necessary to examine the effect of reaction medium on cleavage reactions, such as that of phenyl-*t*-butylcarbinol, which are believed to involve chromium(V).[12] Such studies are planned.

Experimental Section

Stoichiometry. To a solution of chromium trioxide (1.425 mmol) in 100 ml of 97% acetic acid which was 0.09 *M* in perchloric acid was added 1.0 g of isopropyl alcohol. After 1 hr, the solution was diluted with 700 ml of water containing 20 g of sodium acetate, and 40 ml of a 2,4-dinitrophenylhydrazine solution was added. After cooling in a refrigerator for 20 hr, the yellow precipitate was filtered, washed with distilled water, and dried in a vacuum oven at 80°. There was obtained 0.508 mg of acetone 2,4-dinitrophenylhydrazone, mp 124–124.5°, corresponding to 2.14 mmol of acetone. The ratio of acetone to chromium(VI) is 1.502, corresponding to a quantitative yield of acetone.

Kinetics. The kinetic measurements were made using a stopped-flow reactor as described in the previous paper.[2] The rate constants for the two steps in the reaction which could be observed at 510 mμ were calculated as described previously.[3]

Esr Measurements. A Varian E3 esr spectrometer was adjusted for maximum sensitivity. A flow cell in the spectrometer was connected to two 500-ml reservoirs having stopcocks for flow regulation and fitted with a connection for nitrogen pressure. Each reservoir was connected to the cell *via* a calibrated flowmeter. One reservoir contained 2.06×10^{-3} *M* chromium(VI) and 0.250 *M* perchloric acid in 97% acetic acid and the other contained 0.058 *M* isopropyl alcohol in the solvent. Equal flow rates were established for the two solutions, and the intensity of the esr signal was determined as a function of flow rate. Knowing the geometry of the mixer and flow cell, the flow rates could be converted to reaction times. The experiments were carried out at 25°.

The above reaction solutions also were studied spectrometrically at 510 mμ and 25°. The rate constants were caculated as before, and from this the concentration of chromium(V) could be calculated as a function of time.

(12) J. Hampton, A. Leo, and F. H. Westheimer, *J. Am. Chem. Soc.*, 78, 306 (1956); J. J. Cawley and F. H. Westheimer, *ibid.*, 85, 1771 (1963).

Acknowledgment. We wish to thank Professor J. Sturtevant for making his stopped-flow apparatus available for this study and Professor J. Wang for making his esr spectrometer available.

Polynucleotides. I. Use of Sephadex in the Preparation of Thymidine Homodeoxyribopolynucleotides Bearing a 5′-Phosphomonoester End Group

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Abstract: Thymidine tetracosadeoxyribonucleotide has been synthesized from the trinucleotide by the progression: trinucleotide → hexanucleotide → dodecanucleotide → tetracosanucleotide. At each step a cyanoethyl 5′-phosphate fragment bearing a free 3′-hydroxyl group was condensed in the presence of mesitylenesulfonyl chloride with a 3′-acetyl fragment having a free 5′-phosphate group. The desired products were isolated by column chromatography on Sephadex gels of the appropriate grade size and were characterized by paper chromatography with and without the 5′-phosphomonoester end group. The intermediate compounds were further characterized by degradation with snake venom phosphodiesterase treatment after the enzymic removal of the phosphomonoester group.

In recent years two major approaches have been developed for the chemical synthesis of short-chain deoxyribopolynucleotides, (1) stepwise condensation[2] and (2) polymerization.[3] In each case, the product obtained after each condensation was separated by time-consuming procedures involving mainly ion-exchange DEAE-cellulose chromatography. In order to overcome this difficulty, a polymer support for the speedy synthesis of polynucleotides has been investigated in different laboratories.[4] This method has so far shown limited success up to the hexanucleotide.[5]

In this paper we wish to introduce the use of Sephadex (superfine grade) gel filtration technique for the preparation of deoxyribopolynucleotides. 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

(1) National Research Council of Canada Postdoctorate Fellow, 1967–1968.

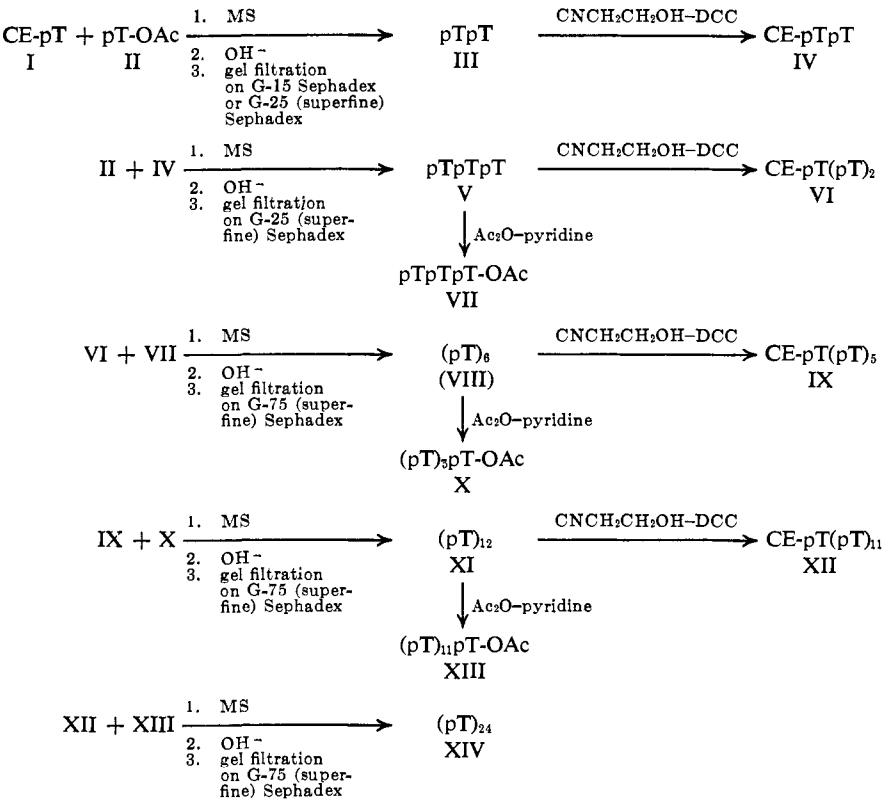
(2) (a) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2971 (1965); (b) S. A. Narang and H. G. Khorana, *ibid.*, 87, 2981 (1965); (c) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, 87, 2988 (1965); (d) H. Kössel, H. Büchi, and H. G. Khorana, *ibid.*, 89, 2185 (1967); (e) E. Ohtsuka and H. G. Khorana, *ibid.*, 89, 2195 (1967).

