SPECIFICITY OF OLIGONUCLEOTIDE SYNTHESIS DIRECTED BY POLYURIDYLIC ACID

By J. Sulston, R. Lohrmann, L. E. Orgel, and H. Todd Miles

THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, SAN DIEGO, CALIFORNIA, AND LABORATORY OF MOLECULAR BIOLOGY, NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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We have shown that the formation of oligoadenylic acids from adenylic acid, using a water-soluble carbodiimide as condensing agent, is facilitated by the presence of a polyuridylic acid template.[1] Here we discuss the base specificity of this reaction.

Materials and Methods.—Paper chromatography was carried out on Whatman 3 MM paper by the descending technique. The solvent systems were: I, isopropanol-concentrated ammonia-water (7:1:2, v/v); II, n-propanol-concentrated ammonia-water (55:10:35, v/v); III, saturated aqueous ammonium sulphate-0.1 *M* sodium acetate-isopropanol (79:19:2, v/v); IV, n-butanol-5 *M* acetic acid (2:1, v/v); V, isopropanol-concentrated ammonia-0.1 *M* boric acid (7:1:2, v/v). Paper electrophoresis was carried out on Whatman 3 MM paper, using varsol or CCl_4 as coolant. The buffers were: VI, formic acid-ammonium formate, 0.05 *M*, pH 2.7; VII, triethylammonium bicarbonate, 0.05 *M*, *ca.* pH 7.5.

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (CDI) could be determined with reasonable accuracy by the oxalic acid-permanganate method,[2] even in the presence of small amounts of nucleosides and nucleotides. The consumption of permanganate by the nucleosides was always estimated in an independent titration.

Pancreatic ribonuclease is an endonuclease which hydrolyzes phosphodiester linkages only at the 3′ position of pyrimidine nucleotide residues. The stock solution contained 1 mg of enzyme (crystalline) in 1 ml of tris(hydroxymethyl)aminomethane-HCl (tris-HCl) buffer (0.1 *M*, pH 7.0). One optical density unit (ODU) of substrate was dissolved in 10 μl of enzyme solution. After incubation at 37° for 1 hr, the products were separated in system VII.

Ribonuclease T_1 is an endonuclease that hydrolyzes phosphodiester linkages only at the 3′ position of guanosine residues. The stock solution contained 0.8 mg of enzyme in 5 ml of tris-HCl buffer (0.1 *M*, pH 7.0). One ODU of substrate was dissolved in 10 μl of enzyme solution. After incubation at 37° for 1 hr, the products were separated in system VII.

Ribonuclease T_2 is an endonuclease which hydrolyzes only 3′-5′ phosphodiester linkages. It was used as before, except that the incubation time was reduced to 30 min.

Other methods and sources have been described.[1]

Results.—Specificity of incorporation on a poly U template: Fourteen reaction mixtures were prepared. Each contained: pA,[3] 0.0125 *M;* $MgCl_2$, 0.075 *M;* NaCl, 0.2 *M;* CDI, 0.2 *M*. In addition, the following reagents were present where indicated by a plus sign in Table 1: poly U, 0.045 *M;* A, C, U, 0.0125 *M;* G, 0.00125 *M* (except solution 5, which was saturated with G). In each reaction mixture, one nucleoside was isotopically labeled (asterisk in Table 1): adenosine-8-C^{14}, cytidine-2-C^{14}, uridine-2-C^{14}, 0.16 μc/μM; guanosine-8-C^{14}, 1.6 μc/μM. The mixtures were initially adjusted to pH 5.5 and kept at 0°C. After three weeks the products were separated by chromatography in system I (see Fig. 1). The radioactive material, with R_f corresponding to the dinucleo-

TABLE 1. *Incorporation on a poly U template.*

Reaction	Poly U	A	G	C	U	Product	Per cent yield[a]
1	+	*+	...	...	...	*ApA	19.3
2	+	...	*+	...	...	*GpA	3.2
3	+	...	...	*+	...	*CpA	0.3
4	+	...	...	...	*+	*UpA	0.5
5	+	*+	+	...	...	*ApA	19.7
6	+	*+	...	+	...	*ApA	18.6
7	+	*+	...	...	+	*ApA	19.1
8	+	+	*+	...	...	*GpA	3.2
9	+	+	...	*+	...	*CpA	0.3
10	+	+	...	...	*+	*UpA	0.5
11	...	*+	...	...	...	*ApA	2.0
12	...	...	*+	...	...	*GpA	2.7
13	...	...	...	*+	...	*CpA	0.3
14	...	...	...	...	*+	*UpA	0.5

Asterisks denote C^{14} label.
[a] Yield of material moving as dinucleoside phosphate in both systems I and VI.

side phosphate, was eluted and subjected to electrophoresis in system VII. The over-all yields are shown in Table 1.

