Oligonucleotides as Primers for Polynucleotide Phosphorylase

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The enzyme polynucleotide phosphorylase, discovered by Grunberg-Manago and Ochoa (1, 2) in extracts of Azotobacter agile, catalyzes the reversible reaction shown in Equation 1.

\[ n \text{Nucleoside-di-P} \rightleftharpoons (\text{nucleoside-P})_n + n \text{P}_1 \] (1)

Ribonucleoside diphosphates react to form polyribonucleotides and inorganic phosphate. Studies of the nature of nucleotide incorporation into nucleic acid in Escherichia coli led to the recognition of a similar reaction by Littauer and Kornberg (3). Beers (4, 5) and Olmsted (6, 7) have investigated the phosphorylase in Micrococcus lysodeikticus.

Heppel et al. (8–10) have elucidated the structure of the polyribonucleotides formed in the polynucleotide phosphorylase catalyzed reaction. The polymers polyadenylic acid, polyuridylic acid, the mixed polymers of adenylc and uridylic acids, and of adenylc, uridylic, guanylic, and cytidylic acids conform to the structure of a normal polynucleotide, and poly AGUC is chemically indistinguishable from natural ribonucleic acid.

With relatively crude preparations of polynucleotide phosphorylase, only nucleoside diphosphate and Mg++ ion are required to demonstrate polymer formation (2–4). Nevertheless Ochoa and Heppel (8), as well as Littauer and Kornberg (3), discussed the possibility that, in analogy with the carbohydrate phosphoglycerate kinase, polymerization requires a polynucleotide primer. They suggested that polynucleotide material contaminating the enzyme preparations served this function. With highly purified enzyme preparations from A. agile, Mii and Ochoa (12) found that polymerization of nucleoside diphosphates occurs only after a lag period and that the lag can be overcome by RNA and the biosynthetic polymers.

The data presented in this report show that oligonucleotides such as pApA, and ApUp, and pApApApA also overcome the lag period and, in sufficient concentration, stimulate the reaction to the same extent as the polymers. Furthermore, they are incorporated into polymers formed in their presence. The polymer chains are built by successive additions of mononucleotide units to the unesterified C-3' hydroxyl of the terminal nucleoside residue in the preformed oligonucleotide chain.

It is shown that oligonucleotides such as ApUp, in which the C-3' hydroxyl of the terminal nucleoside residue is blocked by a phosphomonoester bond, can also overcome the lag in polymerization found with highly purified A. agile preparations. These oligonucleotides are not, however, incorporated into the polymer formed, and the mechanism by which they overcome the lag phase remains obscure.

Preliminary reports of this work have been made (13, 14).

MATERIALS AND METHODS

Enzymes—Polynucleotide phosphorylase was purified from E. coli according to the procedure of Littauer and Kornberg (3). The fraction used, which is described as Ethanol 1 (3), had a specific activity of 15, determined by the "exchange" assay, Assay C, (3), compared with a value of 0.5 for the crude extract. Another preparation was a gel eluate fraction (15) from A. agile which was kindly supplied by Dr. S. Ochoa. This had a specific activity of 40 as measured with the "exchange" assay, Assay 1, (2), compared with a value of 0.5 for the original dialyzed extract. It will be referred to below as "gel eluate." Neither preparation showed a lag period in the polymerization of ADP or UDP. A number of more highly purified fractions, prepared by Mii and Ochoa (12), were also made available to us through the kindness of Dr. Ochoa. All of these displayed a lag period in the polymerization of ADP and UDP (12). They are conveniently designated by their specific activity (S.A.) in the exchange assay (2) as S.A. 150, S.A. 60, and S.A. 113; for these preparations, the ratios of the absorption at 280 nm to that at 260 nm were 0.96, 1.21, and 1.03, respectively. Finally, a preparation of Azotobacter enzyme was made in this laboratory according to the procedure of Mii and Ochoa (12). This fraction, designated S.A. 60, displayed a lag phase in the polymerization of ADP and UDP. The ratio of its absorbancy at 280 nm to that at 260 nm was 1.54.

Snake venom phosphodiesterase, free of 5'-nucleotidase, was prepared by a modification of the procedure of Koerner and Sincheimer (16). Crystalline bovine pancreatic ribonuclease was a commercial preparation (Armour Laboratories, Lot. No. 1044).

nucleotides were prepared from 32P-labeled poly A which was synthesized with the use of ADP32. The ADP32 was labeled in both phosphate groups and was obtained from Schwarz Laboratories, Inc.

An unknown material, extracted from filter paper with water, stimulates the polymerization reaction to a small extent, and therefore, when oligonucleotides prepared by paper chromatography were used to overcome the lag in polymerization, a small correction for this factor was necessary. This extractable material from paper is discussed further below. Although this source of error was not large, it was considered desirable to obtain these compounds by other methods. Accordingly, pApA, pApApA, pApApApA, and pApApApApA were also separated by column chromatography, essentially by means of the procedure of Volkin and Cohn (19). Poly A (60 mg) was digested with a nuclease from guinea pig liver nuclei (17) and the digest treated as described previously (18). After lyophilization, the material was dissolved in water, adjusted to pH 9, and applied to a column (6.3 cm x 0.95 cm²) of Dowex 1-X2, chloride form. The main components of the digest were eluted under the following conditions: adenosine, with 0.005 N HCl; AMP, with 0.005 N HCl; pApA, with 0.01 N HCl; pApApA, with 0.01 N HCl containing 0.0125 N NaCl; pApApApA, with 0.01 N HCl containing 0.05 N NaCl. The fractions containing each oligonucleotide were pooled, and each compound was concentrated by rechromatography on a small Dowex 1-X2 (choloride form) column. The oligonucleotide solutions obtained were freed of chloride by shaking with 5% trioctylamine, in chloroform saturated with water (20). The trioctylamine was removed by ether extraction. It was observed that an inhibitor for polynucleotide phosphorylase was present in fractions eluted from the Dowex 1 column (see "Results") and was only partly removed by treatment of the oligonucleotides with acid-washed Norit A (21). For this reason, the use of columns of the weak anion exchanger, epichlorhydrin-ethanolamine cellulose (22), was investigated, in collaboration with Dr. Gordon Tener (23). Excellent separations were obtained by the same procedure that had previously been used for thymidine oligonucleotides (24, 25). The oligonucleotides were recovered from the pooled column fractions as the lithium salts (24) and were used as such in these experiments. When the preparations were dissolved in water before use in enzyme experiments, it was found that the concentration of oligonucleotide, as determined by ultraviolet absorption, was only 10% of that expected from the weight of material dissolved. The nature of the contaminating material that accounts for this discrepancy is unknown.