(3) (a) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, 87, 2956 (1965); (b) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, 89, 2167 (1967); (c) T. M. Jacob, S. A. Narang, and H. G. Khorana, *ibid.*, 89, 2177 (1967).

(4) (a) R. L. Letsinger and U. Mahadevan, *ibid.*, 87, 3526 (1965); (b) F. Cramer, R. Helbig, H. Hettler, K. H. Scheit, and H. Seliger, *Angew. Chem.*, 78, 640 (1966); (c) L. R. Melby and D. R. Strobach, *J. Am. Chem. Soc.*, 89, 450 (1967); (d) H. Hayastu and H. G. Khorana, *ibid.*, 89, 3880 (1967); (e) G. M. Blackburn, M. J. Brown, and M. R. Harris, *J. Chem. Soc., C*, 2438 (1967).

(5) F. Cramer and H. Köster, *Angew. Chem. Intern. Engl.*, 7, 473 (1968).

Chart I. Synthesis of Thymidine Deoxyribopolynucleotides Bearing 5′-Phosphomonoester End Group



can be separated rapidly and quantitatively by gel filtration, *e.g.*, Sephadex gels with appropriate exclusion limits. An attractive feature of this separation technique is that the product peak 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 or 3′-hydroxyl groups at each step. Preliminary report of this work has already appeared.[6] The general method used is shown in Chart I.

The free 5′-phosphomonoester end group of mono-, di-, tri-, and higher oligonucleotides were protected as β-cyanoethyl esters (CE) by the reaction with an excess of hydracrylonitrile in the presence of dicylcohexylcarbodiimide (DCC).[7] While the introduction of one cyanoethyl group at the 5′-phosphomonoester group was invariably quantitative, there was frequent evidence of further cyanoethylation. This presumably involved partial esterification of the phosphodiester groups to form neutral esters. While the neutral esters could be converted by the usual treatment at slightly alkaline pH to the monocyanoethylated products, the alkaline treatment was obviously not necessary and the products of the cyanoethylation reaction were used directly after isolation for the next step. The corresponding 3′-O-acetyl derivatives of the required compounds were prepared by treatment with distilled acetic anhydride in dry pyridine.[3b]

The reactants for the condensation steps were β-cyanoethylated esters (*e.g.*, CE-pT, CE-pTpT, CE-pT$(pT)_2$, CE-pT$(pT)_5$, or CE-pT$(pT)_{11}$) and the corresponding 3′-O-acetyl derivatives (*e.g.*, pT-OAc, $(pT)_5$pT-OAc, and $(pT)_{11}$pT-OAc).[8] Mesitylenesulfonyl chloride (MS) was used as a condensing agent. To increase the rate of reaction, the anhydrous pyridine solution was concentrated shortly after the addition of the condensing agent. After each condensation step the reaction mixture was usually given a prolonged treatment with aqueous pyridine–triethylamine, followed by alkali treatment to remove the acetyl and cyanoethyl groups. Sephadex gel (superfine) column chromatography was extensively studied for the isolation of the desired product at each stage of synthesis and the results are discussed below. Elution of the column with 0.01–0.05 *M* triethylammonium bicarbonate buffer, pH 7.5, was found to give a poor resolution of the components. The most satisfactory separation was achieved by using 0.1–0.2 *M* triethylammonium bicarbonate eluent but for practical purpose, 0.1 *M* triethylammonium bicarbonate was routinely used.

The dinucleotide $(pT)_2$ reaction mixture was fractionated on Sephadex G-15 and the elution pattern is given in Figure 1. The reaction mixture resolved into three distinct peaks in the order of decreasing molecular weight as examined further by paper chromatographic analysis. Peak I contained a polymerized nucleotide mixture formed during the condensation pro-

(6) S. A. Narang, S. K. Dheer, and J. J. Michniewicz, *J. Am. Chem. Soc.*, **90**, 2702 (1968).

(7) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **89**, 2158 (1967).

(8) The system of abbreviations has been defined in ref 7, footnote 4. Thus, pT-OAc is the abbreviation of 3′-O-acetylthymidine 5′-phosphate. CE is the abbreviation for β-cyanoethyl. MS stands for mesitylenesulfonyl chloride.

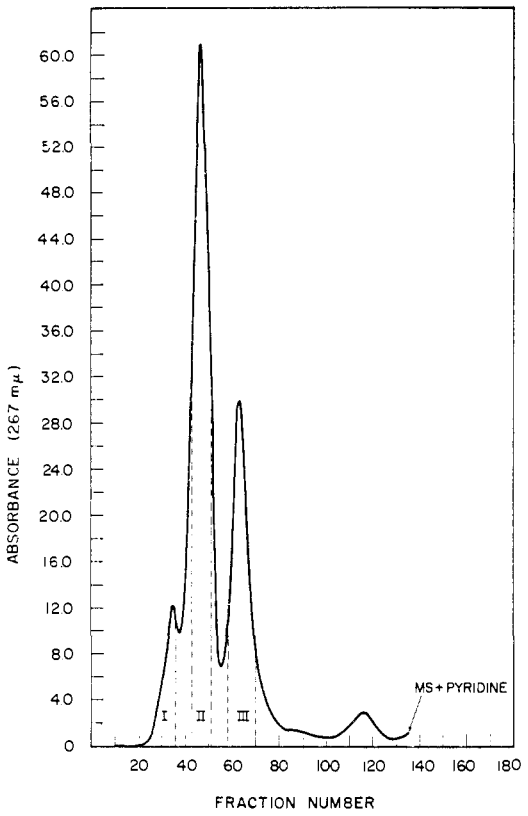


Figure 1. Chromatography of the reaction mixture (one-fourth portion) on a Sephadex G-15 column (150 × 2.5 cm) in the preparation of $(pT)_2$. Fractions of 2 ml were collected every 20 min.