The solubility of guanosine in water is about 0.0013 M at 0°C. In order to test the effect of lowering the nucleoside concentration, reaction 1 was repeated with the concentration of A reduced to 0.00125 M. The yield of ApA was *ca.* 37 per cent. We attribute the increased yield to the greater proportion of A molecules having pA neighbors.

The products from reactions 11, 12, 13, and 14 were eluted and degraded as follows: ApA (reaction 11) was incubated with ribonuclease T_2 in the presence of nonradioactive $A^{3'}$ pA as an internal control and carrier. Electrophoresis in system VII separated Ap from unchanged ApA. The extent of degradation (corresponding to the proportion of 3′-5′ isomer) was estimated by scintillation counting. The unchanged ApA was treated with KOH in the presence of more unlabeled $A^{3'}$ pA; a similar analysis of the products gave the proportion of 2′-5′ isomer. As a further check the adenylic acid formed in this alkaline hydrolysis was subjected to chromatography in system V to confirm that no $p^{5'}$ A was present. The remaining ApA, presumably the 5′-5′ isomer, was degraded by venom phosphodiesterase. The degradation of the other dinucleoside phosphates differed only in the use of ribonuclease T_1 to hydrolyze $G^{3'}$ pA, and pancreatic ribonuclease to hydrolyze $C^{3'}$ pA and $U^{3'}$ pA, with appropriate carriers in each case. The results are summarized in Table 2, together with the isomer ratios of *ApA formed on a poly U template. In view of the small amounts of material available from reactions 12, 13, and 14 these are rough estimates.

Specificity of template for ApA formation: Three reaction mixtures were prepared. Each contained: adenosine-8-C^{14}, 0.0125 M, 0.16 μc/μM; pA, 0.0125 M; $MgCl_2$, 0.075 M; NaCl, 0.2 M; CDI, 0.2 M. Reaction 1 contained no polymer; reaction 2 was 0.05 M in poly U, and reaction 3 was 0.05 M in poly C. Poly A precipitated under these conditions and so could not be used. The mixtures were adjusted to pH 5.6 and kept at 0°C. After ten days the products were separated in system I, and the yield of ApA relative to unchanged A was mea-