Samples of pApA, pApApA, and pApApApA, which were prepared by the digestion of poly A with a partially purified nuclease from A. agile, were kindly made available by Dr. Audrey Stevens, of this laboratory. The oligonucleotide mixture which results from the digestion of poly A was separated into its components by chromatography on diethylaminoethyl cellulose (22) according to an unpublished procedure of Stachelin, Sober, and Peterson. The products were white solids, and the extinction of aqueous solutions was consistent with the dry weight.

The oligonucleotides ApUp and ApApUp, which have a phosphonooester group at C-3', were separated by chromatography on Dowex 1 (19) as well as by the paper chromatographic procedures previously described (10, 18). A ribonuclease digest of 100 mg of poly AU was prepared (18); the deproteinized digest was adjusted to pH 2 with HCl and the insoluble material was removed by centrifugation. The solution was then adjusted to pH 9 and applied to a column (8.8 cm x 0.95 cm²) of Dowex 1-X2, chloride form. The products of the digestion were eluted under the following conditions: 3'-UMP with 0.01 N HCl; ApUp with 0.01 N HCl containing 0.0125 M NaCl; ApApUp with 0.01 N HCl containing 0.025 M NaCl; ApApApUp with 0.01 N HCl containing 0.05 M NaCl. The pooled eluates corresponding to each discrete peak were neutralized for storage. In order to concentrate the oligonucleotides and to remove the salts, the solutions were adjusted to a pH between 2 and 3, and the nucleotides were adsorbed onto acid-washed Norit A. The charcoal was washed extensively with water, and the nucleotides were eluted with 50% ethanol containing 0.3 ml of concentrated NH4OH per 100 ml. These eluates were evaporated to dryness and the residues taken up in a small amount of distilled water. The identity and homogeneity of the preparations were verified by paper chromatography. The recovery of nucleotides in the norite step varied from 50 to 80%.

Adenyllyl-(2'-5')-uridine (27) was a gift from Dr. A. M. Michaelson, Arthur Guinness Son and Company, Ltd., Chemist’s Laboratory, Dublin. Adenosine 5'-benzyl phosphate (28) was a gift from Dr. M. Brown, Cambridge University, England. The equimolar isomeric mixture of adenylnyl-(3'-5')-adenosine (ApA) and adenylnyl-(2'-5')-adenosine was prepared from a sample of synthetic polyadenylic acid kindly supplied by Dr. Michaelson. This polynucleotide, which was prepared chemically (29), consisted of material of various chain lengths, and the internucleo
tide linkages were both 2'-5' and 3'-5' phosphodiester bonds. The chains were terminated by 2',3'-cyclic phosphoryl end groups, which were hydrolyzed by treatment with 0.1 N HCl for 4 hours at 23°. After chromatography of the hydrolysis products in Solvent 1 (see below), the dinucleotide zone (Rv-AMP = 0.59; in the absence of extraneous salt, Rv-AMP = 0.75) was eluted and treated with phosphomonoesterase as described earlier (18). Chromatography in Solvent 1 was repeated, and a new band corresponding to the isomeric mixture of the dinucleo
di-mono
phosphates (Rv-AMP = 1.9; in the absence of extraneous salt, Rv-AMP = 1.5) was found. This band was eluted from paper and the isomers were then resolved by chromatography in Solvent 2 (see below), in this solvent the Rv-AMP values of adenylnyl-(3'-5')-adenosine and adenylnyl-(2'-5')-adenosine are 0.07 and 0.20, respectively. Thymidine oligonucleotides, pTpT and pT-pT (24, 25), were kindly supplied by Dr. H. G. Khorna.

Polymers were prepared from nucleoside diphosphates with the polynucleotide phosphorylase of E. coli or A. agile according to the procedure of Cranberg-Manago et al. (2) with slight modifications (18). ADP and UDP were obtained from the Sigma Chemical Company. Thymine ribonucleoside pyrophosphate (39) was a gift from Professor Sir Alexander Todd, Cambridge University, England. The barium salt was converted to the sodium salt by treatment with Dowex 50-Na⁺.

**Paper Chromatography and Paper Electrophoresis—Descending chromatography was carried out with Solvent 1, isopropanol-water (70:30, volume for volume) with NH₄OH in the vapor phase (30); Solvent 2, saturated ammonium sulfate-isopropanol-1 M sodium acetate (80:2:18, volume for volume) for (31); Solvent 3, isobutyric acid-1 M NH₄OH-0.2 M EDTA (100:60:0.8, volume for volume) for (32); Solvent 4, 170 ml of isopropanol, 44 ml concentrated HCl and water to 260 ml (33); Solvent 5, n-propanol-concentrated NH₄OH-water (60:30:10, volume for volume) (34). Electrophoretic separa-
The molar extinction coefficients of its constituent nucleotides. For temperature, and the concentration of the compound was determined from the absorption of the eluate at an appropriate wave length.3

The approximation does not account for any hypochromic effect.

The area of paper containing the oligonucleotide was located by its appearance of the oligonucleotide. The general procedure was to mixtures were chromatographed in a solvent that resolved the products. The area of chromatography was carried out according to Markham and Smith (30) on strips (57 X 10 cm) of Whatman No. 3MM paper saturated with 0.05 M ammonium formate buffer, pH 3.5, A potential of 1000 volts was applied across the paper. Purine- and pyrimidine-containing compounds were located on the paper strips with an ultraviolet light which was also used to photograph the strips.