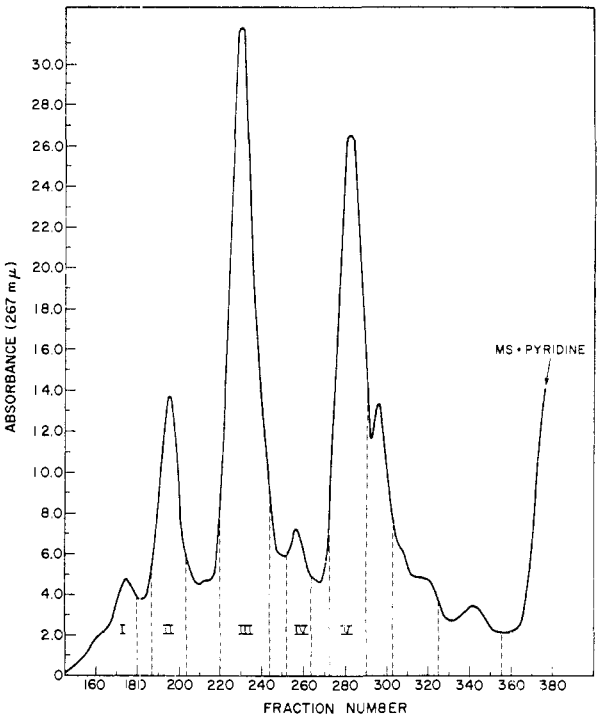


Figure 2. Chromatography of the reaction mixture (one-fifth portion) on a Sephadex G-25 (superfine) K50/100 column in the preparation of $(pT)_2$. Fractions of 4 ml were collected every 6 min.

cess, peak II $(pT)_2$ 100%, and peak III contained pT 90%. However, the left-hand shoulder of peak II was found to be contaminated with polymerized product and similarly the right-hand shoulder contained pyrophosphate of pT. Therefore these end fractions were discarded with 7% loss of the desired product $(pT)_2$. During a large-scale preparation of $(pT)_2$ from CE-pT (containing 20% free pT) and pT-OAc, Sephadex G-25 (superfine) gave better separation between polymerized products, $(pT)_3$ (formed due to the presence of free pT in CE-pT), $(pT)_2$, pyrophosphate of pT, and other degradation products (Figure 2, Table IV). This observation led us to use Sephadex G-25 (superfine) for the chromatography of $(pT)_3$ reaction mixtures and its elution pattern is given in Figure 3. Once again, the reaction mixture was resolved into six main peaks in order of decreasing molecular weight (Figure 3, Table V). The end fractions of the product peak III were discarded with 5% loss of $(pT)_3$. For the fractionation of a $(pT)_6$ reaction mixture, Sephadex G-25 (superfine) was found to give extremely poor resolution of the components but it was satisfactory on G-50 (superfine). The best separation was however achieved on G-75 (superfine) and the desired product was isolated from peak III (Figure 4) in 89% purity. In every case, the product peak was found to be contaminated with pyrophosphate of $(pT)_3$ (6–10%) along with other minor side products. It was therefore considered essential to treat the reaction mixture with acetic anhydride–pyridine[9] to degrade the

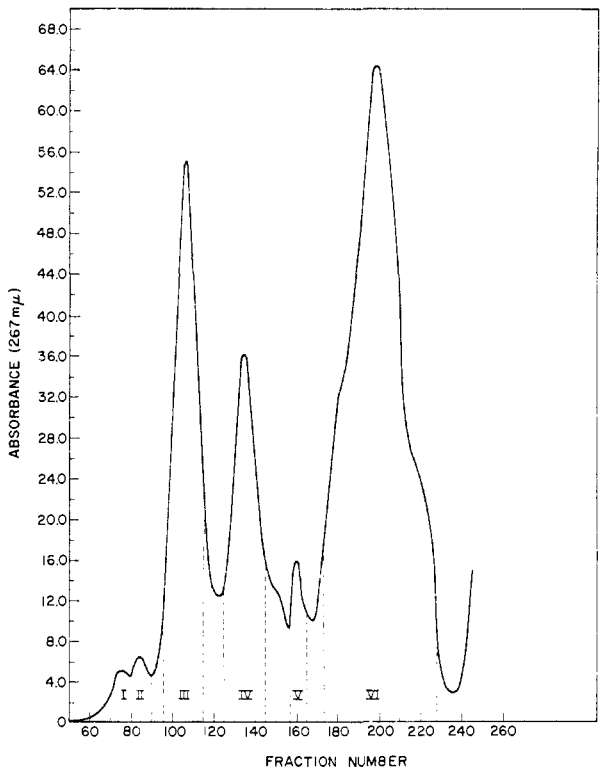


Figure 3. Chromatography of the reaction mixture (one-half portion) on a Sephadex G-25 (superfine) K50/100 column in the preparation of $(pT)_3$. Fractions of 4 ml were collected every 6 min.

pyrophosphate as follows. The crude condensation mixture was rendered anhydrous by its repeated evaporation with added pyridine and the salt of mesitylenesulfonic acid was removed by precipitation from a large excess of chloroform–ether (40:60, v/v). The pre-

(9) M. W. Moon and H. G. Khorana, *J. Am. Chem. Soc.*, 88, 1798 (1966).

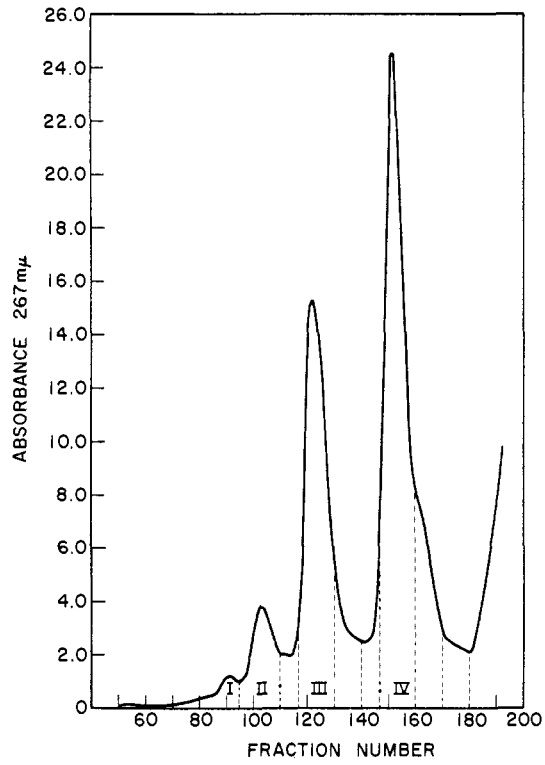


Figure 4. Chromatography of the reaction mixture (one-fourth portion) on a Sephadex G-75 (superfine) K25/100 column in the preparation of $(pT)_6$. Fractions of 2.5 ml were collected every 15 min.

