The Reaction of 5'-Thymidylic Acid with the Condensation Product of Phosphorus Pentoxide and Ethyl Ether[1,2]

By F. N. Hayes and E. Hansbury

A study has been made of the reaction of 5'-thymidylic acid with ethyl polyphosphate and the nature of the high molecular weight material obtained. Although of low R_f and difficultly dialyzable, the polymer was degraded by the acetic anhydride pyrophosphate cleavage method to 3',5'-diphosphates of monomer and small oligomers. No high polymer with genuine phosphodiester linkages was found.

Schramm, et al.,[3] have reported on the use of a "polyphosphoric ester"[4] from the reaction of phosphorus pentoxide and ethyl ether to effect the polymerization of thymidylic acid as well as a group of individual ribonucleotides. From its viscosity, diffusion, sedimentation, and ultraviolet-absorption properties, the product was assigned an average molecular weight ranging from 15,000 to 50,000 (49 to 164 thymidylate monomer units). Polymerization of 5'-thymidylic acid by dicyclohexylcarbodiimide has been reported to give useful amounts of the tetradecamer[5] and the hexadecamer.[6] Picryl chloride[7] has been used to obtain the triacontamer.[8] In hopes of obtaining considerably longer polydeoxyribonucleotides for use in our studies[9] on information transfer in enzyme-catalyzed polymerization, we have set about to repeat the Schramm synthesis with 5'-thymidylic acid and to characterize the products.

Ethyl ether was allowed to react with phosphorus pentoxide under a variety of temperature and stoichiometric conditions. The products were oils whose composition was $C_2H_5O_3P$ and which did not yield orthophosphate[4] on reaction with cold water. The reactivity of the ethyl polyphosphate was examined by a control polymerization with diammonium 5'-thymidylate dihydrate in the absence of added solvent. There was no discernible difference in appearance or reactivity of the ethyl polyphosphate obtained from preparations carried out at 40 to 60°. The product from a 90° reaction had a light brown color and was less efficient in promoting polymerization.

Many reactions were run between 50° ethyl polyphosphate and 5'-thymidylic acid in the form of ammonium salt, pyridinium salt, or free acid, and as a stable dihydrate or lyophilized. The observed lack of dependence on the form of the 5'-thymidylic acid led to use of the diammonium salt dihydrate in further experiments. The early runs did not employ an added

solvent, but subsequently, on advice,[10] hexamethylphosphoramide[11] was used to predissolve the nucleotide.

At first, reaction products were examined superficially for the presence of high molecular weight material recognized as having low R_f in paper chromatography or as being only slowly dialyzable. A time study series run at 50° without added solvent and processed by quantitative paper chromatography exhibited a maximum yield at about 6 hr. (Fig. 1a). The reaction mixture became increasingly brown with time. Another series, also run at 50° but with hexamethylphosphoramide and this time subjected to dialysis, did not reach its peak yield until about 50 hr. (Fig. 1b) and remained clear and almost colorless for 72 hr.

Phosphorus analyses on the high molecular weight fractions of these reactions gave large numbers corresponding to five to fifteen times that to be expected from one phosphorus per mononucleotide unit.

Separation of a contaminating polyphosphate component from RNA has been accomplished[12] using activated charcoal. Application of this method effected at most a twofold decrease in the excess phosphorus.

Cleavage of internucleotide pyrophosphate linkages while leaving phosphodiester bonds intact has been found possible[13] using acetic anhydride. Ethyl polyphosphate offers a medium rich in variously substituted pyrophosphate linkages, in the unreacted form, and particularly after undergoing reaction with itself and the nucleotide in the polymerization reaction. The acetic anhydride cleavage reaction on hydrolyzed ethyl polyphosphate produced only 13% of the potential orthophosphate and thereby left polyphosphate species to hinder subsequent separative and analytical procedures.

In order to obtain enough high molecular weight product for close chemical scrutiny, a large-scale reaction was run and then dialyzed against 2 N sodium chloride for 8 days, whereupon only 15% remained. The retentate was submitted to acetic anhydride–pyrophosphate cleavage and separated into two closely related fractions by precipitation from ethanol. The precipitate was considerably richer in low R_f material. Chromatography on G-50 Sephadex (Fig. 2) gave a distribution for the precipitate which peaked approximately with the tetramer on a scale of elution position calibrated with authentic oligothymidylic

(1) Work was performed under the auspices of the U. S. Atomic Energy Commission.