For Experiments 1 and 2, the incubation mixtures were carried out according to Markham and Smith (30) on strips (57 X 10 cm) of Whatman No. 3MM paper saturated with 0.05 M ammonium formate buffer, pH 3.5, A potential of 1000 volts was applied across the paper. Purine- and pyrimidine-containing compounds were located on the paper strips with an ultraviolet light which was also used to photograph the strips.

Incorporation of Oligonucleotides The incorporation of oligonucleotides into polymers was estimated by measuring the disappearance of the oligonucleotide. The general procedure was to incubate ADP or UDP with the oligonucleotide and polynucleotide phosphorylase. At various times, aliquots of the reaction mixture were chromatographed in a solvent that resolved the oligonucleotide, the nucleoside diphosphate, and the reaction products. The area of paper containing the oligonucleotide was quantitatively eluted with 0.01 N HCl for 6 hours at room temperature, and the concentration of the compound was determined from the absorption of eluate at an appropriate wave length. From the adjacent region of the paper strip, an area of identical size was cut out and eluted in order to correct for the ultraviolet-absorbing material present in the paper itself.

Assay for Polymer Formation—The polynucleotide phosphorylase catalyzed polymerization reaction was generally followed by measuring the release of P_i from nucleoside diphosphates. The reaction mixtures are described in the legends for the figures and tables. At various times during the incubation, aliquots (usually of 0.01 or 0.02 ml) were removed and added to enough cold 2.5% perchloric acid to give a total volume of 1.0 ml. Precipitated protein or polymer was removed by centrifugation, and an aliquot of supernatant fluid was used for the determination of P_i (30).

EXPERIMENTAL AND RESULTS

Incorporation of Oligonucleotides into Polymers

Incorporation of ApA, ApApA, and ApU into Polymers formed with ADP and UDP—Table I contains evidence of the incorporation of these materials into poly A and poly U. Under the conditions used, ApA and ApU were less well utilized than oligonucleotides containing a 5'-phosphomonomer end group (see below). The isomer of ApU containing a 2'-5'-phosphodiester bridge (adenylyl-(2'-5')-uridine) was not incorporated into either poly U or poly A (Experiments 1 and 4, Table I). An equimolar mixture of ApA and adenylyl-(2'-5')-adenosine was tested and 30% (Experiment 1, Table I) was found to be incorporated. The dinucleoside monophosphate which remained was rechromatographed in Solvent 2; it consisted for the most part of adenylyl-(2'-5')-adenosine. This fact indicates preferential utilization of ApA, but the possibility of incorporation of a small amount of the 2'-5' isomer could not be excluded.

In Experiment 2, Table I, which involved UDP and ApA or ApApA, several ultraviolet-absorbing products were separated chromatographically in Solvent 3; (a) polymer, which has an R_f equal to zero and therefore remains at the origin, and (b) several nucleotides of low R_f. The latter were presumed to be small polynucleotides resulting from the addition of 1 to 3 nucleotide units to the original oligonucleotide. These compounds were subsequently investigated in greater detail (see below). Such small polynucleotides are not found as reaction products when polymerization occurs in the absence of added oligonucleotides. Many experiments were carried out under a variety of conditions, but, as shown in Fig. 2 (left), no intermediates of low R_f accumulate unless an oligonucleotide has been included in the reaction mixture.

Incorporation of P_2-labeled pApApA and pApApApA into Poly A—The data presented in Figs. 1 and 2, and in Table II were obtained from one experiment and are concerned with the incorporation of P_2-labeled pApApA into poly A. E. coli polynucleotide phosphorylase was used in this experiment. There is no lag in the polymerization of ADP or UDP with this enzyme, and the addition of oligonucleotide to a reaction mixture has no effect on the rate of P_i release (Fig. 1 A). On the other hand, the formation of acid-insoluble poly A is somewhat slower in the presence of pApApA than in its absence (Fig. 1 A). In other words, the oligonucleotide causes less polymer to be precipitated than is expected from the amount of P_i formed. This is consistent with the initial accumulation of relatively short polynucleotide chains in the presence of oligonucleotide, since it is probable that these chains would not be as readily precipitable by acid.

TABLE I

Incorporation of certain dinucleoside monophosphates into polynucleotides

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound</th>
<th>Initial concentration</th>
<th>Time</th>
<th>Incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ApA</td>
<td>1.0 390 30</td>
<td>1.6 390 0</td>
<td>3.0%</td>
</tr>
<tr>
<td></td>
<td>ApA + adenylyl-(2'-5')-adenosine*</td>
<td>1.6 390 0</td>
<td>3.0%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ApA</td>
<td>1.0 420 50</td>
<td>1.0 420 76</td>
<td>70%</td>
</tr>
<tr>
<td></td>
<td>ApApA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>ApA</td>
<td>2.2 210 200</td>
<td>2.2 210 200</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>ApU</td>
<td>2.2 210 200</td>
<td>2.2 210 200</td>
<td>50%</td>
</tr>
<tr>
<td>4</td>
<td>Adenylyl-(2'-5')-uridine</td>
<td>8.4 210 5</td>
<td>8.4 210 5</td>
<td>5%</td>
</tr>
</tbody>
</table>

* Equimolar isomeric mixture; initial total concentration given.
Fig. 1. The effect of $^{32}$P-labeled pApApA on the polymerization of ADP by E. coli enzyme. The reaction mixture (0.2 ml) contained 25 μg of E. coli polynucleotide phosphorylase per ml and, in μmoles, Tris buffer, pH 8, 38; MgCl$_2$, 3; ADP, 12; and $^{32}$P-labeled pApApA, 0.28 (4500 c.p.m.). At the times indicated, 0.02 ml aliquots were removed and mixed with 0.98 ml of cold, 2.5% HClO$_4$. These suspensions were centrifuged. Samples of the supernatant fluid were used for Pi determination, and others were plated and counted to determine acid-soluble radioactivity. The precipitates were washed with 1.0 ml of 2.5% HClO$_4$ and with 1.0 ml of absolute methanol, after which they were taken up in 0.6 ml of 0.1 M Tris buffer, pH 8 (4). The absorption of these solutions at 260 mμ was calculated as μmoles of “AMP equivalents” per ml.