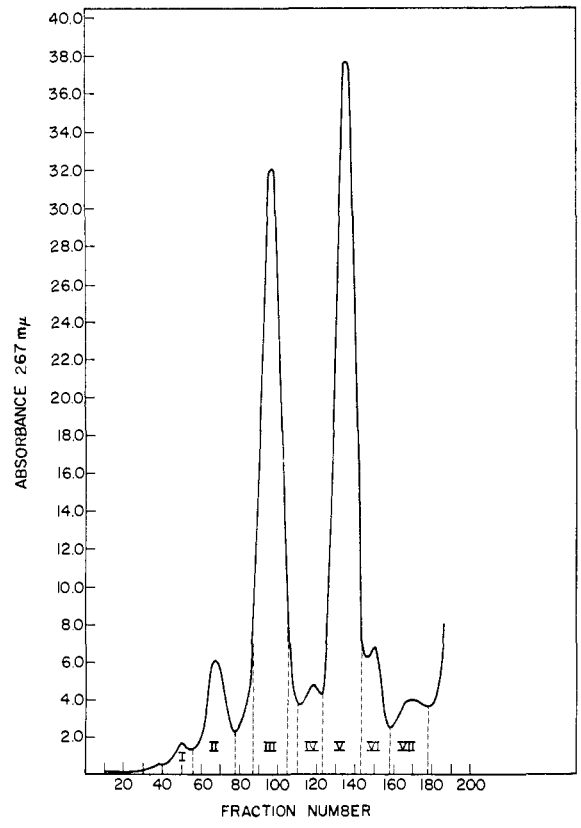


Figure 5. Chromatography of the reaction mixture (one-fifth portion) on a Sephadex G-75 (superfine) K25/100 column in the preparation of $(pT)_6$. Fractions of 1.5 ml were collected every 10 min.

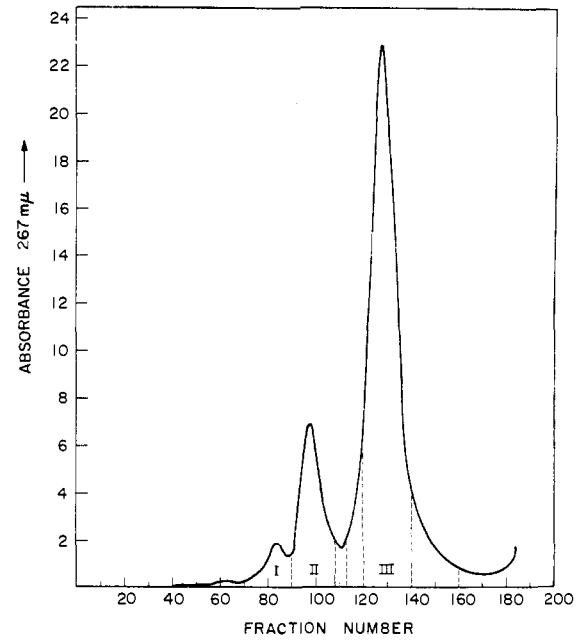


Figure 6. Chromatography of the reaction mixture (1 ml) on a Sephadex G-75 (superfine) K25/100 column in the preparation of $(pT)_{12}$. Fractions of 2.5 ml were collected every 15 min.

cipitate was next dissolved in dry pyridine and treated with a large excess of acetic anhydride–pyridine for 3 days. This reaction mixture was then decomposed with aqueous pyridine and the nucleotide material was isolated by the ether precipitation method. The protected groups were next removed by 2 *N* sodium hydroxide and the reaction mixture was fractionated on Sephadex G-75 (superfine) (Figure 5, Table VII). The isolated desired product $(pT)_6$ from peak III was found to be only contaminated with minor side products, $(pT)_4p$ (5%). Similarly, the dodecanucleotide $(pT)_{12}$ reaction mixture was given a prolonged acetic anhydride–pyridine treatment and then chromatographed on Sephadex G-75 (superfine) (Figure 6, Table VIII). The desired product $(pT)_{12}$ was isolated from peak II with 90% purity.

The elution pattern of the $(pT)_{24}$ reaction mixture on Sephadex G-75 (superfine) is shown in Figure 7. The compound from peak I moved to R_f 0.25 with respect to $(pT)_{12}$ as a single band in solvent C. It was next treated with bacterial alkaline phosphatase and rechromatographed in the same solvent. The homogeneity of $T(pT)_{23}$ was further examined by the selective labeling of the 5′-OH terminal with polynucleotide kinase[10] followed by chromatography on DEAE-cellulose paper with 0.5 *M* triethylammonium bicarbonate, pH 7.5, as eluent.

Invariably, three types of side products were formed under the reaction conditions used in the present syntheses but these were effectively removed by Sephadex gel chromatography: (1) polymerized products appeared generally in the first two minor peaks (Figures 1–7); (2) symmetrical pyrophosphates eluted in the right-hand shoulder of the desired product peak

(10) C. C. Richardson, *Proc. Natl. Acad. Sci. U. S.*, 54, 158 (1965).

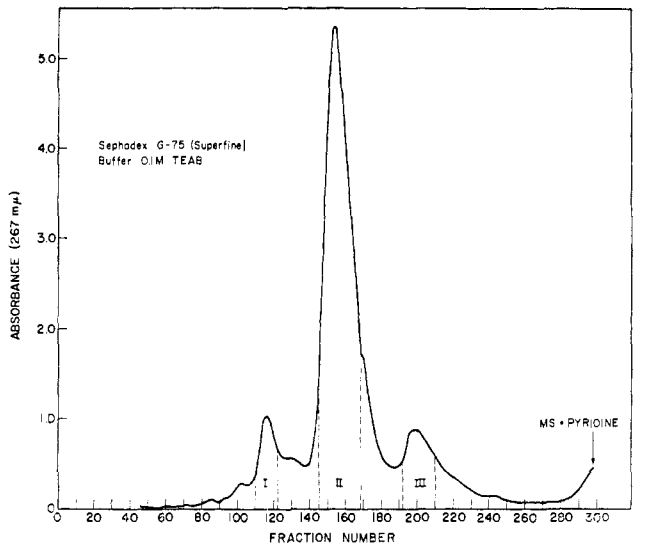


Figure 7. Chromatography of the reaction mixture on a Sephadex G-75 (superfine) K25/100 column in the preparation of $(pT)_{24}$. Fractions of 1.4 ml were collected every 15 min.

due to their comparable size with the products but were eliminated by acetic anhydride–pyridine cleavage; (3) various types of degradation products generally appeared in the latter peaks.

