(b) Coenzyme A (Peak B, Fig. 3).—The ratio of phosphorus to adenosine was found to be 2.96 and the compound was chromatographically and electrophoretically identical with natural Coenzyme A. Degradation with crude rattlesnake venom gave adenosine-3′,5′-diphosphate as the only detectable nucleotide. Enzymatically assayed as above the compound gave a linear response of activity with concentration for 0.0041 and 0.0082 μmole (optical density) and gave an activity of 139% in each case assuming a purity of 75% (by weight) for commercial Coenzyme A. Using the purified commercial product (see below) as the standard and assuming 100% activity on the basis of its adenosine content, the synthetic sample had 96% activity.

Anal. Calcd. for $C_{21}H_{33}N_7O_{16}P_3SLi_3 \cdot 6H_2O$: C, 28.41; H, 5.08; N, 10.98. Found (after drying at 100°): C, 28.52; H, 4.98; N, 9.87.

Purification of Commercial Coenzyme A.—The contents of a freshly opened 25 mg. bottle of "75%" Coenzyme A[83] were dissolved in water (3 ml.) and adjusted to *p*H 6.0 with ammonium hydroxide. 2-Mercaptoethanol (3 ml.) was added and the mixture stored at room temperature for 4 hr. after which time it was diluted with water (15 ml.) and applied directly to a 2 × 22 cm. column of DEAE cellulose in the chloride form. After washing the column with water until no further ultraviolet absorbing material was present in the washing, elution was commenced using a linear salt gradient. The mixing vessel contained 1.5 liters of 0.003 *N* hydrochloric acid and the reservoir contained 1.5 liters of 0.15 *N* lithium chloride in 0.003 *N* hydrochloric acid. Ten ml. fractions were collected at the rate of 1 ml. per minute. Three distinct peaks and two small ones were detected by ultraviolet absorption at 257 mμ (Fig. 4). Peak I had λ_{max} 239 mμ and a second small maximum at 283 mμ (ϵ 239/ϵ 283 = 5.5 at *p*H 2.7) and was obviously not a nucleotide. Peak II contained at least two superimposed compounds one having λ_{max} 243 mμ, and the other λ_{max} 255 mμ. Peak III (273 optical density units at 257 mμ, 18 μmole) was reduced Coenzyme A. Peaks IV and V were too small for identification but, from its position, IV is probably oxidized Coenzyme A.

Peak III was adjusted to *p*H 4.0 with lithium hydroxide and worked up as described for the synthetic material to give 16 mg. of lithium salt which was chromatographically shown (Solvent I) to contain only reduced Coenzyme A and a little of the disulfide form. The material was somewhat hydrated, two preparations having equivalent weights of 960 and 1050 by ultraviolet absorption.

Assayed by the phosphotransacetylase method against the same "75%" standard as used for the synthetic material, it now showed 143% activity on the basis of its adenosine content.

Characterization of P¹,P′Bis-(−2′(or 3′)-phosphoryladenosine-5′) Pyrophosphate (XXVI).—Incubation of the sulfur-free product from peak IV (Fig. 3) with crude rattle snake venom rapidly gave adenosine-2′(3′),5′-diphosphate as the only phosphorus containing product. On incubation with purified prostatic phosphomonoesterase it was slowly (~ 75% in 24 hr.) dephosphorylated to give initially P¹-2′(3′)-phosphoryladenosine-5′ P²-adenosine-5′-pyrophosphate (XXVI, with loss of one phosphomonoester group) and subsequently di-adenosine-5′- pyrophosphate which were isolated in Solvents VI and I, respectively. The chromatographically isolated initial dephosphorylation product was rapidly degraded by crude venom, giving equal amounts of adenosine-2′(3′),5′-diphosphate, adenosine and inorganic phosphate. These results are all consistent with the structure assigned (XXVI) to this product.

(83) Pabst Laboratories, Milwaukee, Wis., Lot 413.

[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER, B. C.]

Studies on Polynucleotides. VIII.[1] Experiments on the Polymerization of Mononucleotides. Improved Preparation and Separation of Linear Thymidine Polynucleotides. Synthesis of Corresponding Members Terminated in Deoxycytidine Residues[2]

BY H. G. KHORANA[3] AND J. P. VIZSOLYI

RECEIVED JULY 18, 1960

Treatment of a molar anhydrous pyridine solution of a mixture of 3′-*O*-acetylthymidine-5′ phosphate (25%) and thymidine-5′ phosphate (75%) with dicyclohexylcarbodiimide at room temperature for six days gives linear thymidine polynucleotides as the major products. Members containing up to eleven units in a chain have been purified and characterized, smaller amounts of somewhat higher polynucleotides also being present in the polymerization mixtures. Procedures developed for the purification include chromatography of the total mixture on a DEAE-cellulose (carbonate) column using the volatile triethylammonium bicarbonate as the eluent and rechromatography of the major peaks under similar conditions. Polymerization of a mixture of N,3′-*O*-diacetyldeoxycytidine-5′ phosphate (25%) and thymidine-5′ phosphate (75%) gives products from which thymidine polynucleotides bearing deoxycytidine residues at one end were isolated pure and characterized. The procedures developed for their purification involved, first, chromatography on DEAE-cellulose (carbonate) columns followed by rechromatography of the major peaks at acidic *p*H using the anion exchanger in the chloride form.

Introduction

The development of methods for the polymerization of mononucleotides and the separation and characterization of the resulting polymers forms a part of the program of synthetic work in the polynucleotide field which is in progress in this Laboratory.[4-6] The range of simple polymers thus obtained offers obvious advantages for a variety of chemical, physico-chemical and enzymic studies in the nucleic acids field. The polymerizations of thymidine-5′ phosphate and the isomeric 3′-phosphate by reaction with dicyclohexylcarbodiimide in anhydrous pyridine have previously been reported.[7,8] While the extension of these initial studies to other mononucleotides and, indeed, in a number of directions is clearly desirable,[9] many

(1) Paper VII, H. G. Khorana, THIS JOURNAL, **81**, 4657 (1959).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service and the National Research Council of Canada, Ottawa.

(3) Institute for Enzyme Research, The University of Wisconsin, Madison 5, Wisconsin.

(4) G. M. Tener, P. T. Gilham, W. E. Razzell, A. F. Turner and H. G. Khorana, *Ann. N. Y. Acad. Sci.*, **81**, 757 (1959).

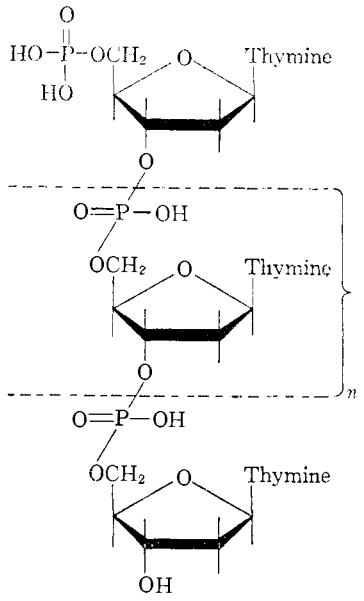
(5) H. G. Khorana, *J. Cellular Comp. Physiol.*, **54**, Suppl. 1, 5 (1959).

(6) H. G. Khorana, in E. Chargaff and J. N. Davidson, eds. "The Nucleic Acids," Vol. III, Academic Press, Inc., New York, N. Y., in press; H. G. Khorana, *Federation Proc.*, **50**, in press (1960).

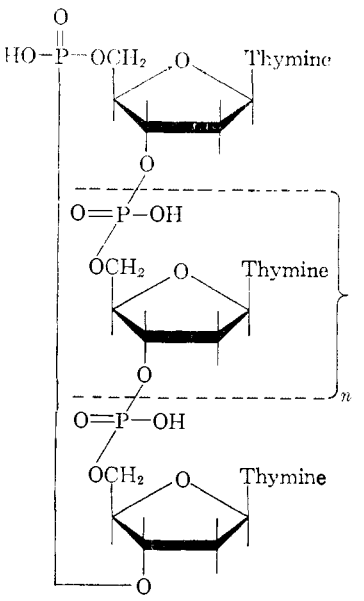
(7) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, THIS JOURNAL, **80**, 6223 (1958).

(8) A. F. Turner and H. G. Khorana, *ibid.*, **81**, 4651 (1959).

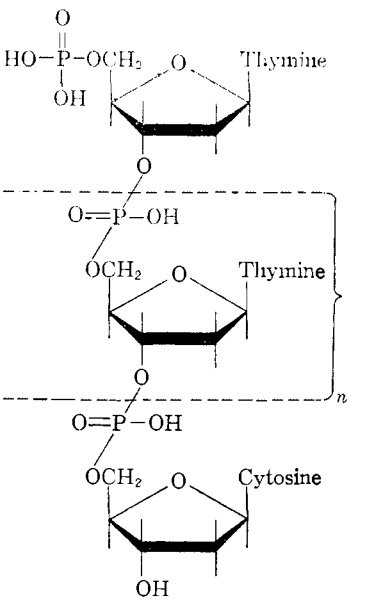
(9) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961).



