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The Prebiotic Synthesis of Deoxythymidine Oligonucleotides

II. Comparison of Condensing Agents*

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Summary. A reaction which oligomerizes nucleotides under possible prebiotic conditions has been characterized. Nucleoside monophosphate in the presence of cyanamide at acid pH condenses to form dithymidine pyrophosphate and phosphodiester bonded compounds. Imidazole compounds and activated precursors such as nucleoside triphosphate are not necessary for this oligomerization reaction which produces primarily cyclic oligonucleotides.

Key words: Prebiotic - Synthesis - Nucleotides - Oligonucleotides - Cyanamide

INTRODUCTION

Several investigators have reported possible prebiotic reactions that oligomerize nucleotides. Ibanez et al. (1971a, b) studied the effects of imidazole and cyanamide on the condensation of mononucleotides at neutral pH at 90 °, and reported the production of extremely small amounts of oligonucleotide up to the pentamer in size. Tapiero & Nagvary (1971), building on work by Sanchez & Orgel (1970), polymerized cytidine 2',3'-cyclic phosphate at 138 ° and obtained 2% of the pentanucleotide. Osterberg et al. (1973), in studying a ureacatalyzed phosphorylation reaction at 100 °, found 7% of the trimer after 11 days of heating. Verlander &

*Abbreviations: CDI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMAPN, dimethylaminopropionitrile; TMP, thymidine-5'-monophosphate; TTP, thymidine-5'-triphosphate; AICA, 4-amino-5-imidazole carboxamide; TLC, thin layer chromatography; DAMN, diaminomaleonitrile.

Orgel (1974) polymerized adenosine cyclic 2',3'-phosphate at room temperature in the presence of various catalysts and obtained about 2% of the heptamer after 40 days of reaction. Stephen-Sherwood et al. (1974) polymerized nucleotide and nucleoside triphosphate at 90 ^{o} in the presence of 4-amino-5-imidazole carboxamide (AICA) and cyanamide for 40 hours to obtain 6% of the trimer and undisclosed amounts of the tetramer.

In all but one of the above studies, moderately high temperatures were employed and either dry or evaporating to dryness conditions prevailed. These conditions were thought to simulate the effects of drying on primeval lakes or ocean shores. Moderately high temperatures have been shown to exist on contemporary earth (references quoted by Bishop et al., 1972).

Mechanisms of nucleotide condensation reactions have been investigated by several authors. Pongs & Ts'O (1969, 1971) in studies on a chemical polymerization system, found that a proton donor (a Lowry-Bronsted acid) such as imidazole and certain imidazole derivatives was necessary for the reaction. Their studies implied that the reaction proceeded through a pyrophosphate intermediate. An alternate possibility for the mechanism of the anhydrous condensation reaction of nucleotides involves the trimetaphosphate as an intermediate (Weimann & Khorana, 1962; Jacob & Khorana, 1964). Glonek et al. (1974) recently synthesized adenosine trimetaphosphate and reported some of its properties, including its immediate hydrolysis on contact with water. Osterberg & Orgel (1972) have reported the production of a trimetaphosphate under possible prebiotic conditions and even found that the nucleoside catalyzed trimetaphosphate formation in a mixture of urea and ammonium phosphate. For the polymerization of 2',3'-cyclic adenosine monophosphate to oligonucleotides, Verlander et al. (1973) found that the best catalysts were bases such as diaminoethane or diaminopropane in the monocationic state.

In the above studies catalysis was mediated only by an acid-base agent. Condensing agents were not used. A condensing agent well known as a prebiotic reagent, cyanamide (Oro, 1963), has been used in some nucleotide oligomerization reactions (Ibanez et al., 1971a,b; Stephen-Sherwood et al., 1974). Steinman et al. (1964) emphasized the fact that the cyanamide dimer, which they used in aqueous solution as a condensing agent, is a tautomer of carbodiimide, and mentioned the fact that carbodiimides are commonly used to effect condensation and dehydration reactions. Carbodiimides have also been used in studies having prebiotic relevance,

albeit no direct connection with prebiotic chemistry (Sulston et al., 1968a,b). Cyanamide, the most plausible prebiotic condensing agent, would exist primarily as a nitrile in aqueous solution. Use of a nitrile as a condensing agent in prebiotic reactions has not been reported in the literature.