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 polynucleotides 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; (5) no elution gradient is required.

Characterization of all the compounds was accomplished by paper chromatography and the data are recorded in Table I. These compounds were further characterized by degradation with snake venom phosphodiesterase after the enzymic removal of the phosphomonoester end group (Table II).

Table I. Paper Chromatography and Thin Layer Chromatography of Thymidine-Containing Deoxyribopolynucleotides

	R_f[a]				
Compound	Solvent A	Solvent B	Solvent C	Solvent D	Sephadex thin layer
pT	1.0	1.0	1.0	1.0	1.0
$(pT)_2$	0.70	0.58	0.89	0.30	1.23[c]
$(pT)_3$	0.48	0.31	0.70		1.41[c]
$(pT)_6$	0.13	0.09	0.35		1.34[d]
$(pT)_{12}$			0.10		1.65[d]
$(pT)_{24}$			0.25[b]		
$(pT)_2pT$-OAc	0.66				
$(pT)_5pT$-OAc	0.16				
TpT	1.57	1.22	1.57	2.9	
$T(pT)_2$	1.20	0.76	1.33	1.6	
$T(pT)_5$	0.39	0.19	0.60	0.15	
$T(pT)_{11}$			0.20		
$T(pT)_{23}$			0.30[b]		

[a] R_f with respect to pT. [b] R_f with respect to $(pT)_{12}$. [c] On Sephadex G-25 (superfine). [d] On Sephadex G-75 (superfine).

Table II. Snake Venom Phosphodiesterase Degradation of Thymidine-Containing Deoxyribopolynucleotides

Compd	OD_{267}, units degraded	Nucleoside:nucleotide ratio— Found	Theor
TpT[a]	6.8	1:0.92	1:1
$T(pT)_2$[a]	6.7	1:2.18	1:2
$T(pT)_5$[a]	8.0	1:5.01	1:5
$T(pT)_{11}$[a]	12.5	1:10.85	1:11
$T(pT)_{23}$	7.6	Completely degraded	

[a] These results represent a typical analysis from three individual degradation experiments.

Experimental Section

General Methods and Materials. The following commercial products were used: thymidine 5′-phosphate (CalBiochem); mesitylenesulfonyl chloride (Aldrich); spleen phosphodiesterase, snake venom phosphodiesterase, and bacterial alkaline phosphatase (Worthington); polynucleotide kinase (gift from Professors Ray Wu, Charles C. Richardson, and Jerard Hurwitz). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell[11] as modified by Wu;[12] Sephadex G-15, G-25 (superfine), G-50 (superfine), and G-75 (superfine) (Pharmacia, Uppsala, Sweden); columns used for gel filtration types K25/100 and K50/100 (Pharmacia, Uppsala, Sweden).

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

In thymidine oligonucleotides, hypochromicity was neglected and for calculation of molar extinction coefficients, a value of 9600 was used for each thymidine residue of the molecule at 267 mμ at neutral pH. The abbreviation OD_{267} refers to the extinction of the nucleotidic solution at a neutral pH in 1 ml of the solution using a 1-cm light-path quartz cell, the number in subscript being the wavelength used.

Pyridinium pT-OAc[13] and CE-pT[3a] were prepared by the usual procedure and the protected derivatives were isolated by ether precipitation.

Cyanoethylation of the 5′-Phosphomonoester End Group in Oligonucleotides. The pyridinium salts of oligonucleotides $(pT)_2$, $(pT)_3$, $(pT)_6$, or $(pT)_{12}$ and hydracrylonitrile (50 equiv/equiv of oligonucleotide) were dissolved in dry pyridine (2–5 ml) and shaken at room temperature for 2–3 days with DCC (10 equiv/equiv of oligonucleotide). Water (three times the volume of pyridine) was then added and the resulting mixture was left at room temperature for 8–16 hr. It was next filtered and the filtrate was extracted with ether (three 20-ml portions). The aqueous layer was then evaporated *in vacuo* with frequent addition of pyridine to remove all traces of water. The final syrupy residue in anhydrous pyridine was added dropwise to anhydrous ether (25–50-fold excess in volume) with stirring to precipitate the β-cyanoethyl protected oligonucleotides. The precipitate was separated by centrifugation and washed with dry ether (four 20-ml portions). The isolated yields were from 70 to 90%.

Acetylation of the 3′-Hydroxyl End Group in Oligonucleotides. An anhydrous pyridine solution of tri-*n*-hexylammonium salts[7] of

(11) I. M. Glynn and J. B. Chappell, *Biochem. J.*, **90**, 147 (1964).
(12) Private communication from Professor Ray Wu.
(13) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).

oligonucleotide $(pT)_3$, $(pT)_6$, or $(pT)_{12}$ was treated with anhydrous acetic anhydride (~10 equiv/equiv of oligonucleotide component) under anhydrous conditions for 6 hr. An equal volume of water was then added with cooling and the resulting solution was kept for 4 hr at room temperature. The aqueous pyridine solution was then concentrated *in vacuo* with frequent addition of pyridine. The syrupy residue obtained finally was dissolved in dry pyridine and added dropwise to anhydrous ether (25–50-fold excess in volume) with stirring to precipitate the acetylated oligonucleotides. The precipitate was separated by centrifugation and washed with dry ether (four 20-ml portions). The yield of the desired product was quantitative.

Synthesis of Deoxyribopolynucleotides Bearing a 5′-Phosphomonoester End Group. Mesitylenesulfonyl chloride was added to an anhydrous pyridine solution of tri-*n*-hexylammonium salts[7] of β-cyanoethylated oligonucleotide and 3′-O-acetyl oligonucleotide. 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) triethylamine. 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 volume.

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 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. 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 $(pT)_2$, $(pT)_3$, $(pT)_6$, $(pT)_{12}$, and $(pT)_{24}$ was checked by paper chromatography and thin-layer gel filtration under the following conditions.

Paper Chromatography. Paper chromatography was carried out by 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); solvent D, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v). The R_f values are given in Table I.

