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Studies of Oligoadenylate Formation on a Poly(U) Template

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Summary. We have studied a variety of condensation reactions involving poly(U) as template and isomeric adenosine dinucleotides as substrates. We find that [3'-5']-linked dinucleotides such as A^3pA and pA^3pA are better acceptors than the corresponding [2'-5']-linked compounds, while $ImpA^2pA$ is a better donor than $ImpA^3pA$. The reaction between A^2pA and $ImpA^3pA$, for example, yields only 4% of product while the reaction of A^3pA with $ImpA^2pA$ yields 86% of product.

The more efficient condensation reactions of dimers are about as efficient as the self-condensation of ImpA. They yield a few percent of material in which five or more substrate molecules are linked together. The percentage of the natural [3'-5']-linkage in the product varies greatly, from as little as 1% to as much as 45%.

Key words: Oligoadenylates – Phosphorimidazolides – Template-directed polycondensation – Isomer ratios – Prebiotic

Introduction

Adenosine and many of its monomeric derivatives form triple-stranded helical aggregates with poly(U), for example, pA:2 poly(U) (Howard et al., 1966; Huang and Ts'o,

Abbreviations. Im, imidazole; MeIm, 1-methylimidazole; A, adenosine; pA, adenosine 5'-phosphate; MepA, adenosine 5'-(phosphoric acid methylester); Ap, adenosine 2',(3')-phosphate; A>p, adenosine cyclic 2',3'-phosphate; ImpA, adenosine 5'-phosphorimidazolide; $(pA)_n$ (n = 2,3...), oligomers of pA; $(Ap)_n$(n = 2,3...), oligomers of Ap; H_2N-pApA, 5'-amidate of a diadenylate pApA; ImpApA, 5'-imidazolide of a diadenylate pApA; $A(pA)_n$, oligoadenylates terminated by a free 5'-OH group; $Me(pA)_n$ (n = 1,2...), 5'-methylester of an oligoadenylate $(pA)_n$. The numbers given as superscripts between a nucleoside and a phosphate indicates the type of internucleotide linkage, e.g. A^2pA^3pA is adenylyl-[2'→5']-adenylyl-[3'→5']-adenosine; poly(U), polyuridylic acid. A star above the symbol for a nucleoside indicates the position of the radioactive label, e.g. $\overset{*}{A}^2pA$, [8-^{14}C]-adenylyl-[2'→5']-adenosine. ODU, optical density units measured at 259 nm. BAP, bacterial alkaline phosphatase (E. coli).

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1966; Shim et al., 1975). These aggregates melt at relatively low temperatures, but are usually stable at 0°C. When an activated derivative of adenosine 5'-phosphate and another derivative of adenosine are incorporated together into a triple-helix, they react together to yield di- and oligonucleotides, often in high yield (Orgel and Lohrmann, 1974). Perhaps surprisingly, these reactions yield an excess of the [2'-5']-linked product rather than the naturally-occurring [3'-5']-linked oligonucleotides (Orgel and Lohrmann, 1974; Lohrmann and Orgel, 1978). In the present paper we present the results of an extended series of studies of the reactions of ImpA, ImpA^2pA and ImpA^3pA with a variety of adenosine derivatives.

Experimental

Materials

Imidazole was purchased from Matheson, Coleman & Bell and was recrystallized from benzene before use. Triphenylphosphine, 2,2'-dipyridyl disulfide, 1-methylimidazole and trimethylphosphate were obtained from Aldrich. 1-methylimidazole was purified by redistillation; trimethylphosphate was shaken with BaO, filtered and redistilled before use.

A and pA were purchased from Terra Marine-Bioresearch, and the corresponding [^{14}C]-labelled compounds from Schwarz-Mann, ImpA and MepA-[8-^{14}C] were prepared as described previously (Lohrmann and Orgel, 1978). A^2pA and A^3pA were synthesized from A > p and A (Sleeper et al., 1978) by an adaptation of a reaction described by Michelson (1959). Radioactive $\overset{*}{A}^2$ pA was prepared by condensation of ImpA with [8-^{14}C]-A on a poly(U) template (Weimann et al., 1968). It was purified by chromatography (System IV). Chromatography of an aliquot in System III showed that the 2'-isomer was 98.8% pure. Radioactive $\overset{*}{A}^3$ p$\overset{*}{A}$ was prepared by partial degradation of [8-^{14}C]-poly(A) as described later. The non-radioactive oligomers, $A(pA)_n$ and $(Ap)_n$ up to the decamer, which were used as chromatographic markers, were prepared in an analogous way. pA^2pA and pA^3pA were synthesized from ApA and $POCl_3$ (Lohrmann, unpublished). The corresponding radioactive dinucleotides were prepared in the same way from $\overset{*}{A}$pA. All dinucleotides were characterized by enzymatic and alkaline hydrolysis. The imidazolides ImpA^2pA and ImpA^3pA, with and without radioactive label, were prepared by a modification of a published procedure (Mukaiyama and Hashimoto, 1971) as described later. Poly(U) and [^{14}C]-labelled poly(A) were prepared by the procedure of Steiner and Beers (1958).

Bacterial alkaline phosphatase (BAPF grade) was purchased from Worthington, venom phosphodiesterase (1 mg/ml) from Boehringer, and Ribonuclease T_2 from Sigma. Prostatic acid phosphatase was prepared according to a procedure developed by Dr. C.K. Biebricher (private communication). The preparation contains both 5'- and 3'-nucleotidase activity.

Chromatography and Electrophoresis

Paper chromatography was carried out on Whatman 3MM paper by the descending technique. The following solvent systems were used:

n-propanol, concentrated ammonia and water (55:10:35) (System I)