During the course of the reaction (Experiment of Fig. 1 A), there was loss of $^{32}$P-labeled pApApA from the acid-soluble fraction and the appearance of radioactivity precipitated by perchloric acid (Fig. 1 B). A control experiment (see legend for Fig. 1 B) in which labeled pApApA was added at the end of reaction indicated that there was very little binding of pApApA to the polymer. It could then be concluded that the oligonucleotide was actually incorporated into polymer during its formation. Further evidence for this was provided by chromatograms run in Solvent 1, ultraviolet photographs of which are presented in Fig. 2. The photograph on the right above the results when pApApA was added at the beginning of the incubation. The trinucleotide (Band D) is still present at 1 hour, but only traces can be seen at 3 hours. Polymer (Band A) is evident, as well as the accumulation of bands (B and C) that have lower $R_F$ values than pApApA but do move from the origin. Bands B and C represent relatively short polynucleotide chains; they do not appear when ADP is incubated with enzyme in the absence of oligonucleotide (Fig. 2, left).

The bands A, B, C, and D were eluted with distilled water, and the amount of radioactivity as well as the amount of nucleotide material in fractions of higher chain length (lower $R_F$ value) increases at the expense of smaller molecules. In other experiments, with the use of lower concentrations of pApApA, it was found that the oligonucleotides of short chain length (bands B and C) disappeared completely during the later stages of the reaction.

The incorporation of $^{32}$P-labeled pApApA into poly A was studied under conditions identical with those described for Fig. 1, except that the pApApA was 0.27 mM. After 5 hours, 50% of the $^{32}$P from the labeled pApApA had disappeared from the acid-soluble portion and accumulated in the acid-insoluble poly A. Neither the formation of acid-insoluble, ultra-
FIG. 2. Chromatographic demonstration of incorporation of P\textsuperscript{32}P-labeled pApApA into poly A. The experiment illustrated is the same as that described for Fig. 1. At the times indicated, 0.03-ml aliquots of the reaction mixture were removed and used for descending chromatography in Solvent 1. Ultraviolet prints of these chromatograms are reproduced here. The photograph on the left shows aliquots removed at 1, 3, and 5 hours from a reaction mixture lacking pApApA; the only reaction product to be seen is polymer, at the origin. The track marked "control" represents a similar reaction mixture, except that pApApA was added 5 minutes before the end of a 5-hour incubation period. The photograph on the right shows the effect of incubating ADP, pApApA, and enzyme for 1, 3, and 5 hours. Note the disappearance of pApApA (Band D), the formation of polymer (Band A), and the appearance of Bands B and C which are considered to be relatively short polynucleotide chains (see text).

violet-absorbing material, nor the incorporation of radioactivity into the acid-insoluble fraction was linear with time, although the release of P\textsubscript{i} was linear. This discrepancy may again reflect the initial formation of polynucleotide chains only a few residues longer than pApApApA, since such chains would be expected to be acid-soluble. In other experiments similar results were obtained with pApA.

The possibility that the oligonucleotides undergo preliminary phosphorolysis to give nucleoside diphosphates was ruled out. A large fraction of the incorporation occurs in the first 20 min-
utes when insufficient $P_1$ is present to allow appreciable phosphorolysis. Furthermore, compounds such as $pApApA$ and $ApA$ are incorporated but cannot undergo phosphorolysis (18). Other points are considered under "Discussion".

Experiments of this type have also been carried out with UDP. Chromatographic investigation of the reaction mixtures showed the disappearance of oligonucleotide primer (in some cases this disappearance was complete) as well as the formation of new, discrete bands (Solvent 3) whose $R_f$ values were consistent with the accumulation of relatively short-chain intermediates.

Direct Evidence for the Incorporation of Oligonucleotides into poly $U$—Evidence for the incorporation of $pApA$ into polymer as well as the demonstration that the $pApA$ forms the first two nucleotide units in the polynucleotide chain was obtained by incubating UDP, $pApA$, and polynucleotide phosphorylase, isolating the polymer formed, and digesting it with pancreatic ribonuclease. Chromatography in Solvent 1 indicated the presence of a ribonuclease-resistant oligonucleotide with a low mobility ($R_f$ of a band $= 0.14$). This would be expected for $pApApUp$.

\[ pApA + n \text{UDP} \xrightarrow{\text{polynucleotide phosphorylase}} \text{pApApUpU} + n \text{P}_1 \]  

\[ \text{pApApUpU} \xrightarrow{\text{pancreatic ribonuclease}} \text{pApApUp} + (n - 2)3'-\text{UMP} + \text{uridine} \]

In another experiment this oligonucleotide was isolated and identified. UDP, $pApA$, purified polynucleotide phosphorylase, and crystalline pancreatic ribonuclease were incubated together. Under these conditions the rate of $P_1$ formation was slower than that observed in a control vessel containing neither $pApA$ nor ribonuclease. The mixture was chromatographed in Solvent 1 and three reaction products were observed, namely, uridine, 3'-UMP, and $pApApA$. Three days were required for complete separation of $pApApUp$ from the origin. It was eluted and subjected to paper electrophoresis at pH 3.5. It moved as a single zone and had a mobility (1.2 times that of 3'-UMP at 14 mA and 800 volts) consistent with the proposed structure. The material was eluted from the electrophoresis strip, and one aliquot was hydrolyzed in 1 n HCl for 1 hour at 100°. The products were separated by chromatography in Solvent 4, and two products, whose $R_f$ values were the same as authentic adenine and uridylic acid (mixed 2' and 3' isomers), were detected in a ratio of 2.4:1. The spectrum of the adenine agreed with that observed in a control vessel lacking oligonucleotide. Ratios $R_f$ for $pAp$, AMP, and UMP were 0.36, 1.00, and 0.24, respectively. The material was eluted and digested with alkali to yield a mixture of 3'-AMP, 2'-AMP, and 3'-UMP.