Thin-Layer Gel Filtration. The swollen gel (superfine grade) was applied with a commercial spreading device to clean, dry 60 × 40 cm glass plates. The gel was equilibrated by flowing 0.1 *M* triethylammonium bicarbonate pH 7.5 buffer through the gel for 10–15 hr. The sample was then applied and developed by descending chromatography in a closed chamber for 8–10 hr. The flow of solvent from the reservoir to the top of the plate was *via* a filter paper wick (Whatman 3MM). The flow rate through the gel was regulated by the angle of the plate to the horizontal line. An angle between 10 and 20° has been found to be the most suitable. Blue Dextran 2000 (Pharmacia) was used as a marker to indicate flow rate through the plate. After completion of a run, the nucleotide material was spotted by ultraviolet light. The R_f values are given in Table I.

Enzymatic Degradations. The structure and purity of $(pT)_2$, $(pT)_3$, $(pT)_6$, and $(pT)_{12}$ intermediates were checked by the analysis of the products formed on degradation with snake venom phosphodiesterase after removal of the 5′-phosphomonoester group. The results are given in Table II.

Synthesis of Dinucleotide $(pT)_2$. a. An anhydrous solution of tri-*n*-hexylammonium salts of CE-pT (4000 OD_{267} units, 0.416 mmol) and pT-OAc (6000 OD_{267} units, 0.521 mmol) in dry pyridine (2 ml) was treated with mesitylenesulfonyl chloride (1.14 g., 5.2 mmol) for 3 hr under standard conditions. After the usual work-up the resulting solution was evaporated *in vacuo* to 8 ml volume. A portion of it (~2 ml) was chromatographed on Sephadex G-15 gel filtration column (150 × 2.5 cm) at 4°. The elution pattern is shown in Figure 1. Each peak and its intermediate fraction were analyzed by paper chromatography in solvents A and C and the distribution of the nucleotide material is given in Table III. The isolated yield was 62%.

Table III. The Analysis of Sephadex G-15 Chromatography of $(pT)_2$ Reaction Mixture (Figure 1)

Peak	Fraction	Total OD_{267} units	Identification (%)
I	20–35	100	Complexed polymerized products
	36–42	150	Polymerized products (50) and $(pT)_2$ (50)
II	43–51	1210	$(pT)_2$ (100)
	52–57	140	$(pT)_2$ (40), pyrophosphate of pT (30), and pT (15)
III	58–70	550	pT (90) and pyrophosphate of pT (5)

b. Large-Scale Preparation of Dinucleotide $(pT)_2$. An anhydrous solution of tri-*n*-hexylammonium salts of CE-pT[14] (1.4 mmol) and pT-OAc (1.6 mmol) in dry pyridine (5 ml) was treated with mesitylenesulfonyl chloride (2.18 g, 10 mmol) for 3 hr under standard conditions. After the usual work-up, the resulting solution was concentrated *in vacuo* to 30 ml volume. A portion of it (~6 ml) was chromatographed on Sephadex G-25 (superfine) column type K50/100 at 4°. The elution pattern is shown in Figure 2. Each peak and the intermediate pooled fractions were analyzed by paper chromatography in solvents A and C and the distribution of the nucleotide material is given in Table IV.

Table IV. The Analysis of Sephadex G-25 (Superfine) Chromatography of $(pT)_2$ Reaction Mixture (Figure 2)

Peak	Fraction	Total OD_{267} units	Identification (%)
I	145–180	139	Polymerized products
	181–187	88	Polymerized products (40) and $(pT)_3$ (60)
II	188–204	602	$(pT)_3$ (97), polymerized products (3)
	205–220	217	$(pT)_3$ (43), $(pT)_2$ (26), and unidentified component (R_f 0.91[a]) (31)
III	221–244	1836	$(pT)_2$ (100)
	245–252	171	$(pT)_2$ (89) and pyrophosphate of pT (11)
IV	253–264	261	Pyrophosphate of pT (70) and unidentified compound (R_f 0.77[a]) (30)
	265–272	154	Pyrophosphate of pT (30), pT (63), and unidentified compound (R_f 0.77[a]) (7)
V	273–290	960	pT (98) and cyclic pT (2)
	291–303	486	pT (94) and cyclic pT (6)
	304–325	281	Mainly pT
	326–355	179	pT (23) and unidentified compound (R_f 1.55[a]) (77)

[a] R_f of the compounds with respect to pT in solvent A.

Identification of the Product $(pT)_2$. The product from peak II of Figure 1 and peak III of Figure 2 was found to be homogeneous in solvents A, B, and C (R_f's given in Table I). A portion of this product was treated with bacterial alkaline phosphatase followed by chromatography in solvent A. A single faster moving band, R_f 1.57 (Table I), was isolated. The homogeneity was further examined by its rechromatography in solvents B and C. Finally, the resulting product TpT (6.8 OD_{267} units) was incubated with snake venom phosphodiesterase. The products which were separated

(14) In this particular preparation of β-cyanoethyl ester of pT, unprotected pT (20%) was found to be present which caused the formation of $(PT)_3$.

by paper chromatography in solvent A were found to be thymidine and thymidine 5′-phosphate. Their relative concentrations were 1:0.92 (theoretical 1:1) (Table II).

Synthesis of $(pT)_3$. An anhydrous solution of tri-*n*-hexylammonium salts of CE-pTpT (0.63 mmol) and pT-OAc (2.0 mmol) in dry pyridine (5 ml) was treated with mesitylenesulfonyl chloride (2.18 g, 10 mmol) for 3 hr under standard conditions. After the usual work-up, the resulting solution was evaporated *in vacuo* to 10 ml volume. One half-portion of it was chromatographed on a Sephadex G-25 (superfine) column type K50/100 at 4°. The elution pattern is shown in Figure 3. Each peak and the intermediate pooled fractions were analyzed by paper chromatography in solvents A and C. The distribution of the nucleotide material is given in Table V. The isolated yield of $(pT)_3$ was 32%.

Table V. The Analysis of Sephadex G-25 (Superfine) Chromatography of $(pT)_3$ Reaction Mixture (Figure 3)

Peak	Fraction	Total OD_{267} units	Identification (%)
I and II	60–90	498	Polymerized products
	91–96	183	Polymerized products (43) and $(pT)_3$ (57)
III	97–115	2970	$(pT)_3$ (97) and polymerized product (3)
	116–125	557	$(pT)_3$ (66), $(pT)_2$ (14), and unidentified component (R_f 0.91[a]) (20)
IV	126–145	1957	$(pT)_2$ (85), $(pT)_3$, (6), and unidentified component (R_f 1.0[a]) (9)
	146–157	672	$(pT)_2$ (65) and pyrophosphate of pT (35)
V	158–165	450	Pyrophosphate of pT (82) and $(pT)_2$ (18)
	166–173	401	Pyrophosphate of pT (44), $(pT)_2$ (4), and pT (52)
	174–228	8204	pT (89) and unidentified component (R_f 1.5[a]) (11)

[a] R_f of the compounds with respect to pT in solvent A.

