). Fractionation was then carried out on Sephadex G-75 (superfine) columns. Figure 4 of $dpA^{Bz}pC^{An}$-$pTpA^{Bz}pC^{An}pA^{Bz}$ represents a typical elution pattern for hexanucleotide reaction mixture. The various hexanucleotides were isolated in 25–33% yield when equimolar ratios of each trinucleotide component were used. The purity of the iso-

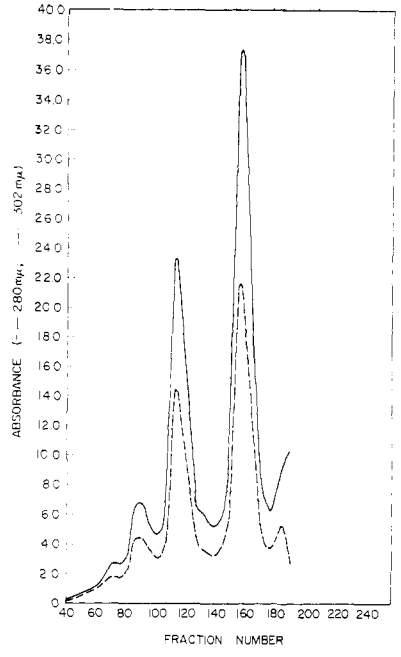


FIGURE 4: Chromatography of the reaction mixture (one-half portion) on a Sephadex G-75 (superfine), K25–100 column in the preparation of $dpA^{Bz}pC^{An}pTpA^{Bz}pC^{An}pA^{Bz}$. Fractions of 2 ml were collected every 15 min. Fractions 108–128 contained the desired product.

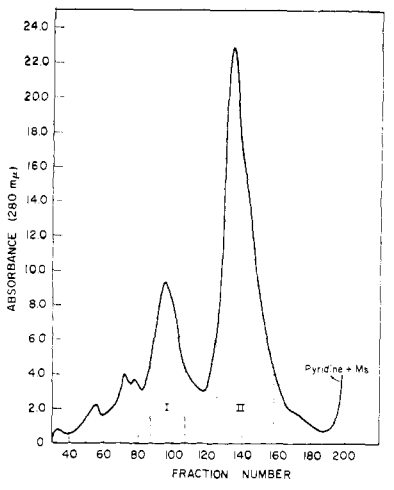


FIGURE 5: Chromatography of the reaction mixture (one-third portion) on a Sephadex G-75 (superfine), K25–100 column in the preparation of $dpTpTpA^{Bz}pA^{Bz}pTpTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpA^{Bz}$. Fractions of 2 ml were collected every 15 min. Peak I contained the desired product.

lated products was about 90%. Similarly, the reaction mixtures of the dodecanucleotide syntheses, after subsequent treatment with acetic anhydride, were fractionated on Sephadex G-75 (superfine) columns. As an illustration, the elution pattern of $pTpTpA^{Bz}pA^{Bz}pTpTpA^{Bz}pC^{An}pA^{Bz}pA^{Bz}pTpT$ is given in Figure 5. By using equimolar proportions of each hexanucleotide, the desired dodecanucleotides were isolated in 15–18% yield. The purity of the compounds ranged from 80 to 90% and they were, therefore, further purified by paper chromatography before further use in enzymatic work.

Invariably, three types of side products were formed under the reaction conditions used in the present synthesis but these were effectively removed by Sephadex gel chromatography. (1) Polymerized products appeared generally in the first two minor peaks (Figures 2–5). (2) Symmetrical pyrophosphates, due to their comparable size with the products, were eluted in the right-hand shoulder of the desired product peak. They were eliminated by acetic anhydride–pyridine cleavage. (3) Various types of degradation products generally appeared in the slower running peaks.

Characterization of all the compounds was accomplished by paper chromatography and the data are recorded in Table VI. These compounds were further characterized by degradation with snake venom phosphodiesterase after the enzymic removal of the phosphomonoester end group (Table VII). Finally, the homogeneity of the dodecanucleotides was further examined by the selective labeling of the 5′-OH terminal with polynucleotide kinase (Richardson, 1965), followed by chromatography on DEAE-cellulose paper with 0.3 M triethylammonium bicarbonate (pH 7.5) buffer as eluent.

In conclusion, it has been demonstrated in the present studies that Sephadex gel (superfine grade) chromatography can be used for the reasonable and rapid separation of deoxyribopolynucleotides of defined sequences provided: (a) the synthesis is designed in such a way that the products and reactants should differ considerably in their molecular size; (b) all the columns are packed with a uniform pressure head with a flow rate slightly higher than desired for actual chromatography (especially G-75, superfine); (c) the applied sample is concentrated to a minimal volume but still flows readily through the sample applicator. The following general features have been experienced with Sephadex column chromatography. (1) The elution pattern is remarkably reproducible; (2) chromatography is generally over within 2–3 days; (3) the desired product is eluted in approximately 20–30 tubes; (4) the same column can be used in a cycle for 4–6 months without repacking; and (5) no elution gradient is required.

Further work is in progress in the joining of these synthetic oligonucleotide chains in the presence of suitable complimentary strands by T_4-phage-induced *Escherichia coli* polynucleotide ligase and the results will be reported in the subsequent papers.

Acknowledgment

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Fate of Reticulocyte Ribosomes During *in Vivo* Maturation*

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ABSTRACT: Changes in the distribution of ribosomes in rabbit reticulocytes have been studied during maturation *in vivo*. ^{32}P was given intravenously to label the ribonucleic acid of these cells and the distribution of the isotope in ribosomes was followed with time. Both the percentage of ribosomes existing as polysomes and the aggregate size distribution of polysomes remained constant during the maturation of the reticulocytes. These findings are particularly noteworthy in view of the observed loss in the total content of ribosomes. The results of this study, employing an autologous system for the maturation of cells *in vivo*, are in agreement with the results of previous studies from this laboratory in which a heterologous system was employed. These investigations suggest that, once formed, each individual polysome aggregate continues to function unchanged until its ultimate destruction during the process of reticulocyte maturation. If 80S monomers are produced during the destruction of polysomes they in turn are short lived since they cannot be detected as an increased percentage of monosomes with increased cell age.

During the course of maturation of the mammalian reticulocyte to the circulating erythrocyte a number of biochemical and morphological changes occur among which are a loss of the capacity to synthesize protein and a loss of ribosomal material. Previous *in vitro* (Marks *et al.*, 1963b; Rifkind *et al.*, 1964) and *in vivo* (Rifkind *et al.*, 1964; Glowacki and Millette, 1965; Rowley, 1965; Danon *et al.*, 1965; Burka and DeBellis, 1967; Danon and Cividalli, 1968) studies have established that reticulocyte maturation involves a progressive loss of polysomes as well as a loss of total cellular ribosomal content. However, studies from different laboratories have suggested three patterns of reticulocyte maturation. The *in vitro* studies demonstrated a preferential loss of polysomes and an ordered shift in the size of the remaining polysome clusters toward single ribosomes (Marks *et al.*, 1963b; Rifkind *et al.*, 1964; Danon *et al.*, 1965). In the second group of studies, there was an increase in the per cent of monoribosomes but the size distribution of the remaining polysomes remained unaltered (Glowacki and Millette, 1965; Rowley, 1965). Finally, in the third study all size classes of ribosomes were lost at proportional rates (Burka and DeBellis, 1967).

The present investigation was designed to further study the fate of ribosomes during cell maturation using a technique

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