Synthesis of Polyadenylate: Polyuridylate Catalyzed by Ribonucleic Acid Polymerase in the Absence of a Template*

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SUMMARY

In the absence of any added template and after a variable lag period, ribonucleic acid polymerase catalyzes the polymerization of adenosine triphosphate and uridine triphosphate to polyadenylate and polyuridylate. The length of the lag period varies inversely with amount of enzyme and substrate present in the incubation mixture. The reaction requires Mn++; it does not occur if Mg++ is the only metal ion present. Unprimed synthesis of polyadenylate and polyuridylate is inhibited in the presence of polynucleotides, spermidine, guanosine triphosphate, and cytidine triphosphate. The product was characterized by nearest neighbor analysis, ultracentrifugal analysis, and thermal denaturation studies.

Enzymatic synthesis of ribonucleic acid from the four ribonucleoside 5'-triphosphates is catalyzed by the enzyme RNA polymerase (EC 2.7.7.6). The reaction requires a polynucleotide template which serves to determine the composition and base sequence of the RNA product. Although the reaction proceeds better when polydeoxyribonucleotides are used as templates, with bacterial RNA polymerase polyribonucleotides also serve to direct RNA synthesis (1–3).

Recent reports indicate that Escherichia coli RNA polymerase can catalyze an unexpected formation of polyadenylate and polyuridylate (4–6). Mehrotra and Khorana have reported that RNA polymerase synthesizes polyadenylate and polyuridylate both in the presence of and in the absence of an oligodeoxynucleotide template (4). Gomatos, Krug, and Tamm have reported that this enzyme catalyzes the formation of polyadenylate and polyuridylate when reovirus RNA serves as a template (5, 6). RNA polymerase from Micrococcus lysodeikticus catalyzes a similar reaction (8).

Since this gratuitous formation of polyadenylate and polyuridylate can interfere with studies in vitro that utilize RNA polymerase, we have studied the characteristics of this reaction with the aim of learning how to inhibit or to circumvent it. In a preliminary communication we demonstrated that unprimed poly A:U synthesis occurs after a lag period and that both ATP and UTP are required for formation of polymer to occur (9). We also showed that the reaction was greatly inhibited in the presence of a high molecular weight polydeoxyribonucleotide template. This report extends these observations and shows that, while unprimed poly A:U synthesis occurs readily with Mn++, in the presence of Mg++ no reaction takes place.

EXPERIMENTAL PROCEDURE

Materials—Radioactive nucleotides were obtained from Schwarz BioResearch. Unlabeled nucleotides were obtained from Calbiochem, Nutritional Biochemicals, and P-L Biochemicals. Spermidine, phosphoenolpyruvate, and pyruvate kinase were purchased from Calbiochem. Poly C and poly I were from Miles Chemical Company. Nucleoside diphosphokinase was prepared according to Ratliff et al. (10). Polydeoxycytidylate oligomers were synthesized by the method of Khorana, Turner, and Vissolyi (11). RNA polymerase was isolated from E. coli by a procedure worked out in this laboratory. The enzyme sedimented as a homogeneous peak in the ultracentrifuge and had a specific activity of over 2000 units per mg of protein when incubated under the conditions of Chamberlin and Berg (12). The enzyme preparation used in each experiment is identified in the legends to the figures.

Methods—Incorporation of labeled ATP or UTP into acid-insoluble material was used as a measure of unprimed poly A:U synthesis. A typical mixture contained 20 pmoles of Tris-HCl, pH 7.9; 0.5 pmoles of MnCl₂; 2.0 pmoles of MgCl₂; 0.2 pmoles each of ATP and UTP, one of which was labeled; 6.0 pmoles of 2-mercaptoethanol; and enzyme in a final volume of 0.5 ml. Reactions frequently were run in the later stages of this work MgCl₂ was usually omitted, and 1.0 pmoles of MnCl₂ was used. Reactions frequently were run in the

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† However, Shatkin has presented evidence which indicates that reovirus RNA does not, in fact, serve as a template for E. coli RNA polymerase (7).

‡ The abbreviations used are: poly A:U, a polymer containing homopolymer chains of adenylate and uridylate; d(pC)ₙ, oligodeoxycytidylate, with a 5'-phosphate end where the subscript refers to the chain length in monomer units; poly C, polyctydylate; poly I, polyinosinate; poly C:I, a polymer containing homopolymer chains of cytidylate and inosinate formed by mixing equimolar amounts of polycytidylate and polyinosinate.