**Table II**

<table>
<thead>
<tr>
<th>Chromatogram band</th>
<th>Counts recovered</th>
<th>Total nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
<td>3 hrs</td>
</tr>
<tr>
<td>$A$</td>
<td>19</td>
<td>31</td>
</tr>
<tr>
<td>$B$</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>$C$</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>$D$</td>
<td>21</td>
<td>7</td>
</tr>
</tbody>
</table>

(500 μg per ml) was added and the incubation continued for 3 more hours. The incubation mixture was chromatographed in Solvent 1, and the products were found to be mainly 3'-UMP, some uridine, and an ultraviolet-absorbing material with the same $R_f$ as authentic $pApApA$. This material was eluted and digested with alkali to yield a mixture of 3'-AMP, 2'-AMP, and 3'-UMP.

**Formation of $pApApApU$ from $pApA$ and $UDP$**—Under the proper conditions it was also possible to demonstrate directly the addition of a uridylic acid residue to $pApA$, and to isolate and characterize $pApApApU$ as the product of such a condensation. The components of the reaction mixture (2.0 ml) were, in μmoles per ml, Tris buffer, pH 8.2, 125; MgCl₂, 10; EDTA, 0.4; UDP, 25; $pApA$, 4.5, and 24 μg of E. coli polynucleotide phosphorylase per ml. The rate of UDP polymerization by this enzyme preparation is only slightly stimulated by the addition of a uridylic acid residue to $pApA$, and to isolate and characterize $pApApApU$ as the product of such a condensation. The components of the reaction mixture (2.0 ml) were, in μmoles per ml, Tris buffer, pH 8.2, 125; MgCl₂, 10; EDTA, 0.4; UDP, 25; $pApA$, 4.5, and 24 μg of E. coli polynucleotide phosphorylase per ml. The rate of UDP polymerization by this enzyme preparation is only slightly stimulated by the addition of a uridylic acid residue to $pApA$, and to isolate and characterize $pApApApU$ as the product of such a condensation.

A similar experiment was carried out with $ApA$. The incubation mixture contained 20 μg per ml of highly purified *Azotobacter* enzyme (S.A. 113), and, in mm concentrations, EDTA, 0.4; MgCl₂, 10; Tris buffer, pH 8.2, 150; UDP, 53, and $ApA$, 0.75. The formation of $P_1$ (in μmoles per ml) amounted to 16 (100 minutes) and 28 (220 minutes) compared with 0.2 and 2.7 for the control lacking oligonucleotide. Pancreatic ribonuclease...
FIG. 3. Time course of the reaction of pApApA with UDP. The incubation mixture (0.2 ml) contained 120 µg per ml of E. coli polynucleotide phosphorylase and, in amoles per ml, Tris buffer, pH 8.2, 125; MgCl₂, 10; EDTA, 0.4; UDP, 24; and pApApA, 4.5. At the times indicated on the chart, aliquots were removed for Pi determination and for chromatography in Solvent 5. The bands corresponding to the compounds indicated on the chart were eluted quantitatively. The results are calculated as amoles of oligonucleotide per ml of reaction. The ordinate on the right refers to polymer material eluted from the origin with 1 N HCl. The concentration of the origin material is expressed as optical density units at 260 nm per ml of reaction.

Effect of Oligonucleotides in Overcoming Lag Phase in Polymerization of Nucleoside Diphosphates

Mii and Ochoa (12) observed that a lag in the formation of Pi occurs during the polymerization of ADP, UDP, and other nucleoside diphosphates by highly purified enzyme fractions from A. agile. These workers also showed that the lag phase can be overcome by the presence of RNA and certain enzymically synthesized polymers (12).

The oligonucleotides discussed in this paper also overcome the lag phase found with purified Azotobacter fractions, and under optimal conditions stimulate the reaction to the same extent as the polymers themselves. As detailed below, the results with nucleotides eluted from paper are subject to a minor source of error because of an unidentified factor in filter paper that has a stimulatory effect. Accordingly, the essential results were confirmed with oligonucleotides separated on polystyrene and cellulose columns.

It should be pointed out that the preparation of polynucleotide phosphorylase from E. coli showed no lag phase in the polymerization of ADP or UDP and no stimulation by oligonucleotides (cf. Fig. 1A, for example). With the A. agile gel eluate, which was not sufficiently purified to show a lag phase, the rate of polymerization of ADP and UDP was, nevertheless, stimulated (about 1.5- to 2.0-fold) by the presence of oligonucleotide. With both of these enzyme fractions, incorporation, in high yield, of compounds such as pApA and ApA into newly formed polymer can be demonstrated (see above).

Effect of Oligonucleotides with 3'-Phosphomonoester End Group on Lag Phase—It can be seen that pApApA and pApA overcome the lag in the polymerization of UDP (Fig. 4 and Table III) as well as ADP (Table V). These compounds also act as primers in the sense that they are incorporated into polymer. In the experiment reported in Table III, the rate of formation of Pi from UDP and the simultaneous disappearance of pApA or pApApA were studied.

FIG. 4. The stimulation of UDP polymerization by pApApA. The reaction mixtures (0.1 ml) contained 33 µg of A. agile polynucleotide phosphorylase (S.A. 150) per ml, 60 mM UDP, and pApApA as indicated. At the times indicated, aliquots were removed for determination of Pi. All values are corrected for Pi present at zero time, usually about 1.5 mM. Concentrations of oligonucleotides are always expressed as amoles of the compound per ml.
In the presence of oligonucleotide, over 50% of the UDP had reacted after 4.5 hours, judging from P₁ release, whereas 70% of the pApA and 75% of the pApApA had disappeared. The rate of P₁ formation in the absence of oligonucleotide was much slower. The rate of polymerization of UDP increases with increasing concentrations of the trinucleotide, pApApA (Fig. 4). A maximal rate appears to be obtained with pApApA concentrations of 0.42 mM or greater. Experiments utilizing ADP as the substrate give similar results and also indicate that 0.42 mM pApApA is an optimal concentration. Experiments were carried out with ADP and UDP, and with the dinucleotide, pApA. Again, the rate of polymerization depends on the concentration of pApA, with a maximum rate at 2.4 mM pApA or higher. Studies of this type also showed that the concentration of pApA-pApA allowing the maximal rate of polymerization of ADP or UDP is about 0.17 mM.