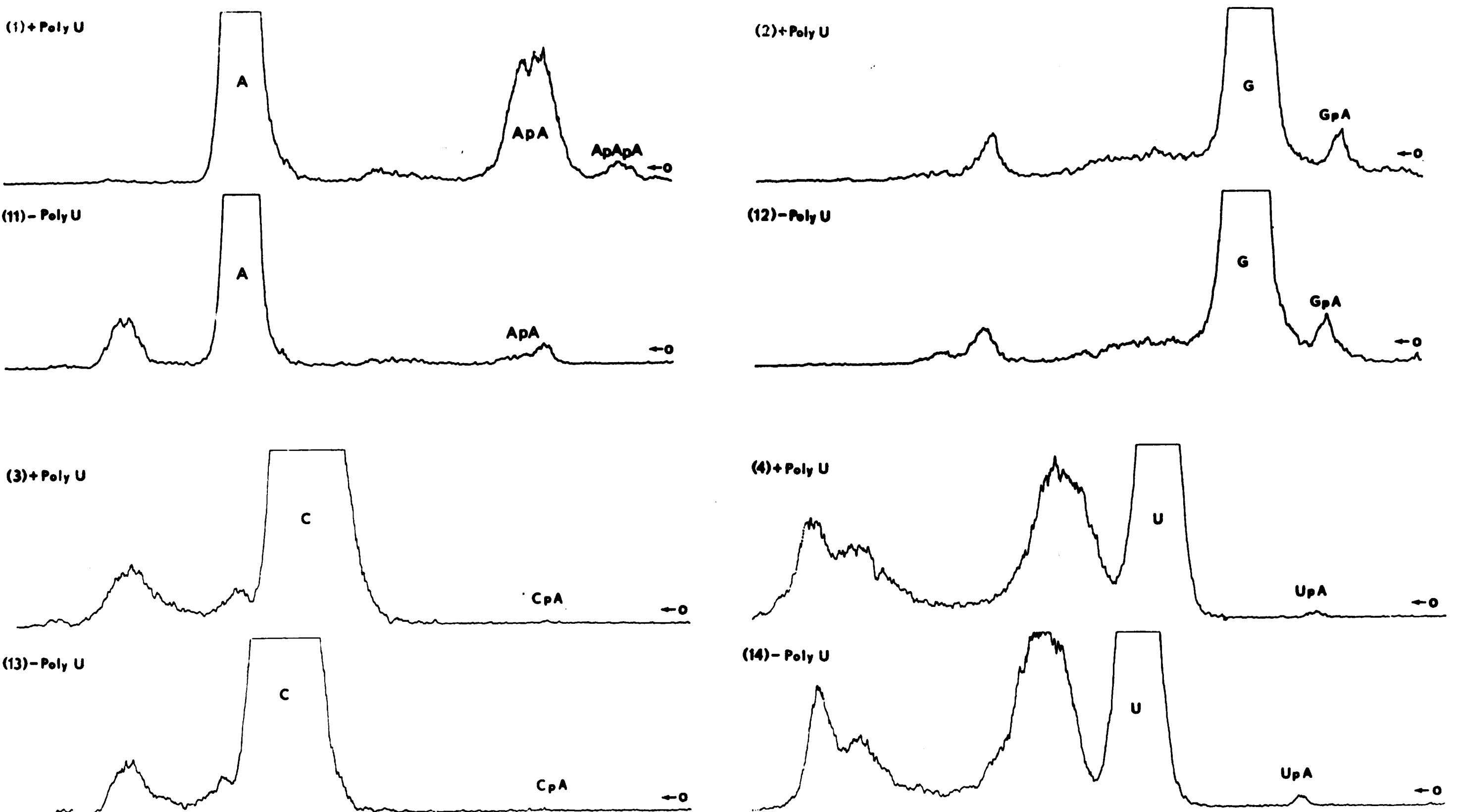

FIG. 1.—Condensation of C^{14}-labeled A, G, C, and U with cold pA, in presence and absence of poly U; chromatography in system I. O = origin.

TABLE 2. *Isomer distributions of crude products.*

Reaction	Product	$(2' \rightarrow 5')^a$	$(3' \rightarrow 5')^a$	$(5' \rightarrow 5')^a$
11	*ApA	30	12	53
12	*GpA[b]	58	15	11
13	*CpA	66	11	16
14	*UpA	66	28	4
*ApA formed on poly U template[c]		64	2	34

Asterisks denote C^{14} label.
[a] Expressed as percentage of crude product (see Table 1).
[b] Of the "GpA," 17% is not accounted for; this fraction was degraded by KOH, but the radioactive product was not mobile in system VII.
[c] Results from previous experiment.[1]

sured by scintillation counting (see Table 3). At this time the pH of each mixture had risen to about 8, and about half the CDI had been consumed.

Condensation in the absence of a template: Eight reaction mixtures were prepared. The following reagents were present where indicated by a plus sign in Table 4: adenosine-5'-phosphate-8-C^{14} (p*A), 0.025 *M*, 0.1 $\mu c/\mu M$; pA, 0.025 *M;* adenosine-8-C^{14} (*A), 0.0125 *M*, 0.16 $\mu c/\mu M$; $MgCl_2$, 0.075 *M;* NaCl, 0.2 *M*. Each reaction mixture also contained CDI (0.2 *M*) and was initially adjusted to pH 5.2. After 3 days at 23° or 12 days at 0° no CDI remained. Samples from reactions 1–4 were chromatographed in system IV to separate pApA (R_f 0.004) and $A^{5'}ppA$ (R_f 0.01) from unchanged pA (R_f 0.07). The slower-moving products were eluted and rechromatographed in system II to distinguish pApA (R_f 0.33) from $A^{5'}ppA$ (R_f 0.44). Reaction mixtures 5–8 were analyzed by chromatography in system I, which readily separates ApA (R_f 0.25–0.35) from A (R_f 0.60). The results are shown in Table 4.

Condensation of adenosine with inorganic phosphate: Two reaction mixtures were prepared. Each contained: adenosine-8-C^{14}, 0.0125 *M*, 0.16 $\mu c/\mu M$; NaH_2PO_4, 0.0125 *M;* NaCl, 0.2 *M;* CDI, 0.2 *M*. In addition, reaction 2 contained: $MgCl_2$, 0.075 *M*. Reaction 1 was titrated initially to pH 6.0 and reaction 2 to pH 5.5; both were kept at 0°C. After five days each mixture was titrated back to the original pH. After a total of seven days, when no CDI remained, the products were separated in system I.