Identification of the Product $(pT)_3$. The product from peak III of Figure 3 was chromatographed in solvents A, B, and C. In each solvent system, it resolved into one major band (97%) and two faint slow-moving components (R_f's given in Table I). The major band was eluted from the paper and treated with bacterial alkaline phosphatase followed by chromatography in solvent A. A single faster moving band, R_f 1.2 (Table I), was isolated. Its homogeneity was further checked by rechromatography in solvents B and C. Finally the product $T(pT)_2$ (6.7 OD_{267} units) was incubated with snake venom phosphodiesterase. The products which were separated by paper chromatography in solvent A were found to be thymidine and thymidine 5′-phosphate. Their relative concentrations were 1:2.18 (theoretical 1:2) (Table II).

Synthesis of Hexanucleotide $(pT)_6$. An anhydrous solution of tri-*n*-hexylammonium salts of CE-$pT(pT)_2$ (2000 OD_{267} units, 0.069 mmol) and $(pT)_2pT$-OAc (2000 OD_{267} units, 0.069 mmol) in dry pyridine (3 ml) was treated with mesitylenesulfonyl chloride (150 mg, 0.69 mmol) for 3 hr under standard conditions. After usual work-up the reaction mixture was concentrated to 4 ml volume. About 1-ml portions were chromatographed on Sephadex G-75 (superfine) K25/100 columns at 4°. The elution pattern is shown in Figure 4. Each peak and the intermediate pooled fractions were analyzed by paper chromatography in solvent C. The distributions of the nucleotide material are given in Table VI.

Synthesis of Hexanucleotide $(pT)_6$ Involving Acetic Anhydride Treatment. An anhydrous solution of tri-*n*-hexylammonium salts of CE-$pT(pT)_2$ (4300 OD_{267} units, 0.149 mmol) and $(pT)_2pT$-OAc (4500 OD_{267} units, 0.156 mmol) in dry pyridine (5 ml) was treated with mesitylenesulfonyl chloride (340 mg, 1.56 mmol). 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 (4 ml) was then added with cooling, followed by excess (0.2 ml) triethylamine. The solution was left at room temperature overnight. The aqueous pyridine solution was then concentrated *in vacuo* with the frequent addition of dry pyridine. The residue obtained finally was dissolved in dry pyridine (5 ml) and added dropwise to 100 ml of chloroform–ether (40:60 v/v) with stirring. The precipitate was separated by centrifugation and washed with chloroform–ether (40:60, v/v) (four 20-ml portions). The precipitate was next dissolved in dry pyridine (6 ml) and treated with redistilled acetic anhydride (2 ml) for 3 days under anhydrous conditions. An equal volume of water was added with cooling, and the resulting solution was kept at room temperature for 4 hr. The aqueous pyridine solution was again concentrated *in vacuo* with frequent additions of dry pyridine. The syrupy residue finally obtained was dissolved in dry pyridine (5 ml) and added dropwise to anhydrous ether (100 ml) with stirring. The precipitate was separated by centrifugation and washed with ether (four 20-ml portions). The precipitate was next dissolved in aqueous pyridine (5 ml) and treated with 2 *N* sodium hydroxide (5 ml) for 10 min at room temperature. An excess of pyridinium Dowex-50 ion-exchange resin was 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 5 ml volume. About 1 ml was chromatographed on Sephadex G-75 (superfine) K25/100 at 4°. The elution pattern is shown in Figure 5. Each peak and intermediate pooled fractions were analyzed by paper chromatography in solvent C. The distribution of the nucleotide material is given in Table VII. The isolated yield of $(pT)_6$ was 33%.

Table VI. The Analysis of Sephadex G-75 (Superfine) Chromatography of $(pT)_6$ Reaction Mixture (Figure 4)

Peak	Fraction	Total OD_{267} units	Identification (%)
I	80–95	25	Polymerized products
II	96–110	79	Polymerized products
	111–117	39	$(pT)_6$ (40) and polymerized products (60)
III	118–130	295	$(pT)_6$ (89), $(pT)_4p$ (4), and pyrophosphate of $(pT)_3$ (6)
	131–140	68	$(pT)_6$ (46), pyrophosphate of $(pT)_3$ (34), and unidentified component (R_f 0.58[a]) (20)
	141–147	57	$(pT)_3$ (45) and unidentified component (R_f 0.60[a]) (55)
IV	148–160	365	$(pT)_3$ (95)
	161–170	109	$(pT)_3$ (50) and three side products R_f 0.50[a] (7), R_f 0.96[a] (30), and R_f 1.48[a] (13)
	171–180	48	Side products R_f 0.96[a] (30), R_f 1.06[a] (23), and R_f 1.47[a] (47)

[a] R_f of the compound with respect to pT in solvent C.

Table VII. The Analysis of Sephadex G-75 (Superfine) Chromatography of $(pT)_6$ Reaction Mixture (Figure 5)

Peak	Fraction	Total OD_{267} units	Identification (%)
I	30–56	27	Polymerized products
II	57–78	116	Polymerized products
	79–87	52	$(pT)_6$ (51) and polymerized product (49)
III	88–105	534[a]	$(pT)_6$ (95), $(pT)_4p$ (4), and unidentified component (1)
	106–109	29	$(pT)_6$ (62) and unidentified component (R_f 0.58[b]) (37)
IV	110–123	77	$(pT)_3$ (71) and unidentified component (R_f 0.46[b]) (29)
V	124–143	692	$(pT)_3$ (93) and unidentified component (R_f 0.55[b]) (7)
VI	144–158	109	$(pT)_3$ (50) and two side products R_f 0.65[b] (20), R_f 1.40[b] (30)
VII	159–178	97	Six components: R_f 0.59[b] (8), R_f 0.79[b] (14), R_f 1.0[b] (25), R_f 1.2[b] (18), R_i 1.44[b] (21), and R_f 1.9[b] (14)

[a] See the Experimental Section. [b] R_f of the component with respect to pT.