The rate of polymerization of UDP is identical in the presence of an optimal concentration of either pApA, pApApA, or pApApApA. Similar data were obtained with ADP as the substrate, but it should be pointed out that the optimal rates differed with the two nucleoside diphosphates.

It was also observed that when oligonucleotides are present in concentrations exceeding those required for maximal stimulation of the rate of polymerization, they are still effectively incorporated. Thus at 0.4 mM as well as at 1.67 mM pApApA, about 90% of the trinucleotide was incorporated into poly A.

One generalization that can be made from the data discussed above is that the longer the oligonucleotide chain, the smaller the amount required to give a maximal rate of reaction. It should be stressed, however, that each of the above values is only approximate. The oligonucleotides used in these experiments were prepared by elution from paper chromatograms (see "Methods"), and the unknown material that also stimulates polymerization can be eluted from paper even if it has been washed extensively with acetic acid or with alcohol-H₂O mixtures. This material is found on extracting various grades of paper directly with water. The factor is not destroyed by boiling for 5 minutes at pH 7 or by treatment with pancreatic ribonuclease.

No accurate correction can be made for the contribution of this factor but it does not exceed 15% of the stimulation obtained with the tr-, tetra-, and pentanucleotides (see also Table IV). In any case this correction could only accentuate the difference found in concentrations of oligonucleotides required for maximal activity, because of the fact that the area of paper used for elution of each oligonucleotide was approximately proportional to the amount of material eluted.

Although the correction for the active material present in filter paper was small, it was considered desirable to confirm the observations concerning the effect of oligonucleotides on the lag period with compounds obtained by procedures other than paper chromatography.

The trinucleotide, pApApA, obtained from a Dowex 1 column, was further purified by adsorption and elution from charcoal. This pApApA overcame the lag in polymerization; at a concentration of 0.4 mM pApApA, a 10-fold stimulation of the initial rate of polymerization of ADP by Azotobacter enzyme (S.A. 150) was obtained. Before treatment with charcoal, however, the preparation was inactive, and this finding has been interpreted as indicating that some inhibitory material accompanied the oligonucleotide upon its elution from the resin. The pApA eluted from the same column was inactive as a primer, even after charcoal treatment. AMP and ADP eluted from a Dowex 1 column were found to be free of any substance which could act like the filter paper extract. Although active samples of pApA, pApApA, and pApApApA were also obtained from synthetic cellulose ion exchange columns, no completely satisfactory method for the preparation of oligonucleotides for primer studies has yet been developed. In experiments with Azotobacter enzyme (S.A. 150), pApApA and pApApApA eluted from a diethylaminoethyl-cellulose column gave maximal priming at concentrations of 0.9 mM and 0.05 mM, respectively. The dinucleotide, pApA, at a concentration of 1.2 mM, gave about one-half the rate of the others. These results are qualitatively similar to those discussed above for oligonucleotides prepared with paper. However, when concentrations higher than those stated above were used, slower rates of polymer formation were observed, again indicating that the preparations might contain some inhibitory material. The results with pApApA eluted from an epichlorhydrinethanolamine-cellulose column were similar to those obtained with the diethylaminoethyl-cellulose preparations. Experiments carried out with the cellulose columns showed that no active material was eluted from the cellulose itself.

**Effect of ApA, ApApA, and ApApApA on Lag Phase**—Similar but less extensive experiments have been carried out with oligonucleotides such as ApA and its homologues. In this case, too, the greater the chain length the lower the concentration of oligonucleotide required for the maximal rate of polymerization. Thus 0.14 mM ApApApA was about as effective as 0.75 mM ApApA with UDP (Table IV). The course of the reaction was determined both by measurement of P₁ formation and by estimation of the formation of ultraviolet-absorbing polymer insoluble in 2.5% HClO₄. The oligonucleotides used in these experiments were prepared by paper chromatography, and a control was carried out to determine the effect of an equivalent amount of paper extract on the reaction rate (Table IV). It can be seen from these data that the contribution made by the paper extract to the stimulation by the oligonucleotide is very small.

**Effect of Other Nucleotides on Lag Period**—Adenosine and AMP did not overcome the lag period found with purified poly-nucleotide phosphorylase, and neither of these compounds was
The experiments were carried out with two different preparations of *Azotobacter* enzyme and in each case the usual stimulation by poly A was observed. The oligonucleotides were tested at concentrations of 5 mM pTpT and 1 or 2 mM pTpTpT.

**Effect of Oligonucleotides on Polymerization of Thymine Ribonucleoside Pyrophosphate**—Griffin et al. (39) have reported that this nucleoside 5'-pyrophosphate is polymerized by polynucleotide phosphorylase. With 21 µg of purified *Azotobacter* enzyme (S.A. 60) per ml of reaction mixture, a lag period was observed in the formation of P; from 0.009 m thymine ribonucleoside pyrophosphate. The reaction conditions were as described for Fig. 4. The lag was overcome by 3 mM pApApA and by 0.12 mM pApApApA. In another experiment, with pApApA, aliquots of the reaction mixture were chromatographed in Solvent A after incubation periods of 50 and 180 minutes. An ultraviolet photograph of the chromatogram is presented in Fig. 5. The fastest moving area in this descending chromatogram is pApApA and its disappearance during the experiment is evident. The major product had an Rf consistent with its being a tetranucleotide formed by the addition of one thymine ribonucleoside phosphate unit to pApApA. Formed in smaller amounts were materials tentatively assumed to represent the addition of two and three such units to the pApApA, and also polymer, which remained at the origin.