The purposes of this study were to (a) determine whether a carbodiimide or a nitrile is more effective in oligomerizing nucleotides under assumed prebiotic conditions, (b) determine whether the triphosphate is necessary in the reaction previously reported by Stephen-Sherwood et al. (1974), (c) determine the consequences of the absence of a proton donor (AICA) and of a condensing agent ($CNNH_2$) in the presumed prebiotic reaction, and (d) determine if the reaction could proceed at neutral pH, a condition which, if met, would enhance the plausibility of the reaction as a simulation of prebiotic conditions.

EXPERIMENTAL

Materials. Cyanamide (Eastman Kodak, mp 42 ^{o}C), 4-amino-5-imidazole carboxamide hydrochloride grade A (AICA) (Calbiochem), deoxythymidine 5'-phosphate dipotassium salt (TMP) (Calbiochem), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (CDI) (Ott Chemical Co.), dimethylaminopropionitrile (DMAPN) (General Biochemicals), diaminomaleonitrile (DAMN) (Terra-Marine Bioresearch), thymidine-2-^{14}C-5' triphosphate (TTP) and thymidine-2-^{14}C-5' monophosphate (Amersham/Searle), venom phosphodiesterase (Worthington code VHP), alkaline phosphatase (Worthington code BAPF), spleen phosphodiesterase (Sigma P-6752), polyethyleneimine cellulose thin layer chromatography plates (Brinkmann or J.T. Baker) (PEI plates), Whatman #1 paper, standard oligonucleotides $(pT)_2$, $(pT)_3$, $(pT)_4$, $(pT)_5$ (Collaborative Research and P-L Biochemicals). Nucleotides were assayed for purity by chromatography in system I prior to use; the melting point of the cyanamide was checked periodically; the alkaline phosphatase was shown to be phosphodiesterase free by its reaction with $(pT)_2$; PEI plates were washed in distilled water and air dryed prior to use. All other chemicals were reagent grade from standard supply houses.

Chromatography. Thin layer chromatography was performed on 20 x 20 cm plastic sheets coated with polyethylene imine cellulose (Brinkmann or J.T. Baker) using two solvent systems: I, 20% formic acid, II, development in 20% formic

Table 1
Rf values of thymidine oligonucleotides and related compounds in 7:1:2 (v/v) isopropanol:con. ammonia:water (after Tener et al., 1958)

Compound	Rf
Thymidine	0.67
TMP	0.15
Linear dimer	0.08
Linear trimer	0.045
Cyclic TMP	0.45
Cyclic dimer	0.28
Cyclic trimer	0.12
Dithymidine monophosphate (TpT)	0.42
Trithymidine diphosphate (TpTpT)	0.21

acid followed by air drying and development in the same direction with 0.5 M ammonium formate pH 4. Rf values of standard compounds for these two solvent systems are given in Table 1. Use of solvent system I enables one to precisely quantitate the yield of nucleoside and mononucleotide of total condensed product including polyphosphates. Use of system II enables one to obtain the values for yields of total monomer (nucleoside plus mononucleotide), the dimer, and, in the absence of polyphosphate compounds, the trimer. Paper chromatography using 7:1:2 v/v isopropanol:con. ammonia:water was performed as in Tener et al. (1958). Rf values are given in Table 1.

Enzymatic Hydrolyses. Specific hydrolyses using venom phosphodiesterase, which attacks oligonucleotides with 3'-hydroxy termini to liberate thymidine-5'-monophosphate, spleen phosphodiesterase, which attacks oligonucleotides with 5'-hydroxy termini to liberate thymidine-3'-monophosphate, and phosphodiesterase-free alkaline phosphatase, which removes any terminal phosphates from a mono- or oligonucleotide, were performed as in the Worthington Enzymes Manual (Worthington Biochemical Corp., Freehold, N.J., 1972). Phosphatase and venom phosphodiesterase digest were performed simultaneously. Phosphatase-spleen phosphodiesterase digestions were performed sequentially with EDTA removal of magnesium ions and pH adjustment from 9 to 6.5 after the phosphatase digest. The reaction products were subjected to five hydrolytic degradations: venom phosphodiesterase, spleen phosphodiesterase, alkaline phosphatase, simultaneous digestion by phosphatase and venom phosphodiesterase, and sequential digestion by phosphatase and spleen phosphodiesterase.