(2) The line abbreviation system uses Th for thymine, t for thymidine (deoxyribonucleosides take the lower case and ribonucleosides the upper case; i.e., ribothymidine is T), pt for 5'-thymidylic acid, ptp for thymidine 3',5'-diphosphate, and (pt)$_n$ for polymers of 5'-thymidylic acid where the integer n is the number of monomer units. Similar examples appear in Fig. 4. Also see F. N. Hayes and D. G. Ott, Los Alamos Scientific Laboratory Report LAMS-3034, 1963, p. 326.

(3) G. Schramm, H. Grötsch, and W. Pollmann, Angew. Chem. Intern. Ed. Engl., 1, 1 (1962).

(4) G. Weill, M. Klein, and M. Calvin, Nature, 200, 1005 (1963).

(5) A. Falaschi, J. Adler, and H. G. Khorana, J. Biol. Chem., 238, 3080 (1963).

(6) A. Rich and I. Tinoco, Jr., J. Am. Chem. Soc., 82, 6409 (1960).

(7) F. Cramer, R. Wittmann, K. Daneck, and G. Weimann, Angew. Chem., 75, 92 (1963); R. Wittmann, Ber., 96, 2116 (1963).

(8) A. Murray, III, unpublished work from this laboratory.

(9) D. G. Ott, et al., Los Alamos Scientific Laboratory Report LAMS-3034, 1963, pp. 329–349.

(10) W. Pollmann, private communication.

(11) Hexamethylphosphorotriamide from Eastman Organic Chemicals.

(12) J. P. Ebel, A. Stahl, G. Derheimer, S. Muller-Felter, and M. Yacoub, "Acides Ribonucleiques et Polyphosphates," Colloques Internationaux du Centre National de la Récherche Scientifique, No. 106, Strasbourg, July 6–12, 1961 (1962), p. 552.

(13) H. G. Khorana and J. P. Vizsolyi, J. Am. Chem. Soc., 81, 4660 (1959).
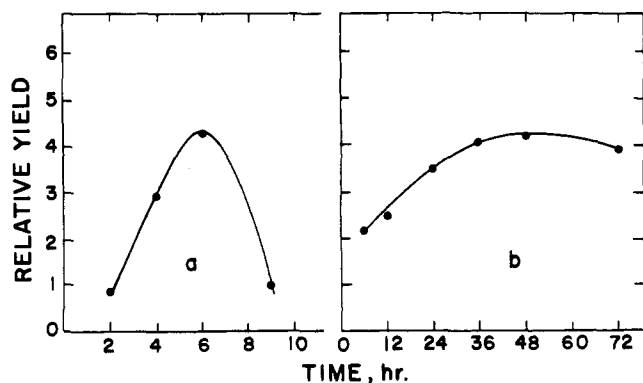
Fig. 1.—Kinetics of polymer formation: (a) without solvent determined by paper chromatography; and (b) with hexamethyl-phosphoramide, determined by dialysis. The yields from the two procedures are not comparable and are expressed in arbitrary units to give information solely on the course of the reaction and the time of peak yield.

acids. DEAE-cellulose chromatography (Fig. 3) of the precipitate (Table I) showed 70% in three major peaks.

TABLE I

DATA ON DEAE-CELLULOSE PEAKS

Peak	%	$\epsilon(P) \times 10^{-3}$	Number of phosphate charges
A	23.8	4.6	3
B	35.7	3.3	4
C	9.8	3.4	5
D	4.3	2.3	6

Phosphorus analyses on individual tubes of peak A gave a constant value when divided by the spectro-photometrically obtained nucleotide content. The combined A had $\epsilon(P)$ 4.6 $\times$ 10^3 and three negative charges which indicated one thymidine, one mono-esterified phosphate, and one diesterified phosphate to be present as one or more structures such as ptpx, pxpt, xptp, and tpxp. Microanalyses of peak A agree with a molecule containing two phosphorus atoms and one thymidine with a remainder of three carbons for x. The analytical sample was a sesqui-calcium salt of unknown degree of hydration and, therefore, a specific set of atoms was not assigned to x.