Table 1. Chromatographic and Electrophoretic Mobilities

Compound	System I[a]	System II[a]	System III[a]	System VI[b]	System VII[c]
dA					0.00
A	1.55	1.86	0.45	0.00	0.38
pA	1.00	1.00	1.00	1.00	1.00
A^2p	1.13	0.95	0.85	1.08	1.01
A^3p	1.11	0.92	0.62	1.04	0.92
A>p	1.57	1.49	0.32	0.61	
MepA	1.58	1.54	0.67	0.58	0.78
pA^2p	0.70	0.40	1.30	1.63	
pA^3p	0.70	0.40	1.09	1.63	
AppA	1.14	0.52	0.43	0.78	
A^2pA	1.28	0.86	0.30	0.37	0.60
A^3pA	1.37	0.92	0.11	0.33	0.58
A^5pA	1.20	0.80	0.35	0.37	0.73
$A(pA)_2$	0.99	0.29	0.04	0.57	
$A(pA)_3$	0.75	0.06			
$A(pA)_4$	0.50	0.02	0.01	0.79	
$A(pA)_5$	0.34	0.01	0.00	0.84	
$A(pA)_6$	0.09	0.01			
$A(pA)_7$	0.08	0.00			
$A(pA)_8$	0.02	0.00			
pA^2pA	0.88	0.41	0.64	1.08	
pA^3pA	0.86	0.41	0.34	1.01	
$(Ap)_2$	0.85	0.31	0.18	1.05	
$(Ap)_3$	0.63	0.08	0.07	1.10	
$(Ap)_4$	0.44	0.03	0.03	1.11	
$(Ap)_5$	0.26	0.01	0.01	1.12	
$(Ap)_6$	0.16	0.01	0.00	1.12	
ImpA	1.62			0.55	
$ImpA^2pA$	1.27				
$ImpA^3pA$	1.37				
$MepA^2pA$	1.23	0.26	2.48		
$MepA^3pA$	1.20	0.26	1.13		

[a] R_f values are given relative to pA.
[b] Electrophoretic mobilities are given taking $R_{adenosine} = 0$ and $R_{adenylic\ acid} = 1$.
[c] Electrophoretic mobilities are given taking $R_{deoxyadenosine} = 0$ and $R_{adenylic\ acid} = 1$

95% ethanol and 1M ammonium acetate made up to 2×10^{-3}M in EDTA and brought to pH 5.0 with glacial acetic acid (7:3) (System II)

saturated $(NH_4)_2SO_4$, 0.1M sodium acetate, pH 6.5, and isopropanol (79:19:2) (System III)

isopropanol, concentrated ammonia and water (7:1:2) (System IV)

95% ethanol and 1M ammonium acetate, pH 7.5 (7:3) (System V)

Paper electrophoresis was performed on Whatman 3MM paper at 3000 volts (55 volts/cm) using varsol as a coolant. The following buffers were used:

0.03M potassium phosphate, pH 7.1 (System VI)

0.05M sodium borate, pH 8.5 (System VII)

The relevant chromatographic and electrophoretic mobilities of various compounds are listed in Table 1.

The nature of the reaction products was confirmed by comparing their chromatographic and electrophoretic characteristics with those of authentic markers, whenever possible. Quantitative estimates of the product yields were obtained by running the paper chromatograms or electrophoretograms through a Baird Atomic RSC 363 scanner with integrator. When necessary, results from several chromatographic and electrophoretic systems were collated to estimate the yields of individual compounds. Yields are expressed as the percentage of the total radioactivity on the paper, after allowing for the background. In some of our degradation studies, the amount of radioactivity on a chromatogram was too low to permit us to make reliable estimates of the isomer ratios using the strip scanner. In these cases, we cut out the radioactive zones and counted their radioactivity more accurately in a Beckman liquid scintillation counter.

Preparations

$[8\text{-}^{14}C]\text{-}\overset{*}{A}^{3}p\overset{*}{A}$

Radioactive poly(A), (100 μmole; 1 mCi/mmole) was partially hydrolyzed in 0.1M NH_4HCO_3 (20 ml), adjusted to pH 10.0 with ammonia, at 100° under pressure. The time of reaction (30 min) was chosen in order to obtain a series of oligomers up to the decamer.

The solution of oligomers was evaporated to dryness *in vacuo*. The residue was dissolved in 0.1M HCl (20 ml) and the resulting solution adjusted to pH 1.0 with 6N HCl. After 3 h at room temperature, the mixture was neutralized with LiOH, lyophilized and the resulting white residues extracted with absolute ethanol. The suspension was centrifuged, washed with ether and dried *in vacuo*.

The mixture of oligoadenylates was dissolved in 0.25M sodium citrate (5 ml, pH 5.6) and incubated with acid phosphatase (1000 units) for 75 min at 37°. The pH was then brought to 7.0 with NaOH and the solution heated for 2 min at 100° in order to denature the enzyme. Next the volume was adjusted to 70 ml and the solution applied on a Sephadex A25 QAE HCO_3^- column (1.5 x 35 cm). The oligomers were eluted with a linear gradient of triethylammonium bicarbonate (0.02M–1M; 2 x 600 ml). A_2 (14.1% yield) was eluted at 0.06–0.08M buffer concentration. Later peaks contained A_3 (15.7%), A_4 (10.8%), A_5 (11.7%), A_6 (9.2%), A_7 (9.8%), A_8 (6.0%), A_9 (4.9%) and A_{10} (0.5%).

$Imp\overset{*}{A}^{2}pA$ and $Imp\overset{*}{A}^{3}pA$

The triethylammonium salt of the dinucleotide ($p\overset{*}{A}^{2}pA$ or $p\overset{*}{A}^{3}pA$; 1500 ODU, ca. 60 μmoles) was dissolved in an anhydrous mixture containing Im (66.3 mg), triethylamine (56 μl), trioctylamine (56 μl) and dimethylformamide (1.3 ml). Triphenylphosphine (64 mg) and 2,2'-dipyridyl disulfide (54 mg) were added to the solution (Mukaiyama and Hashimoto, 1971). After keeping the mixture for 30 min at room temperature, thin-layer chromatography using the same solvent mixture as in System I on Polygram CEL 300 UV_{254} (from Brinkmann) showed that the reaction was complete.

Table 2. Percentage yields and isomer distribution from reactions between ImpA (donor) and $\overset{*}{A}$, p$\overset{*}{A}$ or Mep$\overset{*}{A}$ (acceptor) on a poly (U) template at 0°. The numbers given in parentheses after the yields give the percentage of the material that is exclusively [2'-5']-linked. A Roman numeral followed by a percentage indicates that the bond specified by the Roman numeral contains the indicated percentage of [3'-5']-linkage. The Table indicates, for example, that after 4 days the yield of tetramer from $\overset{*}{A}$ and ImpA is 7.8% and that $\overset{*}{A}^2$ pA^2 pA^2pA, $\overset{*}{A}^3pApApA$, $\overset{*}{A}^2pA^3pApA$, $\overset{*}{A}^2pA^2pA^3pA$ are present in the ratios of 50. 4:9. 0:35. 9:4. 6