The Reactions. Standard solutions containing 30 μmoles TMP per ml, 180 μmoles AICA HCl per ml, and 120 μmoles/ml each of DMAPN, CDI, cyanamide, or DAMN were prepared. The concentration of the radiolabelled TMP and TTP was 0.3 μmoles/ml, 20 μCi/ml. Reaction solutions were prepared by putting 10 μl of the stock solution of each desired reagent in a vial with 100 μl of water. To assure a constant pH throughout the course of the reaction, the pH value was adjusted with HCl solution or NaOH solution, and measured with pHydrion paper. After pH adjustment, 5 or 10 μl of ^{14}C-nucleotide was added and the uncapped vial was incubated at 90-95 °C for 14-18 hours (except in the time of course study). The dried reaction product was dissolved in 100 μl of water. Portions of this solution were used for chromatography and for enzymatic studies.

Pyrophosphate Bond Degradation. Pyrophosphate bonds in the reaction product were degraded by hydrolysis after treatment with acetic anhydride (Khorana et al., 1962).

RESULTS AND DISCUSSION

The solution containing TMP, AICA, cyanamide, and ^{14}C-TTP, which has given reasonable yields of oligonucleotides in previous studies (Stephen-Sherwood et al., 1974), was found to have an initial pH of approximately 3. A reaction using the same proportions of the reactants, but at one-thirtieth the concentration, was found to give the same experimental results as previously obtained.

Solutions containing TMP, AICA, cyanamide, and ^{14}C-TMP were allowed to react at pH 3. The yield of cyclic dinucleotide from these reactions was approximately 28%. The reaction products after pyrophosphate degradation contained 35% monomer. These results imply that the activated precursor, thymidine triphosphate, is not essential for an oligomerization reaction producing cyclic oligomers. Alkaline phosphatase studies did not indicate the presence of significant amounts of polyphosphorylated species of this reaction or of any other reaction where such species were not included in the reactants.

Solutions with TMP, cyanamide, and ^{14}C-TMP at pH 3 yielded as reactions products 28-36% cyclic dinucleotide and 24% higher products. Total yields of oligomeric products was ∿60% after pyrophosphate degradation. Solution containing TMP, AICA, and ^{14}C-TTP at pH 3 yielded no detectable oligomeric product. Thus, neither AICA nor the "activated" precursor TTP is sufficient or necessary for an oligomeriza-

Table 2
Results of enzymatic digests on products of the TMP-cyanamide-^{14}C-TMP pH 3 reaction after pyrophosphate degradation

	Thy	TMP	Products
Reaction	5-16%	25%	57%
Venom	6-16%	84%	
Spleen	6-12%		73%
Phosphatase	20-40%		∿70%
Phosphatase & Venom	96%		
Phosphatase & Spleen	26%		60%

tion reaction to proceed. The necessary and sufficient condition, within the limits of this study, is the presence of cyanamide in an acid solution.

Reference to Table 2 shows that the reaction products of an acid reaction containing TMP and cyanamide but not AICA were completely degraded by venom phosphodiesterase and partially degraded by alkaline phosphatase, but not affected by spleen phosphodiesterase either alone or in combination with the alkaline phosphatase. Because all disubstituted pyrophosphates have already been degraded to monophosphates prior to enzymatic digestion, we are left with only two sets of compounds whose characteristics are those of the reaction products: oligomers with a 5'-5' bond and cyclic oligomers (Tener et al., 1958). Determination of Rf values in the paper chromatography system employed allows us to identify our reaction products as the cyclic oligomers.