I, $n = 0$–9



II, $n = 0$–3



III, $n = 0$–6

basic questions concerning chemical polymerization[10] are being examined by further studying the relatively simple thymidine-5′ phosphate itself. During all this work, marked improvements in the procedures, both for polymerization of this mononucleotide with a view to the preparation of *linear* polynucleotides (general structure, I) and for the separation of the polymers, have been effected. Because of the widespread interest in the thymidine polynucleotides of known size and structure, these procedures are described in this paper with special attention to experimental detail. The general principle used for favoring the formation of the linear polymers over the *cyclo*-oligonucleotides (general structure, II) has been applied to the preparation of thymidine polynucleotides bearing deoxycytidine residues at one end. The preparation and characterization of such compounds (general structure, III) are also described. The following paper[9] records the synthesis of polynucleotides by polymerization of suitably protected deoxycytidine-5′ phosphate.

In the previous work,[7,8] a competing reaction in the linear polymerization was found to be the intramolecular phosphorylation of the 3′-hydroxyl group at one end of the chain by the activated 5′-phosphoryl group at the other end, resulting in the formation of the macrocyclic compounds of the type II. In fact, the cyclic dinucleotide (II; $n = 0$) accounted for 18–20% of the total nucleotidic material, and although the proportion of the higher cyclic members decreased with increase in chain length, it became insignificant only beyond the pentanucleotide level. An increase in the nucleotide concentration would be expected to favor linear polymerization (involving bimolecular reactions) and the present experiments have all been carried out using a much more concentrated (1 molar) solution of the nucleotide than that used previously. The concentration (approximately 30% solution by weight of the nucleotide in pyridine) now used is as high as appears practical (see below). A technique which further reduces the extent of the cyclization reaction consists in the addition of some 3′-*O*-acetylthymidine - 5′ phosphate to thymidine - 5′ phosphate. The protected mononucleotide can only serve as the donor of an activated phosphoryl group and the chains formed with it as the terminal unit cannot undergo the intramolecular reaction. The addition of as much as 50% of 3′-*O*-acetylthymidine-5′ phosphate completely inhibited the cyclization reaction but then, as expected, a large amount of mononucleotide was present in the final products.[11] In the polymerization experiments reported, 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate were used in the ratio of 1:3. This ratio appears to represent a compromise, and although the amount of the cyclic dinucleotide formed is still high, the cyclic tri- and tetranucleotides are only minor products.

The above principle of polymerizing a nucleotide bearing the 3′-hydroxyl group in the presence of a second suitably protected mononucleotide should lead to an interesting general class of polymers, namely, 'homopolymers' terminated in a different nucleotide group. Compounds of this type are clearly useful for studies, such as the determination of the mode of action of phosphodiesterases[12] and nucleases.[5,13] In the present work, homologous series of thymidine polynucleotides bearing deoxycytidine groups at one end (III) have been prepared.

New procedures for the isolation of pure homologous polynucleotides have been developed. A major technical advance is the use of the DEAE-cellulose columns[7] in the bicarbonate form[14] and of

(10) These include studies of the kinetics of polymerization and a comparative study of the efficiency of different chemical polymerizing agents such as "reactive" anhydrides.

(11) The collaboration of Dr. G. M. Tener in the early experiments is gratefully acknowledged.

(12) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959).

(13) H. G. Khorana, R. A. Smith and R. K. Ralph, in preparation.

(14) M. Staehelin, H. A. Sober and E. A. Peterson, *Arch. Biochem. Biophys.*, **85**, 289 (1959). These authors use ammonium carbonate

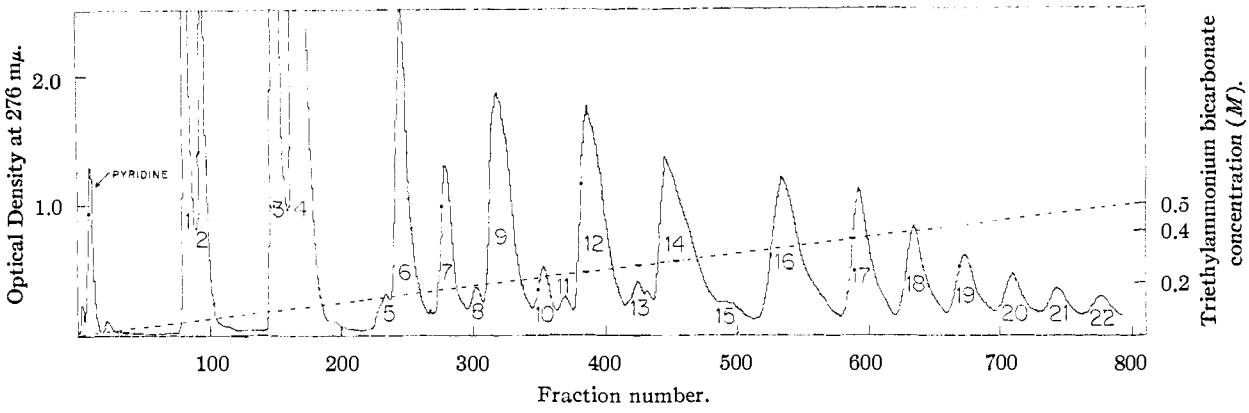


Fig. 1.—Chromatography of thymidine polynucleotides (total polymeric mixture) on DEAE-cellulose (bicarbonate) column. For details of procedure see text; for product distribution and identification, see Table I. Broken line shows triethylammonium bicarbonate gradient.

the volatile triethylammonium bicarbonate[15] as the eluent. Procedures for rechromatography of the initially obtained peaks under altered conditions (of salt gradient or *p*H) have been devised for most of the polynucleotides described here. These procedures enable the isolation of pure compounds on a scale much larger than is conveniently possible by chromatography on paper sheets described earlier.[7,8]

System of Abbreviations.—The basic system of abbreviations for polynucleotides used in this and the following paper[9] is as has been adopted by the *Journal of Biological Chemistry*.[16] These abbreviations have been used widely by different workers in recent years and are very convenient. Thus the trinucleotide III (n = 1) is abbreviated to d-pTpT-pC,[17] the letter "d-" designating deoxyribonucleoside series. In the present work dealing with rather large polymers derived from one kind of mononucleotide, it has been found necessary to develop the existing system of abbreviations further. Penta- and higher polynucleotides of the general structure I and III will be abbreviated to $pT(pT)_n$ pT and $pT(pT)_npC$ respectively. Thus the octanucleotide (III; n = 6) will be designated $pT(pT)_6$-pC. As in the general formulae for full structures (I and III) the basic unit for the present abbreviations is a trinucleotide.[17b] The two end units of a trinucleotide chain, being different from each other, have to be retained and it is only the internal nucleoside-5′ phosphoryl units which can be considered to repeat.

Thymidine Polynucleotides

The elution pattern obtained on initial chromatography of the polymeric mixture (corresponding to 1 mmole of the starting nucleotide) is shown in Fig. 1. The manner of pooling the fractions and the distribution of the nucleotidic material in the different peaks are shown in Table I. The recovery as

TABLE I

CHROMATOGRAPHY OF THYMIDINE POLYNUCLEOTIDES. DISTRIBUTION OF NUCLEOTIDE MATERIAL IN DIFFERENT PEAKS OF FIGURE 1

Peak	Fractions pooled	Total nucleotide material[a] in peaks,[b] %	Remarks, composition of the peak, etc
1	78–87	3.63	Mainly N-pyridinium nucleotide compound
1a	88–93	1.07	Discarded
2	94–102	2.53	Mainly thymidine 3′,5′-cyclic phosphate
3	146–156	6.50	Thymidine-5′ phosphate
3a	157–162	1.45	Discarded
4	163–180	10.19	Mainly cyclic dinucleotide
5	225–238	0.76	Several unidentified components
6	239–265	5.83	Linear dinucleotide
7	272–295	3.45	Cyclic trinucleotide
8	296–308	0.87	Several unidentified components
9	310–345	9.24	Linear trinucleotide
10	346–363	1.42	Cyclic tetranucleotide
11	364–377	0.70	Mixture of unidentified components
12	378–415	8.97	Linear tetranucleotide
13	416–438	1.58	Cyclic pentanucleotide and unidentified compounds
14	439–485	7.80	Linear pentanucleotide
15	486–517	1.36	Not investigated
16	518–570	6.70	Linear hexanucleotide
16a	571–580	0.29	Not investigated
17	581–620	5.20	Linear heptanucleotide
18	621–660	4.12	Linear octanucleotide
19	661–697	3.19	Linear nonanucleotide
20	698–730	2.57	Linear decanucleotide
21	731–763	2.07	Linear undecanucleotide
22	764–793	1.52	Linear dodecanucleotide
1 *M* triethylammonium bicarbonate		4.71	Higher polymers

[a] Total recovery of nucleotide material was 8,833 optical density units at 267 mμ. In view of the hypochromic effect in thymidine oligonucleotides,[19] the recovery is concluded to be practically quantitative. [b] Percentage of the nucleotide material eluted after the tetranucleotide was 41.1%.

for elution. Triethylamine bicarbonate[15] is even more volatile and is used routinely in this Laboratory.