Donor	Acceptor	Time	Monomer	Dimer	Trimer	Tetramer	5-mer	6-mer	7-mer	8-mer
ImpA (2.2×10^{-2}M)	$\overset{*}{A}$ (0.24×10^{-2}M)	5h	56.8	31.6 (98.6) I 1.4	7.4 (78.6) I 16.1 II 5.3	2.1	tr.			
		1d	27.9	44.0 (99.0) I 1.0	17.4 (78.2) I 16.1 II 5.7	7.5 (58.1) I 8.1 II 28.8 III 5.0	2.1	1.1	tr.	
		2d	26.3	43.4	14.3	8.0	2.9	2.0	0.9	tr.
		4d	19.7	50.3	15.6 (78.2) I 16.1 II 5.6	7.8 (50.4) I 9.0 II 35.9 III 4.6	2.9	1.2	0.3	tr.
ImpA (2.2×10^{-2}M)	p$\overset{*}{A}$ (0.24×10^{-2}M)	5h	65.0	23.9 (93.7) I 6.3	8.6 (55.4) I 39.2 II 5.4	2.5	tr.			
		1d	41.9	31.3 (94.5) I 5.5	17.3 (54.2) I 41.4 II 4.3	5.6	2.8	0.8	0.3	
		2d	34.7	34.4	18.5	6.7	3.5	1.9	0.3	
		4d	30.6	32.0 (95.2) I 4.8	19.1 (58.6) I 38.9 II 2.5	9.2 (58.3) I 21.6 II 20.1 III tr.	4.8	2.0	1.4	1.0

Table 2 (cont.)

Donor	Acceptor	Time	Monomer	Dimer	Trimer	Tetramer	5-mer	6-mer	7-mer	8-mer
ImpA (2.2×10^{-2}M)	MepA* (0.24×10^{-2}M)	5h	74.9	17.0 (88.0) I 12.0	7.0 (62.7) I 33.4 II 3.9	1.2				
		1d	49.2	26.2 (92.6) I 7.4	16.8 (64.3) I 31.9 II 3.7	4.7 (56.8) I 22.2 II 11.7 III 9.3	2.1	1.1	tr.	
		2d	50.0	24.4 (89.6) I 10.4	16.0 (62.8) I 32.7 II 4.4	5.8	2.6	1.3	0.6	

In each case the imidazolide was isolated as a sodium salt by dropwise addition of the reaction mixture to a vigorously stirred anhydrous solution containing $NaClO_4$ (30 μmoles), triethylamine (1.9 ml), ether (12 ml) and acetone (25 ml). The white precipitate was collected by centrifugation and washed carefully, first with acetone and then with ether. The yields of isolated material were better than 90%. Chromatography of an aliquot of each reaction product in System I showed it to be better than 98% pure, a trace of dinucleotide being the only UV-absorbing contaminant. The products were stored *in vacuo* over P_2O_5 and solid NaOH.

In order to show that no linkage isomerization occurred during synthesis of the imidazolides, an aliquot (8–10 ODU) of each isomer was hydrolyzed in 0.01M HCl (5 ml) for 1 day at room temperature. Subsequently, the solutions of the dinucleotides were neutralized with aqueous ammonia, evaporated and chromatographed in Systems I and III. Each compound gave a single spot in System I. In System III, which separates pA^2pA from pA^3pA, the mobilities of the two pApA isomers indicated that no linkage isomerization had occurred.

The radioactivel-labelled imidazolides $Imp\overset{*}{A}^2pA$ and $Imp\overset{*}{A}^3pA$ were prepared from the corresponding $p\overset{*}{A}pA$ isomers under strictly anhydrous conditions (dry box). Each isomer (158 ODU, ca. 6.3 μmoles of dimer triethylammonium salt) dried over P_2O_5, *in vacuo*, was dissolved in a mixture containing Im (7 mg), triethylamine (10 μl), trioctylamine (10 μl) and dimethylformamide (150 μl). Subsequently, triphenylphosphine (6.5 mg) and 2,2'-dipyridyl disulfide (5.5 mg) were added, and the resulting mixture kept for 30 min at room temperature. The imidazolides were isolated as sodium salts as described above for the corresponding non-radioactive compounds. Aliquots chromatographed in System I, showed that they were 98% pure, the only contaminants being the corresponding $p\overset{*}{A}pA$'s.

Reactions

Template-Directed Condensations: General Procedure. A solution (200 μl, pH 8.0) containing 0.05M poly(U), 0.20M NaCl, 0.075M $MgCl_2$, 0.2M MeIm-HCl buffer, the donor and the radioactively-labelled acceptor compound (0.1–0.15 mCi/mmole) was kept at 0°. One drop of toluene was added to keep the solutions sterile. Aliquots were taken at various times, mixed with Na_4EDTA and chromatographed in System I. The molarities of donor and acceptor compounds used in each experiment, expressed as the total base present, are given in the appropriate Tables. In arriving at these concentrations we have taken account of the hypochromicity (h) of the A dimers, using a value of 18% for h.

Degradation and Identification of Products

Digestion with alkaline phosphatase (BAP) was carried out for 3 h at 37° in a mixture (100 μl) containing substrate (≤ 5 ODU) 0.1M Tris-HCl (pH 8.5), 10^{-3}M $MgCl_2$, 10^{-6}M $ZnCl_2$ and 0.05 units of enzyme.

Digestion with venom phosphodiesterase was carried out for 1 h at 37° in a mixture (100 μl) containing substrate (≤ 5 ODU), 0.1M Tris-HCl (pH 9.0), 0.01M $MgCl_2$, 0.01M $CaCl_2$ and enzyme solution (20 μl).

Ribonuclease T_2 is an endonuclease specific for [3'-5']-linked phosphodiester bonds and inactive towards [2'-5']-linkages. Digestion was carried out for 1 h at 37° in a

mixture (100 µl) containing substrate (≤ 5 ODU), 0.015M ammonium acetate (pH 4.5) and 1 unit of the enzyme.

In a control experiment we confirmed that a [2'-5']-linked oligomer does not inhibit the enzyme. The presence of A^2pA (2.0 ODU) had no effect on the hydrolysis of $\overset{*}{A}{}^3p\overset{*}{A}$ (3.1 ODU) under our conditions. We also showed that the presence of a [2'-5']-linkage in a molecule does not influence the hydrolysis of a neighboring [3'-5']-bond by demonstrating the complete hydrolysis of the [3'-5']-linkage in $\overset{*}{A}{}^3p\overset{*}{A}{}^2pA$, $\overset{*}{A}{}^2pA^3pA$, etc. Finally, we ruled out the possibility that materials undigestible by RNase T_2 contained [5'-5']-linked phosphodiester bonds by showing that they hydrolyzed completely in 1N KOH (50 h at room temperature).