**Effect of KCl—DeBoer (5), with the use of polynucleotide phosphorylase fractionated from extracts of *M. lysodeikticus*, has observed a considerable stimulation of the rate of P; formation from ADP by high concentrations of KCl. It was considered desirable to determine whether the effects of oligonucleotides observed here would be modified by changes in salt concentration. Incubation mixtures were prepared containing purified *Azotobacter* enzyme (S.A. 113, 7 µg per ml) and, in µmoles per ml, MgCl2, 2; ADP, 6; Tris buffer, pH 8.2, 150; pApApApA, 0.15. Compared with a control, the tetranucleotide stimulated the reaction in the usual fashion. A concentration of 0.13 M KCl inhibited the rate of formation of P; in both the presence and absence of pApApApA. In the former case inhibition amounted to 10%, whereas without oligonucleotide the rate attained after the lag phase was inhibited 90%. Similar results were obtained in other experiments with different concentrations of ADP and MgCl2; 0.13 M KCl never stimulated the reaction; it was inhibitory in all cases.

**Effect of Changes in Ratio of Nucleoside Diphosphate to Magnesium**—The effect of certain variations in the concentrations of nucleoside diphosphate and magnesium ion on the rate of polymerization has been studied. These experiments were carried out with a preparation of polynucleotide phosphorylase from *A. agile* which shows the lag phenomenon. The results of such experiments, with UDP as substrate, are summarized in Fig. 6. Both in the presence and in the absence of pApApApA, the reaction is most rapid at a ratio of UDP to Mg++ equal to about 2. The oligonucleotide stimulates the rate of polymerization over the whole range studied, except when UDP is 0.06 M and the ratio of UDP to Mg++ is 0.5. Similar results were obtained with ADP. A stimulation by pApApApA was observed with concentrations of ADP which varied from 0.006 M to 0.12 M and with ADP to Mg++ ratios which varied from 1:1 to 12:1. The magnitude of the stimulation and the shape of the time curves for P; formation varied widely. The experimental conditions described in preceding sections of this paper were about as favorable as any for demonstrating both a lag period in the polymerization of nucleoside diphosphate and the elimination of this lag by an oligonucleotide primer. It is felt that amplification

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**Table IV**


The incubation mixture (0.2 ml) contained 20.4 µg of purified *Azotobacter* polynucleotide phosphorylase (S.A. 113) per ml and 53 mM UDP; other additions are indicated below. Aliquots were removed for chromatography in Solvent 3; quantitative elution showed that over two-thirds of the primer was incorporated. Other aliquots were used for measurement of P; the values are corrected for a small amount of P; present at zero time.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Formation of P;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>142 min</td>
</tr>
<tr>
<td>None</td>
<td>0.6</td>
</tr>
<tr>
<td>Paper extract*</td>
<td>1.1</td>
</tr>
<tr>
<td>ApApA, 0.75 mM</td>
<td>22.6</td>
</tr>
<tr>
<td>ApApApA, 0.42 mM</td>
<td>26.4</td>
</tr>
</tbody>
</table>

* The oligonucleotides had been isolated by separation on paper and elution with distilled water. In this experiment an amount of blank paper at least equivalent to that used with oligonucleotides was similarly treated and the extract tested.

**Table V**

Effect of adenosine 5'-benzyl phosphate, 5'-AMP, and adenosine on lag period

The incubation mixtures (0.1 ml) contained 21 µg, for Experiments 1 and 2, or 33 µg, for Experiment 3, of purified *Azotobacter* polynucleotide phosphorylase (S.A. 150) per ml and 60 mM ADP, for Experiments 1 and 2, or 57 mM UDP, for Experiment 3. Values for formation of P; (µm) are corrected for a small amount present at zero time.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Additions</th>
<th>Formation of P;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60 min</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>Benzyl ester</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Benzyl ester</td>
<td>12.6</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Benzyl ester</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>5'-AMP</td>
<td>9.2</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Benzyl ester</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* The oligonucleotides had been isolated by separation on paper and elution with distilled water. In this experiment an amount of blank paper at least equivalent to that used with oligonucleotides was similarly treated and the extract tested.

incorporated into polymer (Table V). Adenosine 5'-benzyl phosphate (0.005 M to 0.008 M) showed a small but significant stimulation of the rate of polymerization of ADP and UDP (Table V), but there was no detectable incorporation of this compound into the polymer which was formed.

Experiments with two thymidine oligonucleotides, pTpT and pTpTpT, indicated that these deoxyribonucleotides have no effect on the rate of polymerization of ADP by purified *Azotobacter* fractions. The experiments were carried out with two different preparations of *Azotobacter* enzyme and in each case the usual stimulation by poly A was observed. The oligonucleotides were tested at concentrations of 5 mM pTpT and 1 or 2 mM pTpTpT.
of these studies must await the availability of even more highly purified enzyme with, perhaps, an absolute requirement for primer.

**Effect of Oligonucleotides with Phosphomonoester Group at C-3**

**FIG. 5. Chromatogram showing the products formed during the polymerization of thymine ribonucleoside pyrophosphate in the presence of pApApA.** The incubation mixture (0.05 ml) contained 6.0 μg of A. agile polynucleotide phosphorylase (S.A. 113) per ml and, in μmoles per ml, Tris buffer, pH 8.3, 150; EDTA, 0.2; pApA-pApA, 0.12, where indicated; UDP, 20 or 60; and MgCl₂ to give the ratios of UDP to Mg⁺⁺ indicated on the chart. Aliquots were removed for P₁ determination after 0, 1, 2.5, and 3.7 hours of incubation, and the values obtained were corrected for the amount of P₁ present at zero time. The results plotted in the curve show the μmoles of P₁ formed in the first hour of incubation. In no case was more than 12% of the UDP utilized at this time.

**FIG. 6.** The effect of the ratio of UDP to Mg⁺⁺ on the rate of poly U formation. The reaction mixtures (0.20 ml) contained 6.0 μg of A. agile polynucleotide phosphorylase (S.A. 113) per ml and, in μmoles per ml, Tris buffer, pH 8.3, 150; EDTA, 0.2; pApA-pApA, 0.12, where indicated; UDP, 26 or 60; and MgCl₂ to give the ratios of UDP to Mg⁺⁺ indicated on the chart. Aliquots were removed for P₁ determination after 0, 1, 2.5, and 3.7 hours of incubation, and the values obtained were corrected for the amount of P₁ present at zero time. The results plotted in the curve show the μmoles of P₁ formed in the first hour of incubation. In no case was more than 12% of the UDP utilized at this time.