(15) J. Porath, *Nature*, **175**, 478 (1955).

(16) See under "Instructions to Authors" in current issues of the *Journal of Biological Chemistry*.

(17) (a) According to the nomenclature previously proposed,[6,18] the trinucleotide would be named either 5-*O*-phosphorylthymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′) deoxycytidine or deoxycytidylyl-(5′ → 3′)-thymidylyl-(5′→3′) thymidylic-(5′) acid. (b) The significant shortening and convenience is effected only with the penta- and higher polynucleotides and therefore the abbreviations are introduced in this paper only from the pentanucleotide on.

(18) P. T. Gilham and H. G. Khorana, THIS JOURNAL, **80**, 6212 (1958).

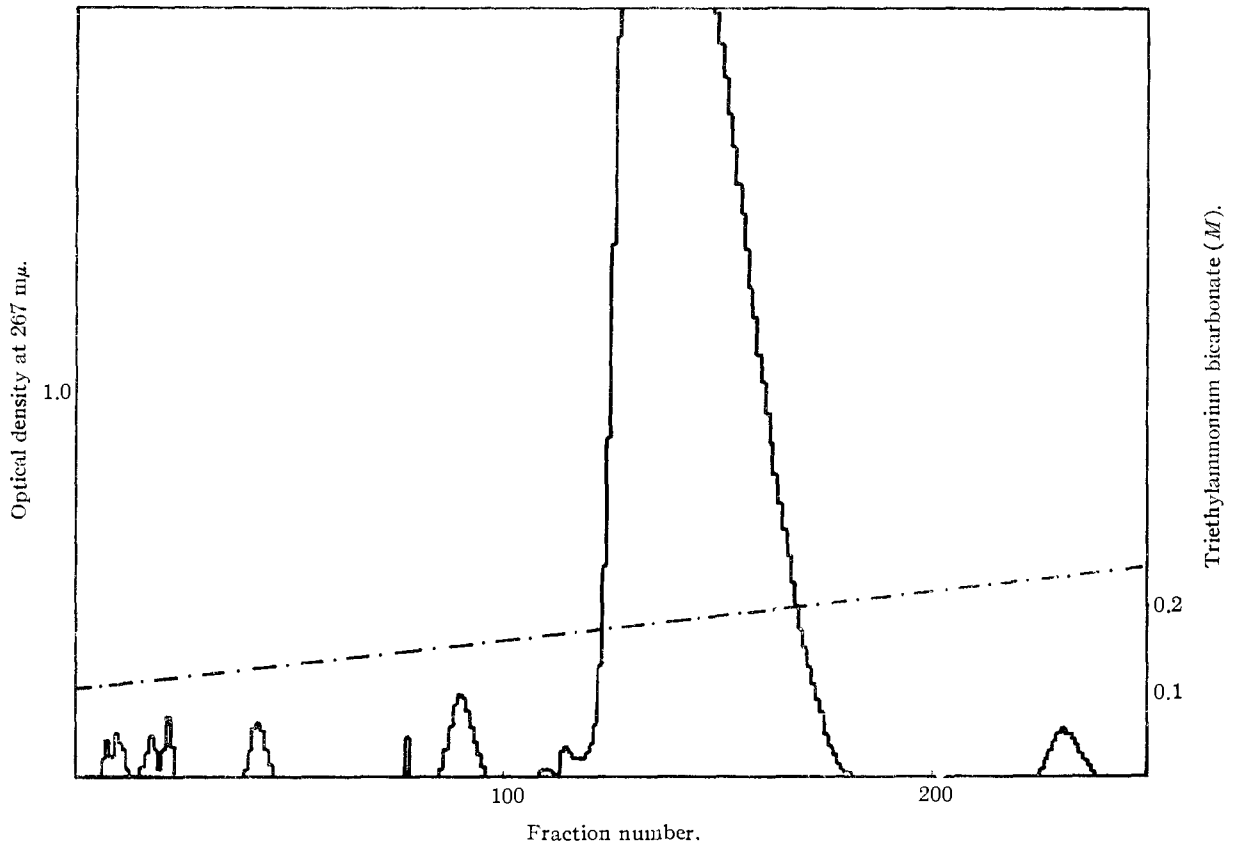


Fig. 2.—Rechromatography of the tetranucleotide peak (peak 12 of Fig. 1) on a DEAE-cellulose (bicarbonate) column (20 cm. × 2 cm. dia.). The column was pre-equilibrated with 0.1 *M* triethylammonium bicarbonate buffer. Elution carried out using a linear gradient (broken line) of the same salt; the major peak is pure tetranucleotide.

judged by the ultraviolet absorption measurements was essentially quantitative.[19] The resolution between the successive homologous polynucleotides was sustained as far as the elution was pursued by the gradient elution technique used. (In the previously published work,[7] elution of pure peaks on a preparative scale was described only as far as the pentanucleotide.) The extent of polymerization achieved in these runs has been markedly higher than was obtained before.[7] Thus 41% of the nucleotidic material appeared after the tetranucleotide (peak 12), about 5% of the total material being eluted after the dodecanucleotide peak (number 22) with 1 *M* triethylammonium bicarbonate. There was no sharp drop at any stage in the yield of the polymers, the amounts decreasing steadily with the increase in chain length after the linear tetranucleotide which accounted for 9.24% of the total nucleotidic material.

Linear Polynucleotides.—The linear polynucleotides were in peaks 6(dinucleotide), 9(trinucleotide), 12(tetranucleotide), 14(pentanucleotide), 16(hexanucleotide and 17-22(heptanucleotide to dodecanucleotide).[20] For further purification, conditions were found for rechromatography of each one of the polynucleotide peaks on DEAE-cellulose (carbonate form) columns. A shallower gradient was now used in each case and the various polynucleotides emerged from the columns at lower salt concentration than that at which they appeared in the initial gross chromatography. During rechromatography of the dinucleotide, it was found that satisfactory results were obtained by pre-equilibrating the column with the concentration of triethylammonium bicarbonate used initially in the mixing vessel and this practice was followed for rechromatography of all of the higher polynucleotides. The conditions used for rechromatography and the yields of pure polynucleotides obtained are shown in Table II. The elution patterns obtained on rechromatography are illustrated with respect to the tetranucleotide (peak 12) and the decanucleotide (peak 20) peaks in Figs. 2 and 3, respectively. It was important to confirm that the increasing number of minor peaks[21] obtained with increase in chain length were not due to any fault in the technique and therefore the main

(19) Some hypochromic effect in the synthetic polynucleotides is probable. Evidence for this at the dinucleotide level[18] and in the higher thymidine oligonucleotides (unpublished experiments of G. M. Tener in this Laboratory) has been obtained.

(20) The skew nature of some of these peaks (tail end) does not indicate heterogeneity, as was shown by rechromatography of the individual peaks and subsequent tests of purity. The elution patterns obtained on this scale have usually given more symmetrical peaks (see *e.g.* Fig. 4, below).

(21) As mentioned below, when the minor peaks (*e.g.*, 5, 8 and 11 of Fig. 1) were examined by paper chromatography, each was found to contain several distinct bands. Since these minor peaks, which just preceded the linear polynucleotide peaks, were not separated beyond the heptanucleotide, they were, presumably, included in the main peaks and this phenomenon accounts for the increased number of small fore-peaks obtained upon rechromatography of the higher polynucleotide peaks.

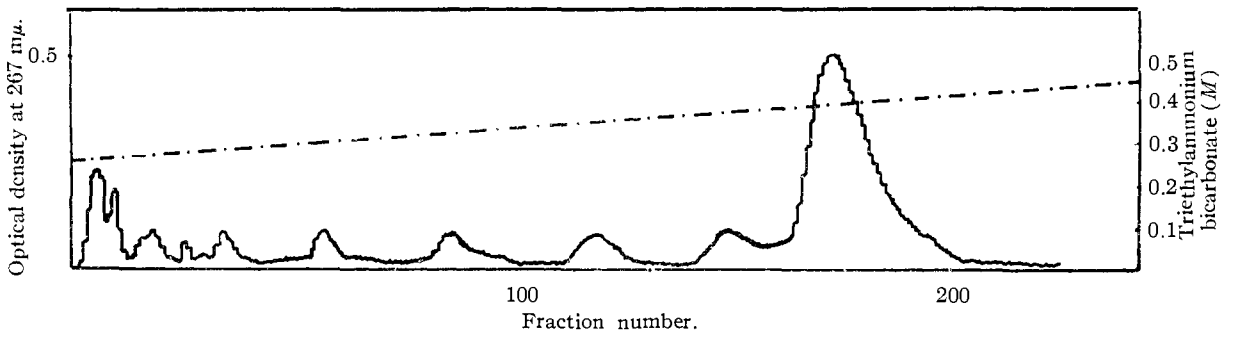


Fig. 3.—Rechromatography of the decanucleotide peak (peak 20 of Fig. 1) on a DEAE-cellulose (bicarbonate) column (20 cm. × 2 cm. diameter). The column was pre-equilibrated with 0.25 *M* triethylammonium bicarbonate buffer. Elution with linear gradient of the same salt as shown by broken line; the peaks in the first ten fractions contain non-nucleotidic material.

peak of pure decanucleotide obtained in Fig. 3 was rechromatographed. A single sharp peak was again obtained (Fig. 4) in the expected region of salt concentration.

taining about 1 μmole of thymidine. Single spots travelling faster than the starting materials were obtained.[23] A tracing of the chromatogram containing results with the octa-, nona- and deca-

TABLE II

RECHROMATOGRAPHY OF INDIVIDUAL PEAKS OF FIGURE 1 (THYMIDINE POLYNUCLEOTIDES) ON DEAE-CELLULOSE (CARBONATE) COLUMNS

(For details of procedure see text.)