The region of R_f 0.00–0.15 (expected to contain Ap and pA) was eluted, and the eluate was subjected to electrophoresis in system VI. The adenosine monophosphate (AMP) was eluted, and portions were chromatographed in systems III and V, together with unlabeled $A^{2'}p$, $A^{3'}p$, and pA as carriers. The isomer ratios were measured by scintillation counting.

The region of R_f 0.27–0.54 (expected to contain adenosine cyclic phosphate, Ap!) was eluted, and the eluate was subjected to electrophoresis in system VII. The Ap! (mobility +0.54 relative to pA) was eluted; 1 *M* KOH at 23° for 15

TABLE 3. *Specificity of template for ApA formation.*

Reaction	Polymer	Per cent yield
1	—	1.2
2	Poly U	14.4
3	Poly C	1.5

Table 4. *Condensation in the absence of a template.*

Reaction	p*A	pA	*A	Mg^{++}, Na^{+}	Temperature (°C)	Product (%) pApA	$A^{5'}ppA$	ApA
1	+	...	...	+	0	1.3	31	...
2	+	...	...	+	23	0.6	36	...
3	+	...	...	...	0	0.7	4	...
4	+	...	...	...	23	0.3	8	...
5	...	+	+	+	0	...	...	6
6	...	+	+	+	23	...	...	3
7	...	+	+	...	0	...	...	7
8	...	+	+	...	23	...	...	3

Asterisks denote C^{14} label.

hours caused complete conversion to $A^{2'(3')}p$ as shown by electrophoresis in system VII followed by chromatography in system III. Adenosine-3',5'-cyclic phosphate is stable under these conditions,[4] and in any case could not yield $A^{2'}p$. The results, summarized in Table 5, show that about 25 per cent of the phosphorylation is at the 2' or 3' position.

Discussion.—General: The data in Tables 1 and 3 establish:

(1) That the presence of poly U under conditions permitting formation of triple helices with A or pA enhances greatly the yields of dinucleoside monophosphates from pA and A, but has little effect on the reaction of pA with other nucleosides.

(2) That the presence of other nucleosides does not affect the condensation of pA with A on a poly U template.

(3) That poly C does not enhance the formation of dinucleoside monophosphates from pA and A. Poly C has some self-structure under the conditions of the experiment. Therefore, this control strictly excludes only totally nonspecific synthesis guided by the polyphosphate backbone.

The simplest interpretation of these facts is that A and pA (or an activated carbodiimide intermediate derived from it) are incorporated into a triple helix with poly U by specific hydrogen bonding, while the other nucleosides are excluded from the helix. pA in the helix reacts more readily with adjacent A in the helix than with A in free solution. On the other hand, pA reacts about as readily with other nucleosides in free solution whether it, itself, is incorporated in a triple helix or is in free solution. A and pA are not incorporated into a helix with poly C.

In a later paper we shall show that, in a similar way, poly C incorporates only G into oligonucleotides. Thus the Watson-Crick base-pairing rules apply to nonenzymatic syntheses using carbodiimide as condensing agent.

The results of experiments on solutions which do not contain polynucleotide are at first sight surprising. The reactivities of different nucleosides with an activated pA molecule would be expected to be very similar. In fact ApA and GpA

Table 5. *Condensation of adenosine with inorganic phosphate.*

Reaction	Mg^{++}	Total phosphorylation %	Per cent of Total Product as: pA	$A^{3'}p$	$A^{2'}p$	$A^{2'(3')}p!$
1	...	1.7	72	2	2	24
2	+	1.6	76	8	4	12

are formed much more extensively than CpA and UpA. We believe that this is due to stacking in free solution. Stacking is known to occur between purine nucleosides and nucleotides, but to a lesser extent between purine nucleotides and pyrimidine nucleosides.[5]

The ratio of isomers obtained in free solution is consistent with this interpretation. Reaction of adenosine with inorganic phosphate and carbodiimide gives the 5′-phosphate as the major product. However, the dinucleoside phosphates formed include a larger proportion of the 2′-5′ and 3′-5′ isomers. Presumably, stacking results in the orientation of the sugars in such a way that the 2′ and 3′ hydroxyls of one sugar are more likely than the 5′ hydroxyl to approach the phosphate of a neighbor in the stack.