Identification of the Product $(pT)_6$. The product from peak III of Figure 5 was chromatographed in solvents A and C. In each solvent system it moved as a single band (R_f's given in Table I). It was next treated with bacterial alkaline phosphatase followed by chromatography in solvent A. It resolved into one major band (95%)

corresponding to $T(pT)_5$, R_f 0.39, and a faster moving band (5%) (R_f 0.88). The compound $T(pT)_5$ (8.0 OD_{267} units) was incubated with snake venom phosphodiesterase. The products separated by paper chromatography in solvent A were found to be thymidine and thymidine 5'-phosphate. Their molar ratios were 1:5.01 (theoretical 1:5.0).

The minor faster moving spot (R_f 0.88, solvent A) on incubation with snake venom phosphodiesterase was chromatographed in solvent D. The compound was degraded up to 90% into thymidine and thymidine 5'-phosphate. Their molar ratios were found to be 0.9:3.0 and thus the structure assigned was $T(pT)_3$. The undegraded portion (10%) (R_f 0.40 with respect to pT) is presumably an isomer of $T(pT)_3$ with one 3'–3' linkage.

Synthesis of Dodecanucleotide $(pT)_{12}$ Involving Acetic Anhydride Treatment. An anhydrous solution of tri-*n*-hexylammonium salts of CE-$pT(pT)_5$ (580 OD_{267}, 0.01 mmol) and $(pT)_5pT$-OAc (650 OD_{267}, 0.011 mmol) in dry pyridine (2 ml) was treated with mesitylenesulfonyl chloride (48 mg, 0.22 mmol) for 3 hr. The reaction mixture was next treated with (1) aqueous pyridine–triethylamine overnight; (2) acetic anhydride–pyridine for 3 days as described above. After the usual work-up the concentrated reaction mixture (1 ml) was applied on Sephadex G-75 (superfine) K25/100 at 4°. The elution pattern is shown in Figure 6. Each peak and the intermediate pooled fractions were analyzed by paper chromatography in solvent C. The distribution of the nucleotide material is given in Table VIII. The isolated yield of $(pT)_{12}$ was 15%.

Table VIII. The Analysis of Sephadex G-75 (Superfine) Chromatography of $(pT)_{12}$ Reaction Mixture (Figure 6)

Peak	Fraction	Total OD_{267} units	Identification (%)
I	70–90	64	Polymerized products
II	91–108	180	$(pT)_{12}$[a] (90)
	109–113	21	$(pT)_{12}$ (50) and unidentified component (R_f 0.25[b]) (40)
	114–120	69	$(pT)_6$ (70), unidentified components R_f 0.25[b] (10), R_f 0.49[b] (20)
III	121–140	663	$(pT)_6$ (85), unidentified component R_f 0.49[b] (15)
	141–160	85	$(pT)_6$ (20), unidentified components R_f 0.45[b] (30), R_f 0.49 (50)

[a] See the Experimental Section. [b] R_f of the component with respect to pT.

Identification of the Product $(pT)_{12}$. The product from peak II moved as a single spot in solvent C (R_f given in Table I). It was next treated with bacterial alkaline phosphatase followed by rechromatography in solvent C. It resolved into one major band (90%) corresponding to $T(pT)_{11}$ and a faster moving band (10%) (R_f 0.31 with respect to pT). The compound $T(pT)_{11}$ (12.5 OD_{267}) was incubated with snake venom phosphodiesterase. The products separated by paper chromatography in solvent C were found to be thymidine and thymidine 5'-phosphate. Their molar ratio was 1:10.81 (theoretical 1:11).

Synthesis of Tetracosanucleotide $(pT)_{24}$ Involving Acetic Anhydride Treatment. An anhydrous solution of tri-*n*-hexylammonium salts of CE-$pT(pT)_{11}$ (105 OD_{267}, 1.0 μmol) and $(pT)_{11}T$-OAc (120 OD_{267}, 1.0 μmol) in dry pyridine (1 ml) was treated with mesitylenesulfonyl chloride (8 mg, 40 μmol) for 3 hr. The reaction mixture was next treated with (1) aqueous pyridine–triethylamine; (2) acetic anhydride–pyridine for 3 days as described above. After the usual work-up the concentrated reaction mixture (1 ml) was chromatographed on a Sephadex G-75 (superfine) K25/100 column at 4°. The elution pattern is shown in Figure 7. The distribution of the nucleotide material is given in Table IX.

Table IX. The Analysis of Sephadex G-75 (Superfine) Chromatography of $(pT)_{24}$ Reaction Mixture (Figure 7)

Peak	Fraction	Total OD_{267} units	Identification
I	110–122	17	$(pT)_{24}$ (mainly)
II	145–167	161	$(pT)_{12}$ and faster moving components
III	192–210	29	Degradation products

Identification of $(pT)_{24}$. A portion of the compound (~9.0 OD_{267}) from peak I was chromatographed in solvent C for 10 days. It moved as a single spot. It was next treated with bacterial alkaline phosphatase and rechromatographed in solvent C for 10 days. The major band (87%) (R_f 0.30 with respect to $(pT)_{12}$) on incubation with snake venom phosphodiesterase got completely degraded as analyzed by paper chromatography in solvent C.

The homogeneity of $T(pT)_{23}$ was further examined by phosphorylation of 5'-hydroxyl end group with [γ-^{32}P]ATP and polynucleotide kinase according to the Richardson[10] procedure. After incubation, the solution was heated in a boiling water bath for 2 min and the reaction mixture was chromatographed on a DEAE-cellulose paper with 0.5 *M* triethylammonium bicarbonate as the eluent. The slow-moving radioactive band was eluted with 1 *M* triethylammonium bicarbonate buffer and the solution evaporated repeatedly in the presence of pyridine to remove the salt and finally evaporated with 0.2 *M* ammonia to remove pyridine. An aliquot of the radioactive product was treated with snake venom phosphodiesterase under standard incubation conditions. The solution was applied on DEAE-cellulose paper and developed with 0.3 *M* triethylammonium bicarbonate buffer. All of the radioactivity was found in an area with mobility corresponding to pT. Similarly, another aliquot of the radioactive product was incubated with bacterial alkaline phosphatase under standard conditions. The solution was next chromatographed on DEAE-cellulose paper with 0.3 *M* triethylammonium bicarbonate buffer. All of the radioactivity was found in the area of inorganic phosphate.