Enzymatic degradations were used to indicate the sequence of units in A. Spleen and venom phos-phodiesterases were unable to cleave A, but E. coli alkaline phosphatase removed a terminal phosphate leaving a fragment which, although still unreactive to venom phosphodiesterase, was further cleaved by spleen phosphodiesterase to 3'-thymidylic acid. From this, A was assigned the partial structure I (Fig. 4).

Inactivity to venom phosphodiesterase may be due to the absence in x of an enzyme binding site. Both acid- and base-catalyzed elimination reactions were in-operative on I. This effectively prohibits the presence on x of a C-2 hydroxyl[14] or a C-3 carbonyl function.[15] Further work on the structure of I and its formation mechanism is in progress.

Phosphorus analyses on individual tubes of peaks B and C showed nonnucleotide contamination most prom-inent in the early tubes. Both peaks were rechroma-tographed on DEAE–cellulose with shallow gradients

(14) D. M. Brown and D. A. Usher, Proc. Chem. Soc., 309 (1963).
(15) C. R. Bayley, K. W. Brammer, and A. S. Jones, J. Chem. Soc., 1903 (1961).
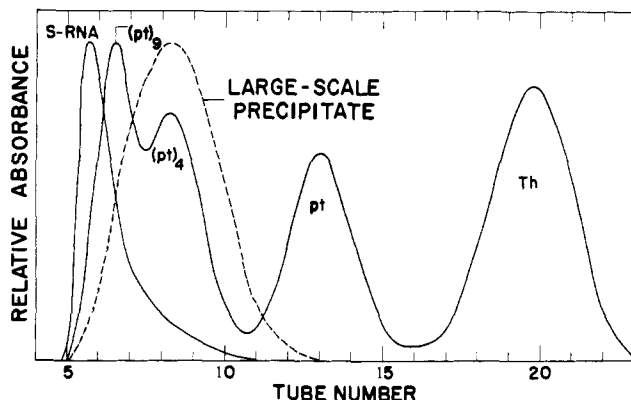

Fig. 2.—Elution profile from G-50 Sephadex of precipitate from large-scale reaction. Also shown are profiles of s-RNA and of a mixture of thymine (Th) and the nonamer, tetramer, and monomer of 5'-thymidylic acid [(pt)$_9$, (pt)$_4$, and pt]. The eluent was 0.005 M triethylammonium bicarbonate.
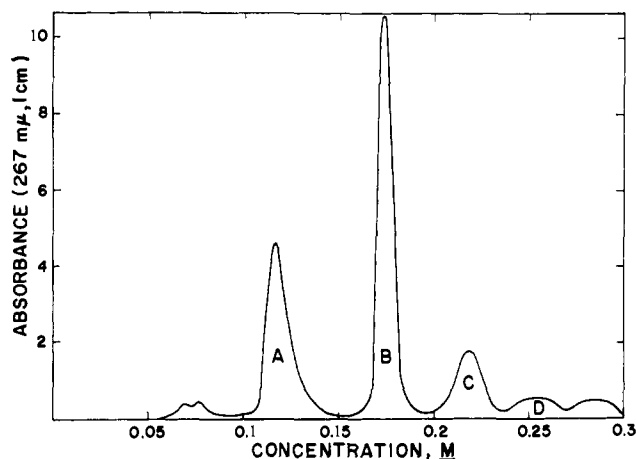

Fig. 3.—Elution profile from DEAE-cellulose of precipitate from large-scale reaction. The eluent was aqueous triethyl-ammonium bicarbonate.

which did not completely eliminate the contaminating phosphorus but which allowed splitting the peaks into a major portion of constant $\epsilon(P)$ following a forerun of low and varying $\epsilon(P)$. Pyrophosphate anion would have the charge to elute between peaks A and B and might be the contaminant in B.

Comparison with authentic compounds both before and after E. coli alkaline phosphatase degradation (III and V) plus analyses for orthophosphate and total phosphate showed peak B to be II and peak C to be IV.