Our analysis of the reaction mixtures always began with a chromatographic separation in System I. When $\overset{*}{A}$, $\overset{*}{A}{}^2pA$ or $\overset{*}{A}{}^3p\overset{*}{A}$ was used as acceptor, the length of the oligomeric products was estimated by comparing their mobilities with those of authentic $A(pA)_n$'s. This procedure is justified because the mobilities of $A(pA)_n$ oligomers is relatively insensitive to the isomeric character of the linkages.

In reactions with pA and pApA's as acceptors and in the self-condensation of ImpApA's, we used $(Ap)_n$ markers for comparison, since $(Ap)_n$'s are readily available and their chromatographic mobilities are similar to those of the corresponding $(pA)_n$ oligomers. In reactions where MepA was the acceptor (Tables 2, 7), $MepA^2pA$ and $MepA^3pA$ were available as markers (Lohrmann and Orgel, 1978). The nature of the higher homologs in this series was deduced from their chromatographic mobilities and from the products they gave on alkaline and enzymatic degradation.

The major radioactive spots from the chromatograms in System I were eluted and degraded in a series of 4 steps. In Figure 1 we illustrate in some detail the application of our procedure to oligomers up to the tetramer, formed in the reaction between $\overset{*}{p}A$ and ImpA.

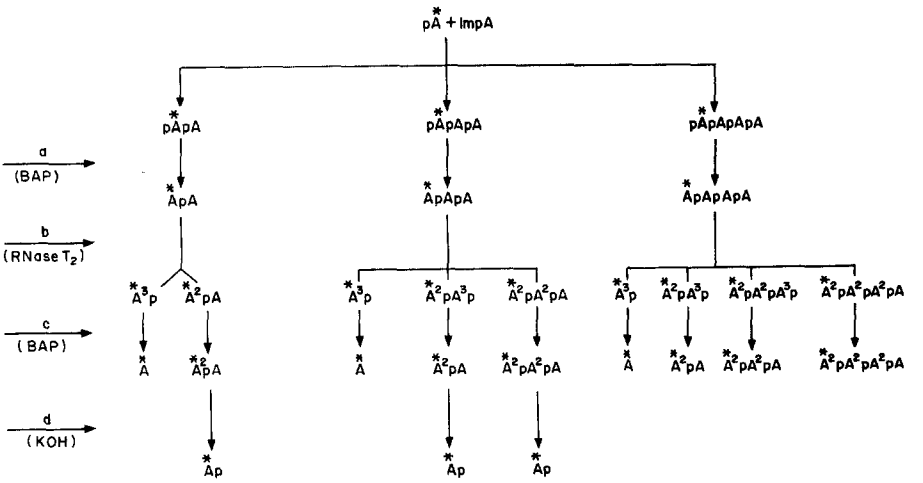


Fig. 1. Degradation and identification of the reaction products, up to the tetramer, formed from ImpA and $\overset{*}{p}A$ (Table 2). Each degradation step (a-d) was followed by a chromatographic separation (see text). Only the radioactive products are shown

In step (a) we incubated the oligomers with BAP. The enzymatic treatment removes 5'-phosphoester groups, both from radioactive and from non-radioactive material that co-chromatographs with it. The latter material is formed by the self-condensation of ImpA. The products from the enzymatic reaction were chromatographed in System I or II.

In step (b) the radioactive spots from the previous chromatography were exposed to RNase T_2. The oligomers containing [3'-5']-linkages hydrolyzed to radioactive fragments of the type $(Ap)_n$ and non-radioactive $A(pA)_n$'s. Oligomers containing only [2'-5']-linkages were not split.

Since $(Ap)_n$ fragments were not always adequately separated from $A(pA)_n$ oligomers, in step (c) the spots were eluted and treated with BAP to obtain $A(pA)_n$'s, which separate well. The amounts of the different isomers could then be deduced from the distribution of radioactivity in the various $A(pA)_n$ spots. Finally, in step (d), the oligomers that were undegradable by RNase T_2 were exposed to 1N KOH. In all cases that could be tested, they gave $\overset{*}{A}p$ as the only radioactive material, indicating that no pyrophosphates were present in the major reaction products.

The procedure outlined in Figure 1 was also applied to other reactions in which $\overset{*}{p}A$, $p\overset{*}{A}^2pA$ or $p\overset{*}{A}^3pA$ served as acceptors. The reaction products derived from $p\overset{*}{A}^3pA$ could not be analyzed for linkage isomerization, because in all cases the [3'-5']-linkage cleaved so that $\overset{*}{A}p$ was the only radioactive fragment.

In a few cases we observed radioactive spots on the chromatogram, which did not fit into the expected chromatographic pattern. They were suspected to be pyrophosphate-linked oligomers of the general type $A^5pA^5pp^5ApA$. In the simplest case, for example, when ImpA reacted with $\overset{*}{p}ApA$'s (Table 4) or when ImpApA's reacted with $\overset{*}{p}A$ (Table 6), we observed a spot moving between $(pA)_2$ and $(pA)_4$ in System I. In our degradation procedure, it was unchanged by BAP. The compound formed from ImpA and $p\overset{*}{A}^2pA$ could not be degraded with RNase T_2, while the compound formed in a similar reaction with $p\overset{*}{A}^3pA$ was hydrolyzed to a new compound which we believe to be AppAp. With BAP the latter compound was converted into AppA, which co-chromatographed with authentic material. The higher numbers of the pyrophosphate series could not be analyzed in detail, since they were formed in extremely low yields.

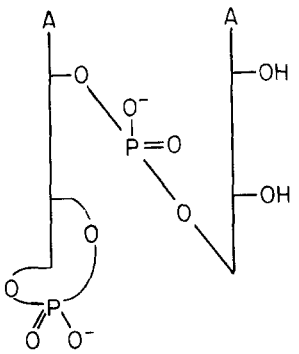
An alternative procedure for the identification of pyrophosphate-linked products was also used. The materials were treated with 1N KOH (50 h, room temperature) and the products subsequently dephosphorylated with BAP. The appearance of AppA indicated that the original compound had contained a pyrophosphate linkage.