Peak no. of Fig. 1	Poly-nucleotide	Conditions of rechromatography: Concentration of salt: Mixing vessel[a]		Conditions of rechromatography: Concentration of salt: Reservoir	Concentration of salt at mid-point of major peak	Yield pure oligonucleotide on rechromatography,[b] %
6	Di-		1 l. of 0.05 *M*	1 l. of 0.1 *M*	0.075	95
9	Tri-		1 l. of .075 *M*	1 l. of .15 *M*	.125	86
12	Tetra-	(1)	1 l. of .1 *M*	1 l. of .2 *M*	.180	84
		(2)	½ l. of .2 *M*	½ l. of .3 *M*		
14	Penta-	(1)	1 l. of .15 *M*	1 l. of .25 *M*	.230	87
		(2)	½ l. of .25 *M*	½ l. of .35 *M*		
16	Hexa-		2 l. of .2 *M*	2 l. of .4 *M*	.320	72[c]
17	Hepta-		2 l. of .25 *M*	2 l. of .45 *M*	.340	69
18	Octa-		2 l. of .25 *M*	2 l. of .45 *M*	.350	70
19	Nona-		2 l. of .25 *M*	2 l. of .45 *M*	.360	52
20	Deca-		2 l. of .25 *M*	2 l. of .45 *M*	.380	56
21	Undeca-		2 l. of .25 *M*	2 l. of .45 *M*	.410	72

[a] The column was pre-equilibrated with the concentration of triethylammonium bicarbonate (*p*H 7.5) used in the mixing vessel at the start of chromatography. [b] This is the % of the total ultraviolet absorbing material that was rechromatographed; the yields of each of the original peaks of Fig. 1 are given in Table I. [c] The actual recovery may be higher, since while investigating the appropriate conditions for rechromatography, the original peak had been put through columns twice. The yield recorded is that obtained in the final column and is based on the total optical density units in the original peak No. 16.

The purity of the oligonucleotides up to the pentanucleotide was checked by direct comparison with samples previously characterized,[7] using extensive paper chromatography and paper electrophoresis. For the characterization of the higher members and for ascertaining their purity, chromatography on paper strips was performed over a period of three days to two weeks in several solvent systems. The pattern of the mobilities (see Table III for R_f's) in all the solvent systems was consistent with their being a homologous series of compounds and single spots were uniformly obtained with all the polynucleotides. The most convincing proof of the homogeneity of the penta- to the deca-nucleotides was provided by dephosphorylation with the bacterial alkaline phosphomonoesterase[22] followed by chromatography on paper using heavy spots con-

(22) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, **38**, 470 (1960). We are very grateful for a sample of Dr. Garen's preparation of this very useful enzyme; the sample was kindly furnished to us by Dr. Leon A. Heppel.

nucleotides is shown in Fig. 5. Finally, the degradation of the products lacking the terminal phosphomonoester groups by venom phosphodiesterase to thymidine-5′ phosphate and thymidine, followed by estimation of the ratios of the two products gave results in good agreement with the size of the starting polynucleotides (Table IV).

Cyclic Oligonucleotides.—Peak 2 contained as the major constituent thymidine-3′,5′ cyclic phosphate,[7] while peak 4 consisted mostly of cyclic

(23) Great emphasis is placed on this criterion of purity of the linear polynucleotides, since the general impurities that would be suspected from all the practical experience with the present method (see also the following paper[9]) are of pyrophosphate type, the pyrophosphate bond being formed between the phosphomonoester groups of different oligonucleotides. These impurities could be eluted with or close to that polynucleotide bearing the 5′-phosphomonoester end group, which has the same net charge at *p*H 7.5. In the ammoniacal solvent systems, too, they may not be resolved from each other. Complete disappearance of the original spots (Fig. 5) on dephosphorylation constitutes the best means at the present time for showing freedom from the pyrophosphates.

TABLE III

R_f'S OF POLYNUCLEOTIDES ON PAPER CHROMATOGRAMS

(Solvent systems and technique as in text)

Solvent	A		B			C		D		E	
Compound[a]	R_f	R_f relative to pT[b]	R_f	R_f relative to pT	R_f[b] relative to pTpTpTpC	R_f	R_f relative to pT	R_f[b] relative to pTpTpTpC	R_f	R_f relative to pT	R_f relative to pTpTpTpC	R_f	R_f relative to pT
pT	0.177	1	0.4	1		0.3	1		0.625	1		0.43	1
d-pC		0.86		1.19			0.66			0.84			
d-pTpC		.47		0.89			.48			.69			
d-pTpTpC		.24		.56			.285			.52			
d-pTpTpTpC		.094		.354	1		.13	1		.32	1		
d-pT(pT)$_3$pC					0.71			0.60			0.79		
d-pT(pT)$_4$pC					.50			.33			.58		
d-pT(pT)$_5$pC					.37			.135			.42		
d-pT(pT)$_6$pC					.24			.05			.276		
d-pT(pT)$_7$pC					.16			.035			.197		

	A		B			C		D		E	
	R_f	R_f relative to pT[b]	R_f	R_f relative to pT	R_f relative to pTpT-pTpT[c]	R_f	R_f relative to pT	R_f	R_f relative to pT	R_f	R_f relative to pTpT-pTpT	R_f	R_f relative to pT
pTpT		0.54		0.68			0.73		0.84			0.37	
pTpTpT		.28		.49			.435		.63			.31	
pTpTpTpT		.125		.285	1		.23	1	.44		1	.27	
Thymidine 3′,5′ cyclic phosphate	0.47	2.65	0.52	1.3		0.58	1.93		0.67				
Cyclo-pTpT[c]	0.24	1.4	0.30	0.75		0.386	1.29		.53				
Cyclo-pTpTpT[c]		0.53		.51			0.79		.40				
Cyclo-pTpTpTpT[c]		0.24		.35			0.43		.31				
pT(pT)$_3$pT					0.695			0.57			0.72	0.22	
pT(pT)$_4$pT					.47			.29			.50	0.17	
pT(pT)$_5$pT					.325			.145			.36		0.34
pT(pT)$_6$pT					.25			.06			.25		.27
pT(pT)$_7$pT					.17			.029			.15		.20
pT(pT)$_8$pT													.14
pT(pT)$_9$pT													.09

[a] All compounds were spotted as ammonium salts; these were prepared by appropriate ion exchange techniques. [b] Chromatograms in these solvent systems, containing d-pTpTpTpC as the marker were run for 3 to 12 days. [c] Cyclo- before these oligonucleotides indicates that they are macrocyclic compounds of the general structure II (n = 0–2).

dinucleotide (II; n = 0). (Peak 3 was identified as thymidine-5′ phosphate.) Cyclic tri- (II; n = 1) and tetra- (II; n = 2) nucleotides were present

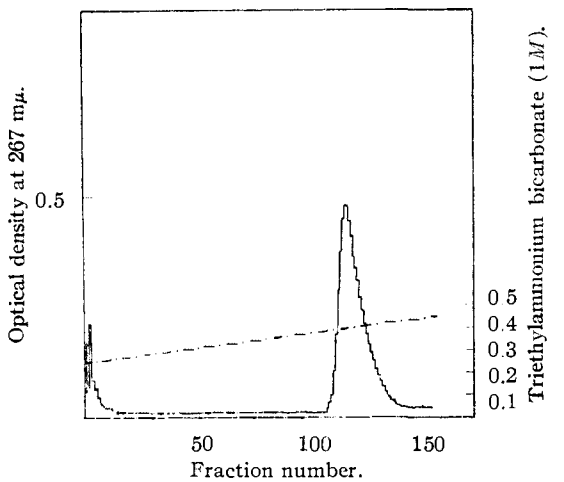


Fig. 4.—Rechromatography of the main decanucleotide peak of Fig. 3; conditions identical to those under Fig. 3. The peak in the first ten fractions contains non-nucleotidic material.