The desired role of ethyl polyphosphate in pro-moting polymerization of 5'-thymidylic acid is to acti-vate the 5'-phosphate such that attack by the 3'-hydroxyl of another molecule will lead to formation of a phosphodiester linkage, a process which should repeat itself until long polynucleotide chains are syn-thesized. A competing reaction (Fig. 4) of ethyl polyphosphate would be phosphorylation of the 3'-hydroxyl of 5'-thymidylic acid to give II, possibly via a stable intermediate, ptp$_4$, whose size[16] with hydration shell could render it difficult to pass through a dialysis membrane. A dinucleotide made in the reaction might be further phosphorylated to ptptp$_4$, later to appear

(16) A study of the Sephadex elution properties of a large group of various phosphorylated derivatives of thymidine has shown that phosphorylation imparts to a molecule a higher effective molecular size than predicted from its molecular formula: F. N. Hayes, E. Hansbury, and V. E. Mitchell, J. Chromatog., in press.
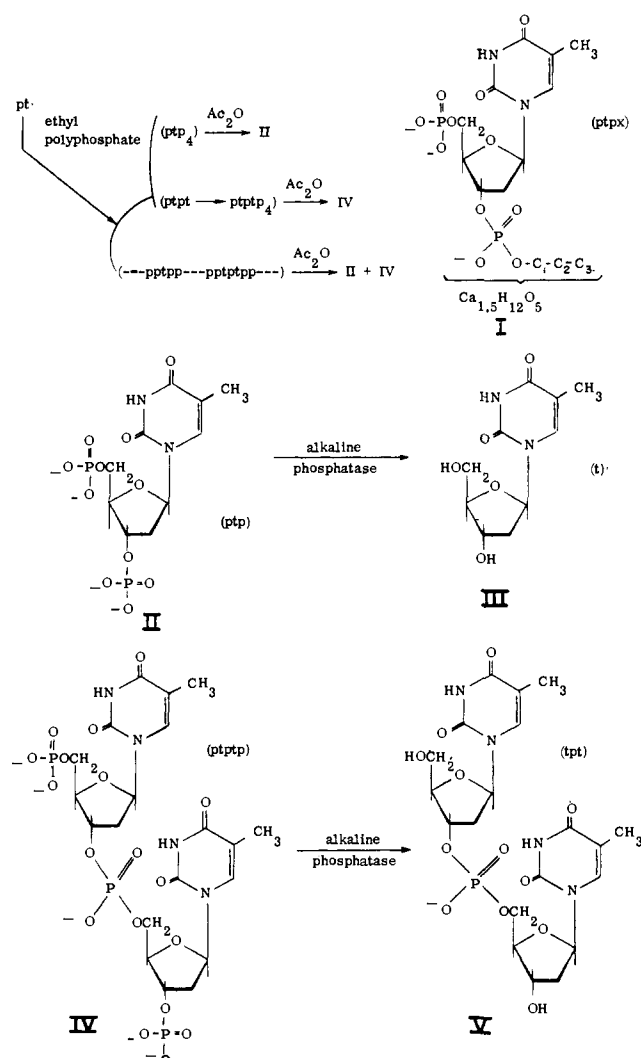
Fig. 4.—Structures and reactions; Ac, acetyl.

as IV on acetic anhydride cleavage. Also possible are longer chains predominantly polyphosphate in nature but with interspersed monomer and genuine small oligomers. Such polymers should also cleave to give II and IV.

All attempts to find long chain polynucleotides in the polymeric material obtained from the reaction of ethyl polyphosphate with 5'-thymidylic acid have been unsuccessful. This was in spite of a great variety of conditions employed in preparing ethyl polyphosphate and in reacting it with both the anhydrous and dihydrate forms of 5'-thymidylic acid—conditions which should have allowed the presence of a variety of distributions of the molecular species[4] of ethyl polyphosphate. After cleavage of pyrophosphate linkages in the polymeric material, small molecules remained possessing complete terminal phosphorylation, such as II and IV.

Experimental

Microanalyses were performed by E. H. Lilly and M. J. Naranjo. Authentic II, IV, V, (pt)₄, (pt)₅, (pt)₉, and (pt)₁₀ were prepared by the methods of Khorana.[17]

Total phosphorus analyses[18] were performed by a modification of the procedure outlined by Bartlett after ashing by the method

of Ames and Dubin. Orthophosphate was measured by the method of Fiske and SubbaRow.[19]

Ultraviolet-absorption measurements were performed with Beckman DK-1 and DB spectrophotometers. The $\epsilon(P)$[20] was experimentally determined as the quotient of the 1-cm. absorbance at the spectral maximum by the phosphorus molarity. Evaporations were carried out with a vacuum rotary flash evaporator. Quantities of oligomeric compounds are expressed as monomer equivalents (spectrophotometrically determined, neglecting hypochromism).