When the acceptor molecule was A or one of the ApA's, the radioactive reaction products were of the general type $A(pA)_n$. They were subjected to step (a) even though the radioactive products contained no 5'-phosphomonoester group. This step served as a purification step in which non-radioactive components, derived from ImpA, were dephosphorylated and subsequently separated from unchanged $\overset{*}{A}(pA)_n$. In cases where A or an ApA was the acceptor, step (d) was particularly important since it established that no [5'-5']-linked phosphodiester had been formed.

When MepA was used as acceptor molecule, oligomers of the type $Me(pA)_n$ were obtained. These compounds remained unchanged in step (a), but were partially degraded in step (b). Step (c) followed by (d) enabled us to determine the isomer distribution.

In the experiments where Imp$\overset{*}{A}^2$pA and/or Imp$\overset{*}{A}^3$pA were allowed to undergo self-condensation, the radiochromatograms were much more complex than in the previous experiments. This was particularly the case when aliquots taken at earlier times were analyzed. The more complex chromatographic pattern is a result of the presence of a series of intermediate oligomeric imidazolides, $Im(pApA)_n$ and their corresponding amido derivatives, $NH_2(pApA)_n$. The latter are artifacts that always form when imidazolides, dissolved in MeIm buffer, are chromatographed in an ammoniacal system (Lohrmann and Orgel, 1976). In the latter stage of the experiment, these compounds are absent, since all imidazolides have hydrolyzed or reacted.

Column I in Table 10 contains the total radioactive material moving faster than the dimers p$\overset{*}{A}^2$pA and p$\overset{*}{A}^3$pA. This includes unreacted ImpApA's and NH_2pApA's formed from them in the chromatographic system. When the radioactive Imp$\overset{*}{A}^2$pA isomer was used, an additional fast-moving compound was formed. This compound has a mobility of 1.07 in System I and 0.83 in System VI. It is insensitive to BAP and, with venom phosphodiesterase, gives a compound that has the same mobility as adenosine cyclic 3',5'-phosphate in Systems V and VI. We deduced from this that the original compound is a dinucleotide containing both a cyclic 3',5'-phosphate and a [2'-5']-internucleotide linkage with the following structure:



I

In the self-condensation of Imp$\overset{*}{A}^2$pA this compound accumulated in 4 days in ca. 15% yield (Table 10). The fact, that a corresponding radioactive spot cannot be detected when Imp$\overset{*}{A}^3$pA is used, gives further support for the proposed structure.

The yields given in Column II (Table 10) include dimers formed by hydrolysis of the starting material and [5'-5']-linked pyrophosphates $A^5pA^5pp^5ApA$ which move slightly behind the dimers in System I. Slower moving products, formed in these reactions, are listed in Columns III, IV and V. The mobilities correspond to the tetramer, hexamer and octamer, respectively, but we believe that the spots also include some pyrophosphate-containing products. No attempt was made to characterize the individual components, since the pattern of products is so complicated.

In one experiment, the self-condensation of Imp$\overset{*}{A}^3$pA, we hydrolyzed the material remaining close to the origin of the chromatogram (ca. 5% of the total radioactivity)

with 1N KOH. Chromatography of the hydrolysis product in System II revealed two radioactive spots coinciding with those from Ap and pAp. The distribution of radioactivity in these spots indicated an average chain length of 10.8 for this material.

Discussion

Yields and Isomer Ratios. In almost all of our experiments we have employed a [^{14}C]-labelled acceptor and a non-radioactive donor. This limits the power of our analytical methods. In an oligomer of the type $\overset{*}{A}^2(pA)_m\,^3pA(pA)_n$, we have no way of determining the proportions of isomers in bonds beyond the first [3'-5']-linked bond, since this bond is cleaved by ribonuclease T_2 leaving a non-radioactive fragment $A(pA)_n$ (or a number of smaller fragments if several [3'-5']-linkages are present). This is a particularly important limitation in the analysis of products from reactions in which $p\overset{*}{A}^3pA$ is employed as acceptor.

The results summarized in Table 2 show that ImpA condenses efficiently with $\overset{*}{A}$, $p\overset{*}{A}$ and $Mep\overset{*}{A}$ to yield oligomers up to the octamer. The yield of the higher material falls off fairly slowly with molecular weight, perhaps by a factor of 2 between successive oligomers, so it seems almost certain that small amounts of material with substantially higher molecular weight are present. The oligonucleotide bonds formed are predominantly [2'-5']-linked.

A more detailed analysis of the data in Table 2 reveals significant differences between the reactions. Thus ImpA reacts most efficiently with $\overset{*}{A}$ and least efficiently with $Mep\overset{*}{A}$. The reaction of ImpA with $\overset{*}{A}$ yields $\overset{*}{A}pA$, which contains about 1% of the natural isomer, while reaction with $Mep\overset{*}{A}$ gives about 10% of $Mep\overset{*}{A}^3pA$. The trimer $\overset{*}{A}pApA$ from $\overset{*}{A}$ contains 16% of $\overset{*}{A}^3pApA$, while the $Mep\overset{*}{A}pApA$ obtained from $Mep\overset{*}{A}$ contains 32% of $Mep\overset{*}{A}^3pApA$.

The system of reactions occurring on the template is clearly complex. Even for the simple case of trimer formation from $\overset{*}{A}$ and ImpA we must consider the following family of reactions (heavy arrows indicate reactions in which ImpA is the donor; light arrows indicate reactions in which ImpA^2pA or ImpA^3 pA is the donor):

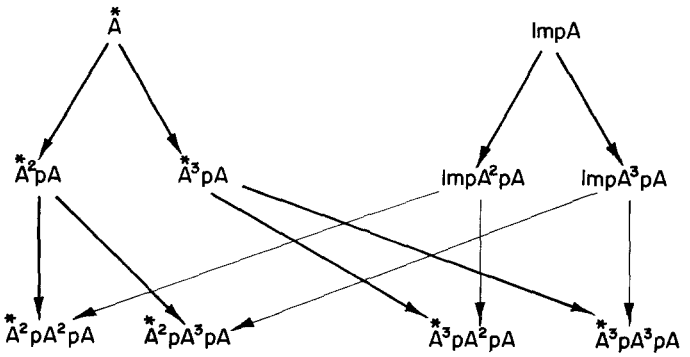


Table 3. Percentage yields and isomer distribution from reactions between ImpA (donor) and $\overset{*}{A}^2pA$ or $\overset{*}{A}^3p\overset{*}{A}$ (acceptor) on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Dimer	Trimer	Tetramer	5-mer	6-mer	7-mer
ImpA (2.2×10^{-2}M)	$\overset{*}{A}^2pA$ (0.24×10^{-2}M)	24h	84.5	7.1	5.8	2.6		
		52h	81.4	6.9	6.9	4.8		
		96h	79.7	7.5 (95.4) II 4.6	8.1 (58.0) II 34.4 III 7.5	4.6		
ImpA (2.2×10^{-2}M)	$\overset{*}{A}^3p\overset{*}{A}$ (0.24×10^{-2}M)	24h	21.7	53.9	14.5	5.7	4.2	
		52h	14.5	55.4	14.5	9.1	6.5	
		96h	11.2	55.2 (96.6) II 3.4	14.5	9.5	5.8	3.9

The interpretation of the results is further complicated by the dependence of the terminal yields of different isomeric oligomers on the rates at which the shorter oligomers are removed by reaction with surviving activated mono- or oligonucleotides.