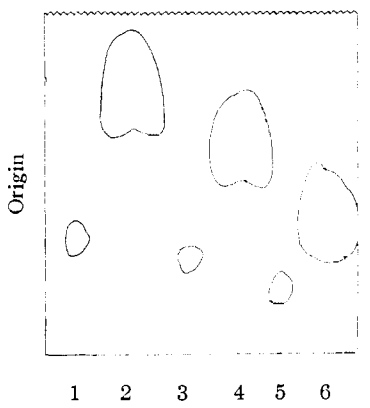


Fig. 5.—Paper chromatography of thymidine octa-, nona-, and decanucleotides before and after treatment with bacterial phosphomonoesterase. Solvent system, n-propyl alcohol–conc. ammonia–water (55-10-35), irrigation by the descending technique for 5½ days. Spots 1, 3 and 5. octa-, nona and decanucleotides before treatment with the phosphomonoesterase; spots 2, 4 and 6, products obtained after action of phosphomonoesterase from the octa-, nona- and decanucleotides, respectively. Each of the spots in the latter set contained a total of 0.7-1 μmole of thymidine.

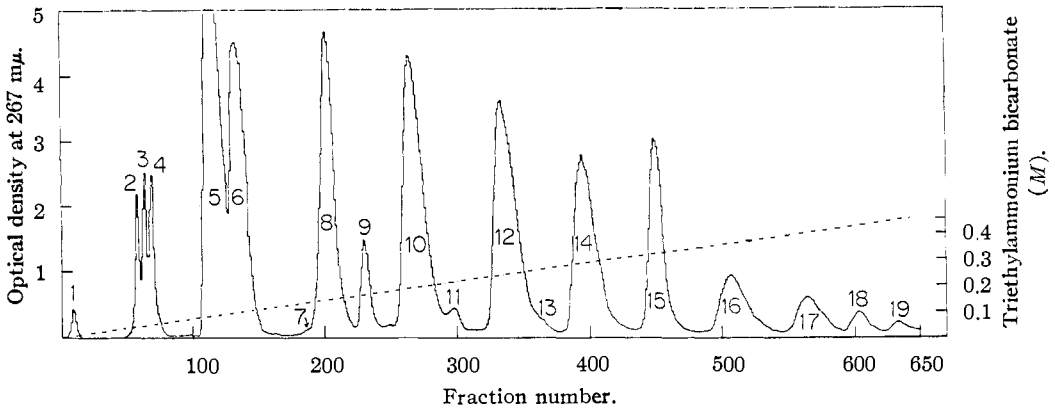


Fig. 6.—Chromatography of total products obtained on polymerization of a mixture of thymidine-5′ phosphate and N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate. For details of procedure, see text; for product distribution and identification of different peaks, see Table V.

respectively in peaks 7 and 10. All these compounds were purified by chromatography on paper sheets in isopropyl alcohol–ammonia–water solvent and they were characterized by the techniques described previously for this class of compounds.[7]

TABLE IV

RESULTS OF DEGRADATION OF THYMIDINE POLYNUCLEOTIDES LACKING TERMINAL PHOSPHOMONOESTER GROUPS BY VENOM PHOSPHODIESTERASE

(Details as in text)

Compound	Products of hydrolysis pT Optical density of spot/ml. at 267 mμ	T Optical density of spot/ml. at 267 mμ	Ratio pT/T Found	Theor.
T(pT)$_3$pT	2.750	0.651	4.22	4
T(pT)$_4$pT	2.605	.525	4.96	5
T(pT)$_5$pT	3.565	.612	5.84	6
T(pT)$_6$pT	3.650	.548	6.66	7
T(pT)$_7$pT	3.940	.471	8.36	8
T(pT)$_8$pT	2.710	.263	10.3	9

Other Minor and Unidentified Products.—Peak 1 contained mostly[24] material which travelled more slowly than thymidine-5′ phosphate on paper chromatograms in the isopropyl alcohol–ammonia–water solvent. Its absorption spectrum was additive of N-alkyl pyridinium and thymidine chromophores. Compounds with the same properties were encountered in previous work (peak A of Fig. 2 in ref. 7 and peak B of Fig. 1 in ref. 8). The structure indicated by the properties reported previously for the similar compound from the polymerization of thymidine-3′ phosphate[8] and now verified with that isolated from peak 1 of Fig. 1 is that it contains a phosphomonoester group and a pyridine residue quaternized at either the 3′ or 5′ carbon of the sugar ring in thymidine. The simpler derivation would be that in which the phosphomonoester group is placed on the 5′-position as in the starting nucleotide and the pyridinium group is formed at the 3′-carbon atom, the configuration being unassigned.[25] (Similar compound isolated in the previous work would have the isomeric structure in which the pyridinium group is formed at the 5′-position and the phosphomonoester group is present at the 3′-hydroxyl group.)

A large number of products present in the polymeric mixture in extremely minute amounts remain unidentified. These are not of any practical significance, since the major desired products can be freed from them as described above, but their formation is of importance in the mechanism of the polymerization reaction. Thus each of the peaks 5, 8 and 11, emerging just before the linear oligonucleotides, gave on prolonged chromatography in isopropyl alcohol–ammonia–water solvent, an average of five bands. The minor fore-peaks obtained on rechromatography of the higher linear polynucleotide peaks gave similarly a series of compounds. The individual bands referred to here represented very small fractions of 1% of the total polymeric mixture and were insufficient for a detailed examination. However, it seems very proable that they are pyrophosphates formed by the linking up of different linear oligonucleotides through their phosphomonoester groups.

Thymidine Polynucleotides Bearing Terminal Deoxycytidine Residues.—The separation of products obtained on polymerization of a mixture of N^6,3′-*O*-diacetyldeoxycytidine-5′ phosphate and thymidine-5′ phosphate is shown in Fig. 6. The fractions were pooled as shown in Table V, which also records the distribution of the nucleotidic material in the different peaks. The elution pattern is similar to that obtained above in Fig. 1, except that the polymerization did not go as far. The major peaks 8, 10, 12 and 14–19 contained the linear homologous polynucleotides and, as might have

(24) There is seen on chromatograms in the isopropyl alcohol–ammonia–water solvent an additional spot with mobility similar to that of thymidine. The ultraviolet absorption characteristics (λ_{max} at 295 mμ) show it to be non-nucleotide. The compound is encountered during acetylation of nucleotides and is apparently a product of reaction between acetic anhydride and pyridine.

(25) A possible mechanism for the formation of such compounds is the prior formation of an *iso*urea ether by an addition reaction between 3′-hydroxyl group and dicyclohexylcarbodiimide and the subsequent attack of pyridine at $C_{3'}$ with cleavage of the C–O bond. Such attack would be expected to occur from the back side and would result in inversion of configuration. In a model experiment *O*-methyl N,N-dicyclohexyl-*iso*urea ether [J. G. Moffatt and H.G. Khorana, THIS JOURNAL, 79, 3741 (1957)] was rapidly cleaved by pyridine at room temperature to form N-methylpyridinium cation. However, all attempts to carry out addition reactions between the hydroxyl groups of thymidine and dicyclohexylcarbodiimide have failed. We are grateful to Drs. G. M. Tener and A. F. Turner for the experiments quoted here.

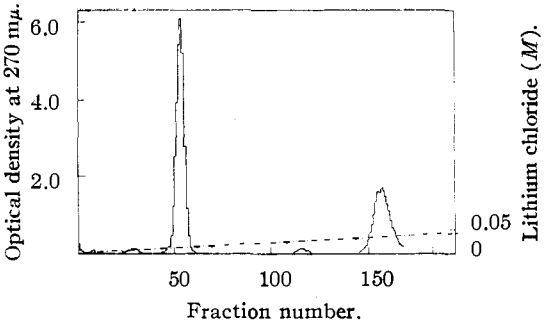


Fig. 7.—Chromatography of the mixture of dinucleotides (d-pTpC and pTpT) (peak 8 of Fig. 6) on DEAE-cellulose (chloride) column. Conditions as in text and Table VI. First peak, d-pTpC, second major peak pTpT.

been expected under these conditions of chromatography (*p*H 7.5), the two members of each size (*e.g.*, pTpT and d-pTpC, and similarly pTpTpT and d-pTpTpC, etc.) were eluted together. In earlier work on these polynucleotides, the initial chromatography of the total mixture was carried out under acidic (*p*H 2.7) conditions and there the separation of all of the expected small-sized compounds (d-pTpC, pTpT, d-pTpTpC, etc.) was satisfactory, but as the size increased, the compounds with approximately equal charge at the acidic *p*H (*e.g.* pTpTpT and d-pTpTpTpC) emerged together and further separation was carried out by paper chromatography. The procedures now preferred and described here take advantage of the use of the volatile eluent for the initial gross separation (Fig. 6) into the pairs of each size and subsequent chromatography under acidic conditions of each one of the major linear polynucleotide peaks. Uniformly successful results were thus obtained, two examples of separation, namely, at the di- and heptanucleotide level, are shown in Figs. 7 and 8. Two major peaks were obtained in every case, the first one being, as expected, that of deoxycytidine-containing polynucleotide. The amount of the ultraviolet absorbing material separating from the major components increased with the increase in size, but the materials recovered from the main peaks were all pure (see