Studies with condensing agents other than cyanamide (i.e., CDI, DMAPN, DAMN) with TMP and ^{14}C-TMP at pH 3 gave negligible yield of oligomers (less than 3% distinct peaks on the chromatograms). A summary of the results from all the reactions is presented in Table 3.

The yield of the reaction was studied as a function of time. TMP-cyanamide-^{14}C-TMP solutions at pH 3 were incubated for 2, 4, 6, 8, 10, 12, 14, 26, 38 and 50 hours. All the water evaporated the fourth hour. At the end of the second hour, a 30% yield of dimer and an 11% yield of higher product was obtained. At the end of the fourth hour and of all subsequent hours, the yields were 28-36% dimer and 69-78% total product (dimer and high oligomers). Thus, the reaction occurred under hydrous conditions and appeared to cease under anhydrous conditions. To test whether the condensation reaction was a function of decreasing water activity, an aqueous solution containing TMP, cyanamide and ^{14}C-TMP was heated at 90 ^{o}C in a sealed tube, i.e. little or no evap-

Table 3
Summary of results

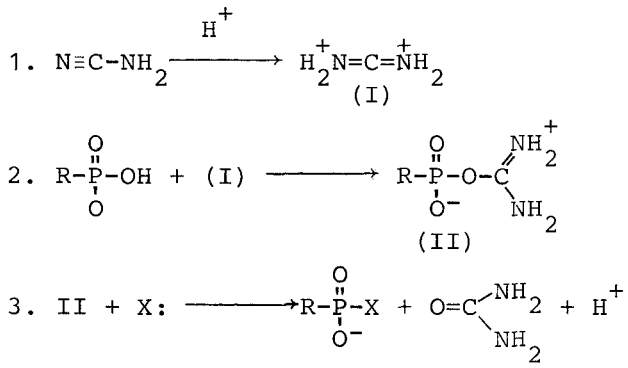
Reactants isotope pH	TMP, Cyan *pT 3	TMP, Cyan *pppT 3	TMP, other[b] *pT pH3	TMP, AICA, Cyan *pT pH3	TMP, AICA, Cyan *pppT pH3	TMP, AICA *pppT pH3	TMP, Cyan w/&w/o AICA *pT or *pppT pH7
T	5-10%	30%	6-10%	10-14%	3%	48%	97%
pT	7-25%		80-91%	42-47%	19%		
Dimer[c]	10-36%	12-14%		27-34%	41%	0%	<2%
Trimer[c]	7-13%	7%		2.5-3%			
Higher products[a]	20-45%		<3%	<10%			
Polyphos-phates	None	45%	<2%	None		50%	
Total yield	69-86%		∿0				

[a]Includes trinucleotide.
[b]Condensing agents include DMAPN, DAMN, Urea or CDI.
[c]Linear and cyclic oligomers, primarily cyclic.

oration took place. Chromatography of the reaction mixture after 15 hours of incubation showed no condensation. It would appear that the concentrative mechanism of evaporation is necessary for the reaction to occur.

The TMP-cyanamide evaporative reaction was run at pH values of 3, 4, 5, 6, 7, 8, 9, 10 and 11. At pH 3, the total yield of oligomeric product was 69-83%. At all other pH values the yields were less than 5%. Thus, the low yields of Ibanez et al. (1971a) in a similar reaction might be attributable to a combination of differing pH and the lack of a concentrative process in that study.

Mechanism of the Reaction. The following mechanism is proposed for the reaction:



The nucleophile X attacks the phosphorus and displaces the oxygen, forming a phosphodiester bond if X is a sugar moiety or a pyrophosphate bond if X is another phosphate moiety. This mechanism follows the suggestions of Steinman et al. (1964) concerning dicyandiamide mediated condensation reactions.

CONCLUSION

A reaction in which nucleotides are polymerized to yield cyclic oligomers at acid pH under drying conditions has been reported. The nucleotide and a condensing agent such as cyanamide are the only reagents necessary for the reaction to proceed. The elevated temperature and acid pH used in the current work render the reaction unsuitable as a strict model for prebiotic pathways.

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