There seems little hope of sorting out the details of these reactions from the results obtained by the condensation of monomers. Instead, we studied reactions involving isomeric dinucleotides, to determine whether there is any general pattern of dependence of the reactivity on the isomeric character of the reactants. The data presented in Tables 3–10 establish that this is the case.

The experiments summarized in Table 3 show that $\overset{*}{A}^3p\overset{*}{A}$ reacts very efficiently with ImpA while $\overset{*}{A}^2pA$ reacts rather poorly. The newly-formed phosphodiester bond in the trimer that is isolated is mainly [2'-5']-linked in both cases. The yields of trimers from the isomeric pApA's follow the same pattern as those from ApA (Table 4), suggesting that a [3'-5']-linked dinucleotide is a much better acceptor than a [2'-5']-linked compound. This conclusion is made even stronger by the discovery that almost half of the trimeric product obtained from $p\overset{*}{A}^2pA$ is the pyrophosphate $A^5pp^5\overset{*}{A}^2pA$ (Table 4).

In Tables 5–7 we give the corresponding data for the addition of the isomers of ImpApA to $\overset{*}{A}$, $p\overset{*}{A}$ and Mep$\overset{*}{A}$. Here the role of the isomers is reversed. ImpA^2pA is a substantially better donor than ImpA^3pA when $p\overset{*}{A}$ is the acceptor, and a slightly better donor when $\overset{*}{A}$ or Mep$\overset{*}{A}$ is the acceptor. In these reactions, the yields after long times are difficult to interpret on account of the variable reaction rates of the initially-formed trimers with the ImpApA's. An interesting feature of these reactions is the production of substantial amounts of [3'-5']-linked material. This varies from 22–44% for ImpA^2pA and from 15–31% for ImpA^3pA.

The data presented in Tables 3 and 5, and in previous papers (Orgel and Lohrmann, 1974; Lohrmann and Orgel, 1978) enable us to interpret our results on the reaction of ImpA with $\overset{*}{A}$ in somewhat more detail. The $\overset{*}{A}pA$, which is formed initially is almost entirely (98.6%) A^2pA. The reaction of ImpA with $\overset{*}{A}^2pA$ would give about 5% $\overset{*}{A}^2pA^3pA$ and 95% of $\overset{*}{A}^2pA^2pA$. The self-condensation of ImpA would yield ImpApA

Table 4. Percentage yields and isomer distribution from reactions between ImpA (donor) and $pA^{*2}pA$ or $pA^{*3}pA$ (acceptor) on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Dimer	Trimer[a]	Tetramer[a]	Pentamer[a]	≥Hexamer[a]
ImpA (1.75×10^{-2}M)	$pA^{*2}pA$ (0.67×10^{-2}M)	5h	89.8	6.8	2.0	1.4	
		1d	81.3	9.3	9.3		
		2d	80.0	9.1	5.5	5.5	
		4d	77.3	10.9 (54.8)[b] II 2.6	7.6	4.2	
ImpA (1.75×10^{-2}M)	$pA^{*3}pA$ (0.67×10^{-2}M)	5h	59.1	23.8	10.6	1.5	
		2d	21.4	46.4	16.7	9.5	5.9
		4d	19.5	46.6	18.6	11.9	3.4

[a] When $pA^{*2}pA$ was used as acceptor, these oligomers contained also pyrophosphates.
[b] In addition to the 54.8% of A^2pA^2pA there was 42.6% of $A^5pp\overset{*}{A}^2pA$

Table 5. Percentage yields and isomer distribution from reactions between $\overset{*}{A}$, as acceptor and ImpA^{2}pA or ImpA^{3}pA (donor) on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Monomer	Trimer	5-mer	7-mer	9-mer	11-mer
ImpA^{2}pA	$\overset{*}{A}$	1d	44.3	46.9 (78.4) I 21.6	7.2	1.0	0.7	
(2.17×10^{-2}M)	(2.2×10^{-2}M)							
		2d	32.4	53.6	11.7	1.9	0.5	
		4d	32.0	55.1	10.2	2.2	0.4	
ImpA^{3}pA	$\overset{*}{A}$	1d	57.3	29.0	9.5	2.9	1.2	0.2
(2.17×10^{-2}M)	(2.2×10^{-2}M)							
		2d	36.5	33.1	18.1	7.9	3.5	0.9
		4d	31.7	35.4 (69.0) I 31.0	19.2	9.3	4.5	

Table 6. Percentage yields and isomer distribution from reactions between $p\overset{*}{A}$, as acceptor and ImpA^{2}pA or ImpA^{3}pA (donor) on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Monomer	Pyro-phosphate	Trimer	Pyro-phosphate	Pentamer	7-mer	⩾9-mer
ImpA^{2}pA (2.1×10^{-2}M)	$p\overset{*}{A}$ (0.24×10^{-2}M)	5h	58.1	1.9	32.5		5.6	1.3	0.6
		1d	39.5	2.6	43.1		10.8	3.6	0.5
		2d	38.6	2.4	42.7		11.3	4.5	0.6
		4d	34.8	3.1	43.6 (55.8) I 44.2		12.4 (59.7) I 22.3 III 18.0	4.3	1.9
ImpA^{3}pA (2.1×10^{-2}M)	$p\overset{*}{A}$ (0.24×10^{-2}M)	5h	84.3	2.1	10.5		2.4	0.7	
		1d	64.4	4.0	17.2	2.3	6.9	4.0	1.2
		2d	60.4	4.9	15.0	3.1	9.4	4.4	2.8
		4d	57.5	4.4	13.3 (84.7) I 15.3	2.2	8.8 (86.2) I 13.8	8.3	5.5