TABLE V

DISTRIBUTION OF NUCLEOTIDE MATERIAL IN DIFFERENT PEAKS OF FIGURE 6

Peak	Fractions pooled	Total nucleotide material eluted from column, %	Remarks, composition of the peak
1	5–13	0.32	Pyridine
2	51–59	1.45	Similar to peaks 1 and 2 of Table I
3	60–65	1.89	
4	66–80	1.89	
5	104–125	13.4	pT + d-pC
6	126–156	12.8	Cyclic dinucleotide
7	180–189	0.26	Discarded
8	190–215	13.0	pTpT + d-pTpC
8a	216–223	0.34	Discarded
9	224–236	2.2	Cyclic trinucleotide
9a	237–253	0.76	Discarded
10	254–288	15.3	d-pTpTpC + pTpTpT
11	289–305	0.93	Cyclic tetranucleotide
11a	306–322	0.37	Discarded
12	323–353	9.8	pTpTpTpT + d-pTpTpTpC
13	354–383	1.08	Discarded
14	384–415	8.25	$pT(pT)_5pT$ + $d\text{-}pT(pT)_5pC$
14a	416–440	0.69	Discarded
15	441–462	5.05	$pT(pT)_4pT$ + $d\text{-}pT(pT)_4pC$
15a	463–493	0.71	Discarded
16	494–532	3.52	$pT(pT)_5pT$ + $d\text{-}pT(pT)_5pC$
16a	533–553	0.47	Discarded
17	554–592	2.14	$pT(pT)_6pT$ + $d\text{-}pT(pT)_6pC$
18	593–620	1.38	$pT(pT)_7pT$ + $d\text{-}pT(pT)_7pC$
19	621–650	0.94	Not investigated
20[a]		0.98	

[a] This is the total material eluted with 1 *M* triethylammonium bicarbonate, *p*H 7.5.

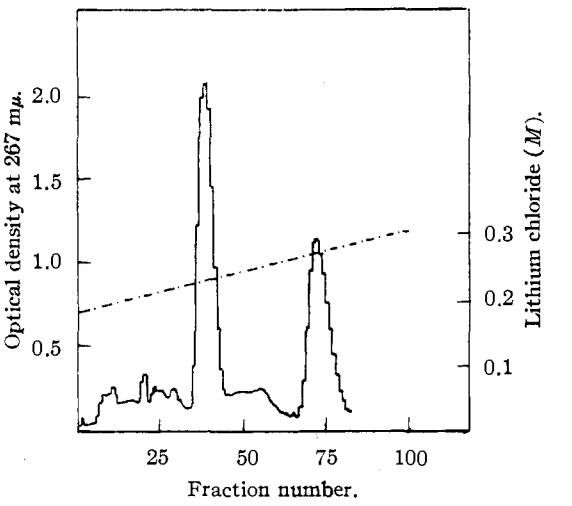


Fig. 8.—Chromatography of the mixture of heptanucleotides (peak 16 of Fig. 6) on DEAE-cellulose (chloride) column. Conditions as in text and Table VI. First major peak, $d\text{-}pT(pT)_5pC$; second major peak, $pT(pT)_5pT$.

TABLE VI

SEPARATION OF THYMIDINE POLYNUCLEOTIDES FROM THE CORRESPONDING POLYNUCLEOTIDES CONTAINING TERMINAL DEOXYCYTIDINE BY RECHROMATOGRAPHY

(Detailed procedure as in text)

Peak no. (Fig. 6, column 1)	Conditions for rechromatography: Concn. salt in mix. vessel 0.003 *N* HCl +	Conc. salt in reserv. 0.003 *N* HCl +	Salt conc. at oligonucl. elution d-(*M*, Cl.)	Polynucl. eluted	%[c]
8		0.05 *M* LiCl	0.015	d-pTpC	65
			.040	pTpT	35
10	0.05 *M* LiCl	.10 *M* LiCl	.025	d-pTpTpC	54
			.070	pTpTpT	46
12	.1 *M* LiCl	.25 *M* LiCl[a]	.120[a]	d-pTpTpTpC	58
			.140[a]	pTpTpTpT	42
14	.1 *M* LiCl	.2 *M* LiCl	.135	$d\text{-}pT(pT)_3pC$	59
			.165	$pT(pT)_3pT$	41
15	.15 *M* LiCl	.25 *M* LiCl	.200	$d\text{-}pT(pT)_4pC$	59
			.220	$pT(pT)_4pT$	41
16	.175 *M* LiCl	.3 *M* LiCl	.225	$d\text{-}pT(pT)_5pC$	59
			.270	$pT(pT)_5pT$	41
17[b]	.2 *M* LiCl	.3 *M* LiCl	.210	$d\text{-}pT(pT)_6pC$	59
			.220	$pT(pT)_6pT$	41
18[b]	.2 *M* LiCl	.3 *M* LiCl	.215	$d\text{-}pT(pT)_7pC$	63
			.230	$pT(pT)_7pT$	37

[a] A lower concentration of LiCl would have been adequate as found during rechromatography of the next higher oligonucleotide mixture. [b] For the relatively small amount of the materials corresponding to these peaks, rechromatography was performed on a smaller column (16 cm. × 1.0 cm. dia.). The flow rate was about 1 ml./min. [c] Percentage of cytosine-containing member and corresponding member containing thymidine only.

below). The detailed conditions used for rechromatography and the proportions of the pure components containing thymidine only to their counterparts containing terminal deoxycytidine recovered at each level are listed in Table VI. The average ratio of the deoxycytidine member to the purely thymidine-containing member was 3:2.

The homogeneity of the polynucleotides bearing terminal deoxycytidine was established by extensive paper chromatography (Table III). Furthermore, paper electrophoresis at acidic *p*H proved especially useful in this series. The mobilities of the homologous members are listed in Table VII.

TABLE VII

RELATIVE PAPER-ELECTROPHORETIC MOBILITIES OF OLIGONUCLEOTIDES AT *p*H 3.5[a]

(Details as in text)

Compound	Mobility relative to[b] pT	d-pTpC	d-pTpTpTpC
pTpT	1.29		
pTpTpT	1.47		
pTpTpTpT	1.58		
pT(pT)$_3$pT	1.64		
d-pTpC		1	
d-pTpTpC		1.44	
d-pTpTpTpC		1.63	1
d-pT(pT)$_3$pC			1.08
d-pT(pT)$_4$pC			1.15
d-pT(pT)$_5$pC			1.19

[a] Electrophoresis run using 3″-wide strips and a potential gradient of 15–16 volts/cm. Each run was performed for about 2 hr. in ammonium acetate (0.05 *M*) buffer. [b] Mobilities are quoted relative to the reference compound run along with the oligonucleotides on the same strip.

(With the members containing thymidine only, the technique as hitherto used in this Laboratory was applicable only as far as the tetranucleotide—all the higher members having about the same mobility.) Degradation of the homologous polynucleotides by venom phosphodiesterase and determination of the ratios of the resulting deoxycitidine-5′ phosphate and thymidine-5′ phosphate gave excellent agreement with the values expected (Table VIII).

TABLE VIII

RESULTS OF DEGRADATION OF POLYNUCLEOTIDES TERMINATED IN DEOXYCYTIDINE BY VENOM PHOSPHODIESTERASE

(Details as in text)

Compound	Products of hydrolysis: pT: Optical density, ml. at 267 mμ	pT: μmole[a]	pC: Optical density, ml. at 280 mμ	pC: μmole[b]	Ratio Found	Ratio pT/pC Theor.
d-pTpC	1.56	0.161	2.15	0.163	0.99	1
d-pTpTpC	4.10	.423	2.86	.2165	1.96	2
d-pTpTpTpC	4.30	.444	1.96	.148	3.00	3
d-pT(pT)$_3$pC	4.90	.505	1.64	.124	4.07	4
d-pT(pT)$_4$pC	3.28	.338	0.882	.067	5.04	5
d-pT(pT)$_5$pC	3.68	.379	0.840	.0636	5.96	6

[a] Using a figure of 9,700 for ϵ_{max} at 267 mμ for thymidine-5′ phosphate. [b] Using a figure of 13,200 for ϵ_{max} at 280 mμ in acid for deoxycytidine-5′ phosphate.

The linear thymidine polynucleotides isolated were all pure when characterized as described in the preceding section. Other features of the elution diagram (Fig. 6) were similar to those of the diagram in Fig. 1. Thus peaks 6, 9 and 11 mainly consisted of thymidine cyclic di-, tri-, and tetra-nucleotides, respectively. The earlier peaks, 2–4, had compositions similar to those described for Fig. 1 and peak 5 consisted of a mixture of deoxycytidine-5′ phosphate and thymidine-5′ phosphate.