§ Manuscript in preparation.
presence of 0.75 \mu\text{ mole} of phosphoenolpyruvate and 20 \mu\text{g} of pyruvate kinase, sometimes with inclusion of 10 \mu\text{g} of nucleoside diphosphokinase. All incubations were done at 37\degree C. At appropriate time intervals 0.05-ml aliquots were removed from the reaction mixture and applied to small discs of Whatman No. 3MM filter paper. The impregnated discs were immersed in cold 5\% trichloroacetic acid-0.01 M sodium pyrophosphate. After 10 to 30 min, the discs were removed and washed three times on a suction flask with cold 5\% trichloroacetic acid-0.01 M sodium pyrophosphate and three times with ethanol-ether (1:1). The discs were then dried, and the radioactivity was determined in a Packard model 3324 Tri-Carb scintillation counter. The reaction also could be followed by the decrease in absorbance at 260 \text{ nm} due to hypochromicity of the polymer. In those experiments in which the product was isolated for ultracentrifugal studies, the reaction was terminated by addition of NaCl and sodium citrate to give final concentrations of 0.2 M and 0.1 M, respectively. The solutions were dialyzed in the cold against 0.2 M NaCl-0.1 M sodium citrate. A model E ultracentrifuge equipped with an ultraviolet light absorption optical system was used for the ultracentrifuge experiments.4

Nearest neighbor frequency analysis of unprimed poly A:U was done by the method of Melvits and Khorana (4). The hydrolysate of the polymer was chromatographed in two different solvent systems; isobutyric acid-concentrated ammonia-water (66:1:33, v/v/v), and 0.1 M sodium phosphate (pH 6.8)-ammonium sulfate-1-propanol (100:60:2, v/w/v). In the first system, 2'(3')-uridylic acid had an Rf similar to that of higher phosphates of adenosine. The second solvent system separated UMP from ATP.

Isolation of the product for determination of absorbance as a function of temperature was accomplished by phenol extraction repeated three to four times on the reaction mixture, followed by centrifugation against 0.1 M sodium phosphate buffer, pH 7.8, with 10^{-4} M EDTA. Prior to use all dialysis tubing was boiled in 0.1\% sodium dodecyl sulfate with 0.1\% EDTA for 10 min, followed by washing in distilled water and dialysis buffer. Determination of absorbance as a function of temperature was done in a Unicam SP 800 spectrophotometer. A Tamson circulating bath, fitted with a temperature control motor and tubing to the Unicam cell carriage, was used to heat the polymer solutions. The temperature in the cell carriage was determined through use of reference and measuring thermocouples. A:A0 (absorbance relative to absorbance at the starting temperature) was plotted as a function of temperature on an X-Y recorder.

RESULTS

Characteristics of Reaction

Effect of Enzyme Concentration—Previously published results showed that, in the absence of template, incorporation of radioactive ATP or UTP into acid-insoluble material depended on the presence of the complementary nucleoside triphosphate (9). This incorporation could be detected after a lag period and was greatly inhibited in the presence of a polydeoxyribonuclease template. The length of the lag period can be varied greatly by varying the enzyme concentration (Fig. 1). If it is defined as the time required for 5.0 mmoles of ATP per ml of reaction mixture to be converted into an acid-insoluble form, then the lag period in this experiment is about 3 hours with 20 units of enzyme and less than 1\frac{1}{2} hours with 80 units. Similar experiments have shown that the lag period can be reduced to less than 10 min when 400 units of enzyme are added to the incubation. A comparison of several different enzyme preparations showed that there was some variation in length of the lag period even though the same number of enzyme units was added to the incubations. The length of the lag period does not vary significantly when a single enzyme preparation is used. Radding, Josse, and Kornberg, when studying unprimed polydeoxyctydylate-polynetoxynucleate synthesis catalyzed by DNA polymerase, also found that the lag period varied with different enzyme preparations (13). We believe that the differences between different preparations of RNA polymerase are due chiefly to different amounts of contaminating nucleases.

Experiments were done to see if the enzyme preparations contained some template which might be used to prime poly A:U synthesis. RNA polymerase was inactivated by heating at 75\degree C for 10 to 30 min. When heat-inactivated enzyme, in 3 to 7 times the usual amount, was added to an incubation mixture containing unreated enzyme, the length of the lag period was not shortened. This indicates that there is no template for poly A:U formation present in the enzyme preparation and that it contributes only its catalytic activity.