Chromatography.—Paper chromatography was performed by the descending technique using Whatman chromatographic paper No. 41H (No. 541) with 1-propanol–concentrated ammonia–water (55:10:35) as the developing solvent. Standard R_f values were: thymidine, 0.73; V, 0.48; 5'-thymidylic acid, 0.41; II, 0.25; (pt)₅, 0.23; IV, 0.22; and (pt)₁₀, 0.11.

Anion exchange column chromatography was performed on a DEAE–cellulose column (1.6 × 14 cm.) by applying the sample at pH 8.5 and eluting with 1 l. of a linear gradient of aqueous triethylammonium bicarbonate at pH 7.5.

Gel filtration column chromatography was carried out on a G-25 Sephadex column (1 × 165 cm.) and on a G-50 Sephadex column (2.3 × 32 cm.) with 0.005 M triethylammonium bicarbonate as the elution solvent. With both columns the order of elution is oligomer–monomer–nucleoside–base, but G-50 was considerably more capable of resolving short oligonucleotides whereas G-25 gave better resolution of nucleoside from base.

Enzymatic Procedures.—Cleavage of phosphate ester linkages was performed according to the following procedures, each of which was run with 0.5 to 5 μmoles of substrate and terminated by heating at 70° for 5 min.

E. coli **alkaline phosphatase**[21] and substrate (0.2 unit of enzyme per μmole of terminal phosphate in substrate) with Tris (100 μmoles, pH 8.0) in a final volume of 1 ml. were incubated at 37° for 4 hr.

Venom 5'-nucleotidase[22] and substrate (enzyme to liberate 10 μmoles of orthophosphate per hr. for each μmole of terminal phosphate in substrate) with Tris (100 μmoles, pH 8.9) in a final volume of 1 ml. were incubated at 37° for 4 hr.

Venom phosphodiesterase[23] and substrate (30 units of enzyme per μmole of phosphodiester linkage in substrate) with Tris (100 μmoles, pH 8.9) in a final volume of 1 ml., overlaid with toluene (50 μl.), were incubated at 37° for 20 hr.

Spleen phosphodiesterase[21] and substrate (0.1 unit enzyme, initially and in three subsequent hourly additions, per μmole phosphodiester linkage in substrate) with sodium succinate (125 μmoles, pH 6.5) in a final volume of about 1 ml. were incubated at 37° for 4 hr.

Preparation of Ethyl Polyphosphate.— Ethyl ether dried over sodium, phosphorus pentoxide,[24] and chloroform dried over calcium chloride (1 ml.:1 g.:2 ml.) were heated with efficient stirring under anhydrous conditions until the solution became clear. Temperatures of 40, 45, 50, 55, 60, and 90° were used. All volatile material was evaporated, and the resulting oils were stored under dry conditions in a deep freeze. The weight ratio of product to phosphorus pentoxide was consistently about 1.5 compared to 1.522 for the product to be $C_2H_5O_3P$.

Anal. Calcd. for $C_2H_5O_3P$: P, 28.7. Found: P, 28.5.

Another sample of ethyl polyphosphate was kindly contributed by Dr. W. Pollmann.

The Extent of Acetic Anhydride Cleavage of Pyrophosphate Linkages in Ethyl Polyphosphate.—In parallel reactions, two samples (100 μl.) of ethyl polyphosphate were heated at 50° for 6 hr., followed by addition of water with efficient stirring. Each product was treated with Dowex-50W X-4 (H⁺), followed by pyridine, and evaporated repeatedly from pyridine. The final oil was allowed to stand at room temperature for 3 days with pyridine (6.83 ml.), acetic anhydride (2.29 ml.), and triethylamine (0.19 ml.). Water (13.6 ml.) was stirred in with cooling, and after 1 hr. the solution was evaporated repeatedly with additions of water. The product was treated with 8 N ammonia (14

(17) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961.

(18) G. R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959); B. N. Ames and D. T. Dubin. *ibid.*, **235**, 769 (1960).

(19) C. H. Fiske and T. SubbaRow, *ibid.*, **66**, 374 (1925).

(20) E. Chargaff and S. Zamenhof, *ibid.*, **173**, 327 (1948).

(21) Purchased from the Worthington Biochemical Corporation.