General Remarks.—The method of polymerization as it stands is satisfactory for the preparation of linear polynucleotides containing up to about twelve units in a chain. However, further studies are required in order to induce the chemical polymerization to go much further. These studies will be concerned with a comparison of the efficiency of different polymerizing reagents. With dicyclohexylcarbodiimide, the mixtures are heterogeneous at the start of the polymerization reaction. With toluenesulfonyl chloride and other reactive anhydrides[7,26] clear solutions result, and this factor may make kinetic studies of the polymerization reaction simpler. However, detailed analysis of products obtained by using reagents other than dicyclohexylcarbodiimide is necessary before further studies with these reagents can be undertaken. In the previous work,[7] the results obtained using dicyclohexylcarbodiimide were cleaner than when *p*-toluenesulfonylchloride was used.

Much effort continues to be expended in this Laboratory on the techniques for the separation of the synthetic polynucleotides. The procedures as now evolved are satisfactory for the purification of all of the products encountered, and it is hoped that the information that is being gained with the relatively simple polymeric mixtures will be of use in the formidable problems of separation of polynucleotides of natural origin.

Experimental

Preparation of N^6,3′-*O*-Diacetyldeoxycytidine-5′ Phosphate and 3′-*O*-Acetylthymidine-5′ Phosphate.—Deoxycytidine-5′ phosphate (free acid) and thymidine-5′ phosphate used in the present work were commercial samples. They were checked carefully for their purity by (a) paper chromatography (solvents B and F, see below) on double-acid washed paper strips, using at least 2 μmole of material for each spot; (b) paper electrophoresis; and (c) spectral characteristics. Paper chromatography in solvent B detects deoxyuridine-5′ phosphate which may be present in thymidine-5′ phosphate, while solvent F is suitable for detecting any ribonucleoside-5′ phosphates.

Deoxycytidine-5′ phosphate (0.5 mmole, 165 mg. of free acid) was dissolved in a mixture of 10 ml. of water and 1 ml. of pyridine and the solution lyophilized. The finely divided material thus obtained was suspended in 5 ml. of dry pyridine and 1.5 ml. of acetic anhydride added. The stoppered flask was kept in the dark at room temperature and shaken frequently. Clear solution resulted within a few hours. After a total of about 18 hr., water (20 ml.) was added to the essentially colorless solution in ice bath. The solution was kept at room temperature for about 1.5 hr. and then concentrated to a syrup *in vacuo* at low temperature (bath temperature below 20°) using a rotary evaporator. Water was added to the syrupy concentrate and the solution re-evaporated as above. The procedure was repeated twice when most of pyridinium acetate was removed. Finally an aqueous solution (about 50 ml.) of the product was lyophilized to give a fine white powder which was stored at 3° as a solution in pyridine and used directly. Paper chromatography in solvents C, D and G as well as paper electrophoresis at *p*H 3.5 showed the absence of any unacetylated material. A weak fast-travelling spot was frequently seen on chromatograms. This was evidently non-nucleotidic in character (λ_{max}, 295 mμ) and, as noted above, appears to

(26) Unpublished work of H. G. Khorana and J. P. Vizsolyi.

result from the reaction of acetic anhydride with pyridine alone. Another very weak spot possessing N^6-acetyldeoxycytidine spectrum was also seen on chromatograms run in solvent D. This could be unhydrolyzed mixed anhydride between acetic acid and phosphate group of N,*O*-diacetyldeoxycytidine-5′ phosphate. No attempt was made to remove this minor by-product.

The procedure for the acetylation of thymidine-5′ phosphate was identical, except that the starting material, usually as ammonium salt, was first converted to the pyridinium salt by passage through a column of pyridinium Dowex-50 ion exchange resin and the total effluent was evaporated and the residue rendered anhydrous by repeated evaporation of its solution in pyridine. The reaction time usually given for this acetylation was around 7 hr. at room temperature in the dark.

Polymerization of a Mixture of 3′-*O*-Acetylthymidine-5′ Phosphate and Thymidine-5′ Phosphate.—A mixture of pyridinium 3′-*O*-acetylthymidine-5′ phosphate (as obtained by acetylating 1 mmole of thymidine-5′ phosphate) and pyridinium thymidine-5′ phosphate (3 mmole) was taken up in dry pyridine (10 ml.) and the solution evaporated to a gum *in vacuo* (oil pump) at low temperature. Dry air was admitted to the system, the residue redissolved in 10 ml. of dry pyridine and the solution re-evaporated as above. The whole procedure was repeated at least three times and the resulting anhydrous foam was taken up in 2 ml. of dry pyridine. To the clear solution was added under agitation from a pressure-equalizing flask a solution of dicyclohexylcarbodiimide (1.65 g.; 8 mmole) in dry pyridine (2 ml.). The stoppered reaction vessel was vigorously shaken for some five minutes, during which time the initially separated mobile liquid turned into a gum. The two-phase mixture was shaken mechanically at room temperature in the dark for a total of six days. The gum progressively hardened and at the end the total reaction mixture turned into a solid mass because of the crystallization of additional amount of dicyclohexylurea. To the reaction mixture was then added rapidly under shaking an aqueous solution of sodium hydroxide (9 ml. of water + 6 ml. of 2 *N* sodium hydroxide) and the sealed mixture was shaken thoroughly and the solid lump broken with a glass rod. The alkaline solution was shaken with ether (50 ml.) and the total mixture filtered from dicyclohexylurea. The clear aqueous layer was washed twice with ether and kept for a total of 1 hr. at room temperature to remove the acetyl group. Amberlite 1R-120 (H^+) resin was then added gradually until the *p*H dropped to neutrality and the solution was then filtered from resin and the latter washed thoroughly with water. The total aqueous solution was concentrated at low temperature and made up to a standard volume and stored at 3°.

Polymerization of a Mixture of N^6,3′-*O*-diacetyldeoxycytidine-5′ Phosphate and Thymidine-5′ Phosphate.—The polymerization was carried out exactly as described above, except that one-half the scale was used. After working up by the addition of appropriate amount of aqueous alkali to the polymerization mixture, and extraction with ether, the aqueous alkaline solution was passed through a column (6 cm. × 2 cm. dia.) of Amberlite-120 resin (ammonium form) and the total effluent and washings evaporated to dryness. The residue was dissolved in 10 ml. of conc. ammonia and the solution kept at room temperature for 2.5 hr. to ensure complete removal of the N-acetyl group. The solution was then evaporated and the residue made up to a standard volume in water and the solution stored at 3°.

Large-scale Separation of Polymers on DEAE-cellulose (Carbonate) Columns.—A portion of the solution (corresponding to 1 mmole of nucleotide) of the polymer mixtures obtained above was adjusted to *p*H 8–9 with ammonia and applied to the top of a DEAE-cellulose column (carbonate form) (30 cm. long × 4 cm. dia.) and carefully washed in with water (total volume of water wash, 300 ml.). Elution was begun using a linear gradient elution technique. In the case of polymers obtained from the mixture of 3′-*O*-acetylthymidine-5′ phosphate and thymidine-5′ phosphate, the mixing vessel contained initially 4 l. of water and the reservoir 4 l. of 0.25 *M* triethylammonium bicarbonate (*p*H 7.5). When this eluent had passed through the column, elution was continued by maintaining the linear gradient (4 l. of 0.25 *M* triethylammonium bicarbonate in the mixing vessel and 4 l. of 0.5 *M* salt in the reservoir). In the case of polymers from the mixture of N,*O*-diacetyldeoxycytidine-5′ phosphate and thymidine-5′ phosphate, the same column was used but the linear gradient was a little different: first, 4 l. of water in the mixing vessel and 4 l. of 0.3 *M* triethylammonium bicarbonate (*p*H 7.5) in the reservoir, and, then, 2 l. of 0.3 *M* triethylammonium bicarbonate in the mixing vessel and 2 l. of 0.45 *M* solution of the same salt in the reservoir. In both experiments, a flow rate of 2–2.3 ml./min. was maintained and approximately 20 ml. fractions were collected using an automatic fraction cutter. The elution pattern obtained from the polymers containing thymidine only is shown in Fig. 1, and the pooling of fractions and product composition is shown in Table I. The elution pattern obtained in the polymerization of a mixture of diacetyldeoxycytidine-5′ phosphate and thymidine-5′ phosphate is shown in Fig. 6 and the product distribution in Table V. The recovery of the nucleotide material in both experiments was essentially quantitative.

Each of the pooled peaks was evaporated *in vacuo* at low temperature and the residual syrup obtained, especially with higher oligonucleotides, was redissolved in water and the solution re-evaporated. The process was repeated several times to ensure complete removal of triethylammonium bicarbonate. Further processing of the peaks is described below. The concentrated solutions of those peaks that were to be purified by chromatography in ammoniacal solvents on paper strips were passed through small columns of ammonium Dowex-50 ion exchange resin to obtain ammonium salts of materials.