Table 4 shows that the formation of pyrophosphate is strongly favored by the presence of Mg^{2+} and Na^{+} ions and is somewhat more extensive at high temperatures than at low. On the other hand, the formation of pApA is only slightly enhanced by Mg^{2+} and Na^{+} and is more extensive at *low* temperatures. This suggests that stacking is important for nucleotide formation but does not enhance pyrophosphate formation. This interpretation is supported by the observation that the formation of ApA from pA and A occurs more readily at low temperatures.

Relevance to prebiotic synthesis: The reactions which we have described in this and a previous paper differ from biochemical or potentially prebiotic oligonucleotide syntheses in two important respects. Firstly, they involve triple rather than double helices. Secondly, we have used a complex organic condensing agent rather than a preformed activated nucleotide (nucleoside triphosphate), or a nucleotide in the presence of a simple condensing agent. The following discussion is therefore tentative, and would require revision if it turned out that under other conditions the 3′-5′ linkage is the predominant one formed nonenzymatically in homogeneous solution.

Our results suggest that conditions exist under which the inherent base-pairing properties of the four nucleosides are sufficient to ensure an adequately accurate complementary replication of polynucleotides without the intervention of enzymes. They suggest, on the contrary, that it is the specificity of the 3′-5′ linkage which may be difficult to achieve under prebiotic conditions. The same conclusion applies to syntheses at low temperatures in the absence of templates when stacking is important. Preliminary experiments show that this is equally true for condensations involving *double-stranded* and triple-stranded poly C–G helices or triple-stranded poly U–deoxy A helices. All of these reactions result, predominantly, in the formation of 2′-5′ or, where this is not possible, 5′-5′ linkages.

There are two obvious escapes from this dilemma. We may believe that a solid surface of nonbiological origin, a mineral, or perhaps some simple organic polymer such as a random polypeptide, functioned in the same way as a contemporary enzyme. We are designing experiments to test this possibility, but we can never exclude it since the number of possible surfaces is unlimited.

We may alternatively believe that, in the early stages of evolution, 2′-5′ and 3′-5′ linkages (and perhaps very occasional chain-reversing 5′-5′ linkages also)

occurred indiscriminately. This does not seem implausible, since it has been shown that oligo 2′-5′ adenylates form stable helices with poly U[6]. In general, the melting temperatures of these complexes are only a few degrees lower than those of the corresponding complexes formed with the oligo 3′-5′ adenylates. It seems likely that complexes formed from mixed 2′-5′, 3′-5′ oligomers would melt at intermediate temperatures.

Perhaps the sugar component of the nucleoside was also capable of some variation. Clearly polymers could form containing both ribotides and deoxyribotides. We plan to investigate the incorporation of isomers of adenylic acid, particularly L-ribosides, and derivatives of other sugars, particularly D-arabinosides, into oligonucleotides on standard polynucleotide templates.

The emergence of the standard nucleic acids made up of ribotides or deoxyribotides linked by 3′-5′ bonds and of the standard synthesis from mononucleotide derivatives may thus be the end result of a long evolutionary process involving natural selection for *replication* at the level of oligonucleotides. A great variety of oligomers may have formed, but only those capable of directing effective template synthesis could have won out in the long run. Oligonucleotides as well as mononucleotides may have undergone condensation reactions since the former would displace the latter from the templates. It may well be that the final restriction to the synthesis of 3′-5′-linked oligomers from activated monomers required the evolution of an enzyme. The slightly greater stability of the complexes formed by the 3′-5′ oligomers could account for their selection.

Summary.—Polyuridylic acid facilitates the formation of nucleotide bonds between adenylic acid and adenosine, but not between adenylic acid and guanosine, cytidine, or uridine. Polycytidylic acid has no effect on the reaction of adenylic acid with adenosine.

The results of experiments carried out in the absence of templates suggests that stacking plays a key role in the reactions between nucleosides and nucleotides in aqueous solution at 0°C.

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[1] Sulston, J. E., R. Lohrmann, L. E. Orgel, and H. T. Miles, these PROCEEDINGS, **59,** 726 (1968).

[2] Zetsche, F., and G. Baum, *Chem. Ber.*, **75B,** 100 (1942).

[3] For notation, see ref. 1.

[4] Lipkin, D., W. H. Cook, and R. Markham, *J. Am. Chem. Soc.*, **81,** 6198 (1959).

[5] Schweizer, M. P., A. D. Broom, P. O. P. Ts'O, and D. P. Hollis, *J. Am. Chem. Soc.*, **90,** 1042 (1968).

[6] Michelson, A. M., and C. Monny, *Biochim. Biophys. Acta*, **149,** 107 (1967).