Table 7. Percentage yields and isomer distribution from reactions between Mep$\overset{*}{A}$, as acceptor and ImpA^{2}pA or ImpA^{3}pA (donor) on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Monomer	Trimer	Pentamer	7-mer	9-mer	11-mer
ImpA^{2}pA	Mep$\overset{*}{A}$	5h	73.0	23.4 (59.4) I 40.6	3.6	tr.		
(2.17×10^{-2}M)	(0.22×10^{-2}M)							
		1d	56.4	31.7 (64.0) I 36.0	9.0 (55.7) I 19.4 III 24.9	2.5	0.4	
		2d	52.7	33.2	9.8	3.3	1.1	
		4d	52.4	34.0 (63.5) I 36.5	9.5 (56.1) I 22.3 III 21.6	3.4	0.7	
ImpA^{3}pA	Mep$\overset{*}{A}$	5h	85.6	10.4 (88.0) I 12.0	2.9	0.5		
(2.17×10^{-2}M)	(0.22×10^{-2}M)							
		1d	66.3	17.4 (83.0) I 17.0	9.6 (80.0) I 20.0	3.9	2.8	tr.
		2d	54.8	18.5	12.7	7.3	4.5	2.2
		4d	49.8	19.5 (91.0) I 9.0	14.4 (83.9) I 16.1	7.9	4.7	2.5

Table 8. Percentage yields and isomer distribution from reactions between $\overset{*}{A}^2pA$ or $\overset{*}{A}^3p\overset{*}{A}$, as acceptor, and $ImpA^2pA$ or $ImpA^3pA$, as donor on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	Dimer	Unknown	Tetramer	6-mer	8-mer
$ImpA^2pA$	$\overset{*}{A}^2pA$	5h	90.7		9.3		
(2.1×10^{-2}M)	(0.4×10^{-2}M)	24h	83.8		15.0	1.2	tr.
		55h	76.9		18.0	4.5	0.6
		5d	74.3		19.7 (54.6) II 45.4	4.4	1.6
$ImpA^2pA$	$\overset{*}{A}^3p\overset{*}{A}$	5h	50.4	tr.	44.6	5.0	
(2.1×10^{-2}M)	(0.4×10^{-2}M)						
		24h	25.5	2.8	53.9	15.6	2.1
		55h	15.2	2.8	56.6	20.7	4.8
		5d	13.8	2.5	51.3 (93.1) II 6.9	26.3	6.3
$ImpA^3pA$	$\overset{*}{A}^2pA$	5h	99.0		1.0		
(2.1×10^{-2}M)	(0.4×10^{-2}M)	24h	98.8		1.2		
		55h	97.5		1.9	0.6	
		5d	95.6		2.2 (20.0) II 80.0	1.7	0.6
$ImpA^3pA$	$\overset{*}{A}^3p\overset{*}{A}$	5h	88.1		11.2	0.8	
(2.1×10^{-2}M)	(0.4×10^{-2}M)	24h	65.7	2.2	23.9	6.7	1.5
		55h	50.8	1.6	30.3	12.3	4.9
		5d	38.7	1.9	29.7 (98.1) II 1.9	16.8	12.9

with 95% [2'-5']-linked. The [2'-5']-linked phosphorimidazolides would react with $\overset{*}{A}$ to give $\overset{*}{A}pApA$, which is 22% [3'-5']-linked.

Since the $\overset{*}{A}pApA$ that is found contains 16% of $\overset{*}{A}^3pA^2pA$, we may conclude more than half is formed by the reaction of ImpApA with $\overset{*}{A}$.

The superiority of $ImpA^2pA$ over $ImpA^3pA$ as a donor and of A^3pA over A^2pA as an acceptor is demonstrated dramatically in Table 8. The most favorable combination, $ImpA^2pA$ and $\overset{*}{A}^3p\overset{*}{A}$, reacts efficiently to give 86% of product, while the least favorable combination, $ImpA^3pA$ and $\overset{*}{A}^2pA$ gives only 4% of product. The reactions of $ImpA^3pA$ with $\overset{*}{A}^3p\overset{*}{A}$ and of $ImpA^2pA$ with $\overset{*}{A}^2pA$ give intermediate yields.

The isomer ratios of newly-formed bonds in these experiments are also strongly dependent on the isomeric character of the reactants. $ImpA^2pA$ with $\overset{*}{A}^2pA$ gives 45% of $\overset{*}{A}^2pA^3pA^2pA$ while $ImpA^3pA$ and $\overset{*}{A}^3p\overset{*}{A}$ give only 2% of $\overset{*}{A}^3pA^3pA^3pA$. The very high proportion of $\overset{*}{A}^2pA^3pA^3pA$ apparently formed from $ImpA^3pA$ and $\overset{*}{A}^2pA$ is subject to great uncertainty, since so little material was available for analysis.

The overall efficiency of the self-condensation of the ImpApA's is not very different from that observed in the corresponding reactions of ImpA. In the case of the self-

Table 9. Percentage yields and isomer distribution from reactions between $p\overset{*}{A}^2pA$ or $p\overset{*}{A}^3pA$, as acceptor, and $ImpA^2pA$ or $ImpA^3pA$, as donor on a poly(U)template at 0°. (For nomenclature see Table 2.)

Donor	Acceptor	Time	$(pA)_2$	Pyrophosphate	$(pA)_4$	$(pA)_6$	$\geqslant(pA)_8$
$ImpA^2pA$ (1.72×10^{-2}M)	$p\overset{*}{A}^2pA$ (0.67×10^{-2}M)						
		5h	84.4	4.7	9.4 (72.9) II 27.1	1.6	tr.
		1d	74.7	7.5	13.8 (65.1) II 34.9	2.9	1.2
		2d	70.5	5.7	17.1	4.8	1.9
		4d	71.9	7.8	15.6 (72.4) II 27.6	3.1	1.6
$ImpA^2pA$ (1.72×10^{-2}M)	$p\overset{*}{A}^3pA$ (0.67×10^{-2}M)						
		5h	56.0	2.7	34.7	6.7	
		1d	35.5	5.3	42.1	15.3	2.0
		2d	32.6	4.3	41.3	17.4	4.4
		4d	28.2	5.9	44.4	17.8	3.7
$ImpA^3pA$ (1.72×10^{-2}M)	$p\overset{*}{A}^2pA$ (0.67×10^{-2}M)						
		5h	93.3	6.7	tr.		
		1d	85.1	9.2	2.1	2.1	1.4
		2d	75.6	14.6	3.7	2.4	3.7
		4d	73.1	13.1	13.4	4.5	4.5
$ImpA^3pA$ (1.72×10^{-2}M)	$p\overset{*}{A}^3pA$ (0.67×10^{-2}M)						
		5h	90.1	tr.	9.9	tr.	
		1d	65.0	4.0	21.0	6.3	3.8
		2d	56.6	3.8	22.6	11.3	5.7
		4d	52.3	4.6	23.1	12.3	7.7

condensation of $Imp\overset{*}{A}^3pA$ we determined the chain length of the products and found 5% of the material with a mean chain length of 10.8. The highest efficiency observed in dimer condensations is, therefore, similar to that observed in the self-condensation of ImpA. In the latter reaction a few percent of material corresponding to pentamer and higher oligomers is obtained.