Further Purification of Thymidine Polynucleotides by Rechromatography on DEAE-cellulose (Carbonate Columns.—The peaks from Fig. 1, corresponding to linear thymidine polynucleotides, were rechromatographed on DEAE-cellulose columns (20 cm. × 2 cm. diameter, average size) (carbonate form). Satisfactory results were obtained by pre-equilibrating the column with the triethylammonium carbonate concentration used in the mixing vessel and by applying the polynucleotide as a concentrated solution in the same salt solution. The elution was carried out by the linear gradient method, using concentrations in the mixing vessel and reservoir as listed in Table II for each polynucleotide. On the average, 10–15 ml. fractions were collected, the flow rate being about 1 ml./min. The major peak was again recovered by evaporation of the combined eluate and repeated evaporation after addition of water. The triethylammonium salts of the polynucleotides were exchanged to ammonium salts by passage through small columns of ammonium Dowex-50 ion exchange resin.

The number of minor peaks obtained on rechromatography, in general, increased with the increasing size of the polynucleotide. The resolution was throughout satisfactory, as far as tried (undecanucleotide). The typical patterns obtained on rechromatography are illustrated with respect to the peak 12 (tetranucleotide) and peak 20 (decanucleotide) (Fig. 2 and 3 respectively). Rechromatography of the major peak of Fig. 3 gave a single sharp peak (Fig. 4).

Separation of Individual Polynucleotides Containing Terminal Deoxycytidine from Corresponding Polynucleotides Containing Thymidine Only.—Each of the major peaks from Fig. 6 corresponding to linear polynucleotides was rechromatographed on a DEAE-cellulose column (15 cm. × 2 cm. dia., average size) in the chloride form by using linear gradient elution technique. The volume used in the mixing vessel and in the reservoir was 1 l. each in all the experiments and the salt concentrations used are listed in Table VI. The flow rate was about 1.5 ml./min. and 15 ml. fractions were collected. Each of the original peaks gave two major peaks and often other minor peaks which were discarded. The first major peak was invariably the oligonucleotide containing terminal deoxycytidine, while the second major peak was the analogue containing thymidine only. The ratios of the optical density of the two major products obtained on rechromatography are also listed in Table VI. The typical elution patterns obtained are illustrated with respect to the dinucleotides (pTpC and pTpT) and heptanucleotides (pT$(pT)_5$pC and pT$(pT)_5$pT) in Fig. 7 and 8, respectively.

Recovery of Linear Polynucleotides.—The combined pure peak fractions obtained above from the chloride columns were neutralized with lithium hydroxide and then evaporated down to a completely solid white residue by first using a rotary evaporator and then sucking on an oil pump. The solid cake was dissolved in minimum of methyl alcohol

(2–4 ml.) and acetone (25–30 ml.) and then diethyl ether (5–10 ml.) added to precipitate the nucleotidic material. All the centrifuge tubes containing the precipitated materials were kept at 0° for some hours to ensure complete precipitation and the precipitates then centrifuged down. The precipitates were restirred by adding 1 ml. of methyl alcohol and then acetone (about 15 ml.) added. The precipitates were again collected by centrifugation. Finally they were washed with acetone and dried *in vacuo* at room temperature. The lithium salts of the polynucleotides thus obtained were dissolved in small amounts of water and converted to the ammonium salts by passing through columns (2 cm. × 1 cm. dia.) of ammonium Dowex-50 (200–400 mesh) ion exchange resin. The combined effluents and washings were concentrated to small volumes and the solutions stored frozen. Some loss was observed during recovery by the precipitation procedure with the lower thymidine oligonucleotides, but in general the recoveries, especially with the polynucleotides containing terminal deoxycytidine, were practically quantitative.

Purification of Cyclic Oligonucleotides.—Cyclic oligonucleotides (cyclic di- to penta-nucleotide) were purified for comparison with samples characterized earlier[7] by prolonged paper chromatography in solvent A. The samples were applied by first exchanging the triethylammonium cation present in the concentrated solutions of the peaks (*e.g.*, 4, 7, 10 and 13, of Fig. 1) for the ammonium ion by passing through ammonium Dowex-50 ion exchange resin columns.

Paper Chromatography.—Paper chromatography was carried out by the descending technique using double acid-washed paper (Whatman paper 40 or 44). The solvent systems used are: solvent A, isopropyl alcohol–concd. ammonia–water (7–1–2, v./v.); solvent B, isobutyric acid–1 *M* ammonia–disodium ethylenediamine tetraacetate (0.1 *M*) (100–60–1.6, v./v.); solvent C, ethyl alcohol–1 *M* ammonium acetate (*p*H 7.5), (5–2, v./v.); solvent D, ethyl alcohol–0.5 *M* ammonium acetate (*p*H, 3.8) (5–2, v./v.); solvent E, *n*-propyl alcohol–conc. ammonia–water (55–10–35, v./v.); solvent F, isopropyl alcohol–concd. ammonia–0.1 *M* boric acid (7–1–2, v./v.); solvent G, *n*-butyl alcohol–acetic acid–water (5–2–3, v./v.). The R_fs' of different polynucleotides in the solvents A–E, which are the more useful, are listed in Table III.

Paper electrophoresis was carried out in an apparatus similar to that of Markham and Smith.[27] The buffers used routinely were 0.05 *M* ammonium acetate (*p*H 3.2–3.5) and 0.05 *M* triethylammonium bicarbonate (*p*H 7.5.). Thick double-acid washed paper (Whatman #31) strips were used, the paper being soaked in the buffer and then blotted before application of the spots. The relative mobilities of different oligonucleotides are given in Table VII.

Enzyme Experiments. (a) Removal of Terminal Phosphomonoester Groups.—The prostatic phosphomonoesterase prepared by the method of Boman[28] was first used in early work, conditions of concentration, buffer and incubation as standardized with the dinucleotide pTpT being used.[7] More recently the alkaline phosphatase of *Escherichia coli*, as prepared by Garen and Levinthal, has been used. The preparation containing about 2 mg./ml. of protein was diluted fivefold with 0.05 *M* trihydroxylmethyl aminomethane buffer (*p*H 8) and the conditions used for complete removal of phosphomonoester groups from mono- to deca-nucleotides were: substrate containing approximately 0.1 μmole of phosphomonoester end group was contained in a final volume of 0.04–0.05 ml. of water. To it 0.002 ml. of 1 *M* tri-hydroxymethyl aminomethane buffer (*p*H 8) was added and the *p*H of the resulting solution checked to be around 8. 5 μl. of the above diluted enzyme was added and the mixture incubated at 37° for 4 hr. Dephosphorylation of the phosphomonoester groups was complete in all the polynucleotides, and no contamination of any phosphodiesterase activity in the enzyme preparation was detected. The results obtained are illustrated with thymidine octa- to deca-nucleotides in Fig. 5, the chromatograms traced having been developed in solvent E for $5^1/_2$ days.

(b) Degradation of Polynucleotides by Venom Phosphodiesterase.—Aliquots of solutions of the purified polynucleotides (homologous series of general structure III) containing a total of about 0.5 μmole of the nucleoside) were lyophilized in small reaction tubes. To the residue in each tube was added 0.01 ml. of 2 *M* ammonium carbonate, *p*H 9, buffer and 0.04 ml. of venom phosphodiesterase preparation. The enzyme preparation was combined peak tubes from DEAE-cellulose column chromatography after acetone fractionation, as described previously.[29] The incubations were carried out at 37° for 6–7 hr. in all cases. Under these conditions, degradation was complete in every case. (Previously the venom diesterase preparation had been standardized with respect to pTpT and TpT: the incubation time given in the degradation of polynucleotides and the amount of enzyme used were each double that necessary for the complete hydrolysis of the reference substrates.) The total incubation mixtures were then applied on paper chromatograms which were developed in solvent C. The spots and appropriate blanks were eluted by soaking in 0.1 *N* hydrochloric acid for at least 18 hr. The ratios of deoxycytidine-5′ phosphate to thymidine-5′ phosphate are listed in Table VIII.

Exactly the same procedure was used for degradation of thymidine polynucleotides of general structure $T(pT)_npT$ to thymidine-5′ phosphate and thymidine, the total incubation mixtures being applied on paper chromatograms which were developed in solvent A. The ratios of the nucleotide to the nucleoside are listed in Table IV.

N-Pyridinium Nucleotide (Peak 1, Fig. 1).—The major constituent of this peak showed the following characteristics. Its mobility on paper electrophoresis at *p*H 3.5 was nil. At *p*H 7.5 it had a net negative charge, electrophoretic mobility being 0.46 that of thymidine-5′ phosphate. On paper chromatograms in solvent A, its R_f was 0.55 relative to thymidine-5′ phosphate. It was dephosphorylated by prostate phosphomonoesterase and the resulting ultraviolet absorbing material had R_f in solvent A of 0.38 (R_f of thymidine run as marker, 0.65). The substance moved toward the cathode on paper electrophoresis, showing positive charge. The ultraviolet absorption spectrum of the substance showed a peak at 260 mμ with a shoulder (almost a second peak) at 267 mμ.

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