(22) Purified from *Crotalus adamanteus* venom by the method of I. R. Lehman, G. G. Roussos, and E. A. Pratt, *J. Biol. Chem.*, **237**, 819 (1962).

(23) Purified from *Crotalus adamanteus* venom by the method of R. L. Sinsheimer and J. F. Koerner, *ibid.*, **198**, 293 (1952).

(24) Analytical reagent grade from Mallinckrodt Chemical Works, and Hfrom Riedel de Haën, A.G., anover.

ml.) and allowed to stand at room temperature for 1 day, whereupon it was evaporated and redissolved in water. Orthophosphate analyses were 13.2 and 13.5% of the total phosphorus.

Reaction of Ethyl Polyphosphate with 5'-Thymidylic Acid.—Diammonium 5'-thymidylate dihydrate (20 μmoles) was well mixed with 55 mg. of ethyl polyphosphate (from 50° preparation) in a 15-ml. centrifuge cone. The reaction mixture was slowly rotated in a 50° oil bath. At the end of the desired time, water (300 μl.) was added with cooling and stirring, followed by concentrated ammonia (75 μl.). An aliquot of the mixture (about 10%) was chromatographed on paper along side a 5'-thymidylic acid marker for a solvent front travel of about 30 cm. The region from 0.5 cm. behind the origin to 0.75 times the distance traveled by the marker was cut out and repeatedly extracted with 0.01 N HCl until no more ultraviolet-absorbing material was removable. The yield (Fig. 1a) was determined spectrophotometrically.

Reaction of Ethyl Polyphosphate with 5'-Thymidylic Acid in Hexamethylphosphoramide.—Diammonium 5'-thymidylate dihydrate (31.5 μmoles) was dissolved in hexamethylphosphoramide (100 μl.) with warming, and ethyl polyphosphate (100 μl.) was mixed in. The reaction was run and terminated as before. The product was put in 0.94-in. flat-width cellulose dialysis tubing and dialyzed at 4° for two 12-hr. periods against 2 N sodium chloride followed by one period vs. water. The yield (Fig. 1b) was determined by spectrophotometric analysis of the retentate.

Large-Scale Reaction.—Diammonium 5'-thymidylate dihydrate (10 mmoles) was dissolved in hexamethylphosphoramide (30 ml.) and then further mixed with ethyl polyphosphate (33 g.). The solution was heated at 50° for 71 hr., after which the reaction was terminated by addition of water (50 ml.) with cooling and stirring. Ammonia was added to bring the pH to 7, and the solution was transferred into 5.75-in. flat-width cellulose dialysis tubing. Dialysis at 4° against 2 N sodium chloride was carried out for 8 days with five changes, whereupon the dialysate medium was changed to water for eight 24-hr. periods. By the end of dialysis vs. water no more chloride was issuing from the bag. The retentate (1.48 mmoles, 7.70 mmoles P) was treated with Dowex-50W X-4 (H$^+$), followed by pyridine, evaporated to a glass, re-evaporated twice from dry pyridine, and dissolved in pyridine (100 ml.), tributylamine (4.6 ml.), and acetic anhydride (34 ml.). After standing at room temperature in the dark for 3 days, the solution was treated with water (200 ml.) while being cooled and agitated. The resulting solution was evaporated to dryness, and the residue was repeatedly dissolved in water and re-evaporated and finally dissolved in water (30 ml.) and concentrated ammonia (20 ml.). After 3 days at room temperature followed by evaporation, the oil was treated with absolute ethanol, yielding a precipitate and a light brown supernatant which deposited another crop of crystals on cooling. The combined preicipitate contained 1.14 mmoles of 267 mμ-peaking material. The supernatant was evaporated and redissolved in water. Most of the color was removed by extraction with chloroform followed by treatment with Dowex-50W X-4 (H$^+$). About 0.3 mmole was present in the slightly yellow solution. Paper chromatography revealed that both products were complicated mixtures. The major spot from the supernatant had R_f 0.42. The precipitate gave a minor component (R_f 0.42) with nothing intervening down to the major spot (R_f 0.26). A G-50 profile of the precipitate is shown in Fig. 2 along with standards. The precipitate (0.1 mmole) was chromatographed on DEAE–cellulose with a 0.005 to 0.3 M triethylammonium bicarbonate gradient (Fig. 3). Table I contains data on the major peaks: their recovered yields, ϵ(P) values, and phosphate-negative charges according to calibration runs on the column. The profile of phosphorus analyses from individual tubes tracked well with the absorbance profile for peak A but was skewed to slightly lower molarity for peaks B through D.