Table 10. Percentage yields from condensations involving ImpA^{2}pA and ImpA^{3}pA on a poly(U) template at 0°. (For further information see Text.)

Donor	Acceptor	Time	I	II	III	IV	V
Imp$\overset{*}{A}^2$pA	Imp$\overset{*}{A}^2$pA	5h	48.2	38.2	13.8		
(0.12 x 10^{-2}M)	(0.12 x 10^{-2}M)	1d	23.7	46.4	21.4	8.5	
		2d	18.9	48.4	32.5		
		4d	15.2	48.9	23.4	12.5	
Imp$\overset{*}{A}^3$pA	Imp$\overset{*}{A}^3$pA	5h	41.4	35.9	13.9	8.7	
(0.12 x 10^{-2}M)	(0.12 x 10^{-2}M)	1d	13.9	34.1	18.8	17.3	15.9
		2d	7.4	24.6	16.0	22.3	29.7
		4d	3.8	20.9	22.2	20.9	32.2
ImpA^{3}pA	Imp$\overset{*}{A}^2$pA	5h	47.8	35.9	12.0	4.4	
(0.12 x 10^{-2}M)	(0.12 x 10^{-2}M)	1d	25.4	46.9	22.3	13.1	
		2d	17.9	44.8	19.4	17.9	
		4d	11.0	38.0	20.0	17.0	14.0
ImpA^{2}pA	Imp$\overset{*}{A}^3$pA	5h	46.0	34.5	12.6	6.9	
(0.12 x 10^{-2}M)	(0.12 x 10^{-2}M)	1d	21.0	31.9	25.4	21.7	
		2d	12.6	25.2	20.5	20.5	21.3
		4d	tr.	22.0	21.9	21.9	34.2

Chemical Implications. Our studies of the reaction of activated nucleotides with MepU showed that the 2'-OH is 6–9 times more reactive than the 3'-OH group (Lohrmann and Orgel, 1978). In the present studies the proportion of [3'-5']-linked isomers obtained was as low as 1–2% in some cases (Table 1) or as large as 45% in others (Table 8). Clearly orientation on the template can favor the formation of 2'- or 3'-linked isomers depending on the detailed structures of the donor and acceptor.

We find that a substantial proportion of the product consists of pyrophosphates in a few cases (Table 4). We believe that small amounts of pyrophosphates are often formed, but that they represent a significant fraction of the product only when the yield of oligonucleotides is low. We are confident that the general conclusions drawn from the incompletely analyzed experiments described in Table 10 are not falsified by the presence of substantial amounts of pyrophosphates, since the degradation of the products with alkali yielded p$\overset{*}{A}$p and $\overset{*}{A}$p as the only radioactive products. It should be noted that, in any case, only one pyrophosphate bond can be present in any oligomer, no matter how great its molecular weight.

The formation of the cyclic phosphate I in substantial amounts (Table 10, Column I) was unexpected. We would have anticipated that the presence of a bulky substituent on the 2'-OH group would have depressed the yield of the cyclic product.

Prebiotic Significance

The present studies show that the efficiency of template-directed condensation of imidazolides is very sensitive to the isomeric character of both the activated donor and the acceptor. In the most favorable case, the product includes an oligomer produced by seven successive condensations in a yield of about 1%, but contains little material with higher molecular weight. If we suppose that prebiotically interesting polynucleotides must have contained 50 or more monomers, it is clear that reactions of monomers under our conditions is inadequate to account for the production of such polynucleotides.

Independent arguments suggest that template-directed *replication* of molecules could not have occurred using exclusively monomeric substrates. Pyrimidine nucleotide monomers do not stack on complementary purine-containing templates and consequently do not take part in template-directed reactions. We believe, therefore, that non-enzymatic replication of polynucleotides must have involved activated oligomers (Orgel and Lohrmann, 1974). It is encouraging that the efficiency of condensation of the imidazolides of pA^2pA or pA^3pA is similar to that of ImpA.

We think it unlikely that the substrates of prebiotic polynucleotide replication were long oligomers since such oligomers are likely to stack on templates even when their sequences fail to match everywhere. We presume, therefore, that simple catalysts must have improved the efficiency of synthesis of long oligomers from short oligonucleotides by at least a factor of 10. We are searching for such catalysts, and have some preliminary indications that certain metal salts display promising activity.

The isomer-dependence of our reaction is surprising. Unfortunately [2'-5']-linked poly(U) is not available, so we cannot study the effect of the isomeric structure of the template. However, the results which we have accumulated already suggest that there will be very substantial differences in the rate of synthesis of superficially similar oligomers, e.g. between A^2pA^3pA and A^3pA^2pA (Table 2). Usher and coworkers have already shown that [2'-5']-linked molecules hydrolyze much faster than [3'-5']-linked molecules when they are organized in helical structures (Usher and McHale, 1976). Taken together, these facts suggest that the sequence of [2'-5']- and [3'-5']-bonds as well as the base-sequence must be taken into account in considering the pre-enzymatic evolution of polynucleotides.

It is impossible to establish the relative abundances of [2'-5']- and [3'-5']-linkages that would have been present in prebiotic polynucleotides, since our knowledge of the relevant chemistry is fragmentary, and we cannot estimate the relative importance of synthetic and hydrolytic processes in determining the steady-state concentrations. However, it seems likely that a good deal, and perhaps a preponderance, of [2'-5']-linked product must have been present. If this is so, we may anticipate that many of the functions performed by RNA, with the help of enzymes, occurred spontaneously, but with low efficiency, when polymers containing both [2'-5']- and [3'-5']-links were present.

Finally, we emphasize that all of the reactions we have described in this paper occur in triple-helical structures. Reactions involving double-helical structures were probably more important on the primitive earth. We are beginning to study such reactions.

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