**on Lag Phase**—Although they are not incorporated into the polymer produced, compounds such as ApUp and ApApUp nevertheless do overcome the lag in the polymerization of ADP or UDP that is catalyzed by purified A. agile preparations. The experiments shown in Fig. 7 demonstrate the effect of ApUp on UDP polymerization as an example. Quite similar results were obtained with ApApUp in stimulating the polymerization of ADP, except that here 2.6 mM ApApUp was as effective as a concentration of 1 mg per ml of poly A, and 0.7 mM ApApUp led to an initial rate that was 50% as high. The values are also comparable to those obtained with compounds such as pApA, pApApA, and ApApA. The ability of poly A and poly U to overcome the lag in the polymerization of ADP and UDP, respectively, has been reported previously (12, 13). The experiments reported above were carried out with ApUp and ApApUp prepared by chromatography on Dowex-1 (see "Methods"). The oligonucleotides obtained from paper chromatograms were also active in overcoming the lag period, and this effect was significant even when corrected for the contribution of an equivalent amount of the active agent eluted from filter paper.

Studies were carried out in order to determine whether either ApUp or ApApUp was utilized during the process of polymerization. The design of these experiments was similar to those described above for studying the incorporation of compounds like
pApA into polymer. The results, given in Table VI, indicate that no ApUp or ApApUp disappears even after extensive polymerization has occurred. In addition, chromatographic investigation of the reaction mixtures did not indicate the formation of relatively short polynucleotide chains, as was seen with oligonucleotides such as pApA. These observations, in addition to the fact that ApUp and ApApUp are not utilized, show that these oligonucleotides do not serve as centers for chain extension.

The ability of these compounds to overcome the lag in the polymerization of ADP or UDP was slight, although it was difficult to assess their quantitative effect because of the presence of the paper factor. Michelson’s synthetic polyadenylic acid, the chains of which are terminated by 2',3'-cyclic phosphodiester groups (see “Methods”), was effective in overcoming the lag when used at a concentration of 1.4 mg per ml.

RNA “core”, the polynucleotides obtained from exhaustive digestion of RNA with pancreatic ribonuclease, was only slightly effective in shortening the lag period. Mononucleotides, including 3'-UMP, 3'-AMP, and uridine 2',3'-cyclic phosphate were completely without effect.

**DISCUSSION**

The data presented above show that oligonucleotides, such as pApA, that have a nonesterified C-3' hydroxyl group on the terminal nucleoside residue can take part in the formation of long chain polyribonucleotides catalyzed by polynucleotide phosphorylase. The evidence shows that a nucleoside 5'-diphosphate transfers a nucleoside monophosphate to the free C-3' hydroxyl of the oligonucleotide to form a new, 3'-5'-phosphodiester bond. Several experimental observations indicate that the oligonucleotide molecules are incorporated intact into the polymer and also demonstrate that they occur at the beginning of the chains. The possibility that the oligonucleotides undergo preliminary phosphorolysis to give nucleoside diphosphates was ruled out by several lines of evidence. (a) With pApA, ApA and ApU phosphorolysis does not occur (18). Furthermore, the compound pApApUp has been isolated from a ribonuclease digest of poly U synthesized in the presence of pApA. (b) The direct formation, in high yield, of pApApApU from pApApA and UDP, as well as the analogous synthesis of pApApGpG, has been demonstrated (38). (c) In the early stages of the polymerization reaction, when most of the primer is incorporated, the concentration of P1 is too low to allow appreciable phosphorolysis. (d) The concentrations of oligonucleotides affording optimal rates of polymerization decrease with increasing chain length, a finding which would not be expected if, for example, pApApApA were broken down to pApA before utilization. (e) Serial chromatograms show that such oligonucleotides as pApApA, pApApApApA, and ApApU, remaining in the reaction mixture, appear to be unchanged and homogeneous. However, when pApApA was not

**TABLE VI**

<table>
<thead>
<tr>
<th>Nucleoside diphosphate</th>
<th>Oligonucleotide</th>
<th>Solvent system</th>
<th>Oligonucleotide recovered (max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP</td>
<td>ApUp</td>
<td>3</td>
<td>0 hr 1.5 hr 4 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>UDP</td>
<td>ApApUp</td>
<td>3</td>
<td>0 hr 1.5 hr 3.4 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>UDP</td>
<td>ApUp</td>
<td>2</td>
<td>0 hr 1.5 hr 3 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>UDP</td>
<td>ApApUp</td>
<td>1</td>
<td>0 hr 1.0 hr 3.5 hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.8</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Oligonucleotides serve as primers for the polymerization of adenosine 5'-diphosphate, uridine 5'-diphosphate, and thymine ribonucleoside pyrophosphate catalyzed by polynucleotide phosphorylase. Highly purified preparations of enzyme from Azotobacter agile (12) catalyze the polymerization reaction only after an initial lag period. This lag can be eliminated by oligonucleotides that have an unesterified, terminal, C-3' hydroxyl group. These oligonucleotides serve as starting points for chain proliferation; in their presence polymer chains are extended by successive additions of nucleoside 5'-monophosphate units to the free C-3' hydroxyl groups of the preexisting chains, starting with the addition of a unit to the added oligonucleotide. These primers have little or no effect on the rate of polymerization when relatively crude enzyme preparations (from A. agile or Escherichia coli) are used, but even with these fractions, oligonucleotides are incorporated into polymer and serve as starting points for chain extension.

The lag in polymerization that is found with highly purified A. agile preparations is also overcome by oligonucleotides in which the C-3' hydroxyl of the terminal nucleoside residue is blocked by a phosphate group. However, these compounds are not incorporated into the polymer formed and their mode of action is not understood.

References

Oligonucleotides as Primers for Polynucleotide Phosphorylase
Maxine F. Singer, Leon A. Heppel and Russell J. Hilmoe


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