Peak A was also obtained in 80% yield from DEAE–cellulose chromatography of the supernatant solution. Peak A (R_f 0.44) was eluted from G-25 in the oligomer peak. An aqueous solution of A (60 μmoles) was evaporated to dryness, and the residue was dissolved in absolute ethanol (300 μl.) and mixed

with 0.5 M calcium bromide (200 μl.) in absolute ethanol. The white precipitate was centrifuged and washed successively with three portions of acetone followed by two of ethyl ether and then placed in a vacuum desiccator to dry (yield, 31.6 mg.).

Anal. Calcd. for $C_{13}H_{24}O_{16}N_2P_2Ca_{1.5}$ (586.4): C, 26.62; H, 4.13; N, 4.78; P, 10.56. Found: C, 26.83; H, 4.11; N, 4.85; P, 10.30.

Ultraviolet spectrophotometry (pH 7) on the calcium salt gave: λ_{max} 267 mμ; λ_{min} 234.5 mμ; A_{max}/A_{min} = 3.65; A_{250}/A_{260} = 0.66; A_{280}/A_{260} = 0.72; ϵ(P) 4.81 × 10^3.

Peak A was recovered unreacted[25] from treatment with venom phosphodiesterase and with spleen phosphodiesterase.

A mixture of equal volumes of 0.01 M peak A and 1 N potassium hydroxide was heated at 40° for 17 hr. After cooling, 70% perchloric acid was added with stirring to bring the pH to 7. The supernatant was separated from the potassium perchlorate precipitate by centrifugation. Peak A was recovered unreacted.[25]

A mixture of equal volumes of 0.01 M peak A and 0.1 M sulfuric acid was heated at 85° for 30 min. After cooling, the solution was neutralized with ammonia. Peak A was recovered unreacted.[25]

The product from *E. coli* alkaline phosphatase treatment of peak A (R_f 0.73) was eluted from G-25 in the monomer peak, and analyzed to show the appearance of orthophosphate equimolar to peak A reacted. This alkaline phosphatase product did not react with venom phosphodiesterase (as judged by no change in both R_f and G-25 elution position) but was altered by spleen phosphodiesterase treatment to a product (R_f 0.41) which eluted from G-25 in the monomer peak. It was established to be 3'-thymidylic acid by its resistance to 5'-nucleotidase and reaction with *E. coli* alakaline phosphatase to give orthophosphate and an ultraviolet-absorbing product (R_f 0.77) which eluted from G-25 in the nucleoside peak, characteristic of thymidine.

Peak B (30 μmoles) was rechromatographed on DEAE–cellulose with a 0.05 to 0.2 M triethylammonium bicarbonate gradient. A single peak with maximum at 0.14 M resulted which had ϵ(P) values lowest at the onset of the absorbance peak, increasing along the rising slope, and reaching a value of 4.6 × 10^3 just before the maximum. This value remained constant for the maximum and the whole downslope. The peak which contained 27.2 μmoles was cut into a forerun (5.5 μmoles, ϵ(P) 2.5 × 10^3) and a major fraction (21.7 μmoles, ϵ(P) 4.6 × 10^3), which chromatographed as a single spot (R_f 0.26) and reacted with *E. coli* alkaline phosphatase to give a product (R_f 0.71) with phosphorus entirely as orthophosphate.

Peak C was rechromatographed on DEAE–cellulose with a 0.15 to 0.2 M triethylammonium bicarbonate gradient. The elution profile had a single peak with maximum at 0.18 M. Again, low but gradually increasing ϵ(P) values occurred along the rising portion of the peak and reached a constant value of about 5.6 × 10^3 beginning at the maximum and remaining constant along the downslope. The major fraction with constant ϵ(P) (R_f 0.24) had a trace contaminant (R_f 0.44) and was further purified on G-25 from which the high R_f substance emerged considerably later than the main material. Treatment with *E. coli* alkaline phosphatase gave a product with an orthophosphate to total phosphate ratio of 0.65 and R_f 0.46 for its ultraviolet-absorbing component (V).

(25) A sufficient and necessary test for recovery of peak A was that the product have both identical R_f and same elution position from G-25 as peak A.