Effect of Substrate Concentration—The length of the lag period is also influenced by substrate concentration. Fig. 2 shows the results of an experiment in which the amount of ATP and UTP was varied from 50 to 400 mmoles (each) at constant enzyme concentration. Under these conditions, the length of the lag period is shortened as substrate concentration is increased.

In some early experiments no unprimed poly A:U synthesis was observed at low substrate concentrations. This result apparently was due to nucleases in the enzyme preparation since, with more highly purified RNA polymerase, a reaction was observed with all substrate concentrations tested (down to 0.05

![Fig. 1. Effect of enzyme concentration on length of the lag period.](http://www.jbc.org/)

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polydeoxyguanylate with DNA polymerase required very high sensitive to the presence of nucleases. These enzymes seem to ported that formation of unprimed polydeoxycytidylate : reaction has been observed with all enzyme fractions tested, but prevent the formation of small initiating oligonucleotides. The were used.

amounts of RNA polymerase were used. No reaction was enzyme concentrations (13). In our experiments, no unprimed order to obtain an acid-insoluble product.

The reaction mixtures and conditions were similar to those of Fig. 1 except that UTP-2-14C (3.4 × 10^6 cpm per pmole) and unlabeled ATP were used. The amount of each nucleotide added to the incubation mixture is indicated. MgCl2 was omitted, and 1.0 pmole of MnCl2 and 56 μg of enzyme (Preparation 85-G) were used in the reaction.

Fig. 2. Effect of substrate concentration on length of lag period. The reaction mixtures and conditions were similar to those of Fig. 1 except that UTP-2-14C (3.4 × 10^6 cpm per pmole) and unlabeled ATP were used. The amount of each nucleotide added to the incubation mixture is indicated. MgCl2 was omitted, and 1.0 pmole of MnCl2 and 56 μg of enzyme (Preparation 85-G) were used in the reaction.

mm). As might be expected, poly A:U synthesis is rather sensitive to the presence of nucleases. These enzymes seem to prevent the formation of small mutating oligonucleotides. The reaction has been observed with all enzyme fractions tested, but when relatively impure fractions were used considerable manipulation of substrate and enzyme concentrations was required in order to obtain an acid-insoluble product.

Substrate specificity experiments, similar to those in Fig. 2, were run in which GTP and CTP replaced ATP and UTP. Likewise, no reaction took place when either ATP or UTP was replaced by its corresponding deoxynucleotide analogue. When ATP-8-14C was incubated in a standard reaction mixture for 2 hours, followed by addition of UTP at that time, the lag period was the same length as when UTP was added initially. The same was true when UTP-2-14C was incubated for 2 hours prior to ATP addition, i.e. no shortening of the lag period was observed. Also prior incubation of the enzyme followed by addition of ATP and UTP did not affect the lag period.

Effect of Metal Ions—Initial studies (9) of unprimed poly A:U synthesis were done at those Mn++ and Mg++ concentrations (1 mM and 4 mM, respectively) utilized by Chamberlin and Berg (12). It was subsequently found that the reaction proceeded better if Mg++ was omitted and 2 to 4 mM MnCl2 was used. Furthermore, the reaction did not proceed at all if Mg++ was the only metal ion present (Fig. 3). Other experiments in which the MgCl2 concentration was varied from 0.1 to 20 mM showed no reaction at any concentration. The Mg++ concent-

Further evidence for this structure of the unprimed product was obtained by studies of absorbance changes induced by heating. The melting curve obtained when the product was heated showed a sharp transition with a midpoint (Tm) at 56.1°. This is the reported Tm for poly A:U in 0.1 M sodium phosphate, pH 7.8-10^-4 M EDTA (16). Furthermore, the extent of thermal hyperchromicity is the same as that observed for poly A:U melting, and the spectral changes (Fig. 4) are similar to those seen when poly A:U formed by mixing equimolar amounts of poly A and poly U is heated (16).

As noted previously (9), when parallel reactions are run with labeled UTP and labeled ATP, it is frequently observed that UMP is incorporated at a greater rate than AMP. In addition, the total amount of UMP incorporated is frequently larger than that of AMP. However, in several experiments essentially all of both substrates have been converted to an acid-insoluble form. The data in Fig. 4 indicate that the polymer, in this case, must consist of essentially equimolar amounts of A and U.

In their study of unprimed deoxyadenylate:deoxythymidylate
copolymert synthesis by DNA polymerase, Schachman et al. (17) noted that use of higher enzyme levels led to production of smaller polymers. We have found a similar result with unprimed poly A:U synthesis. ATP and UTP were incubated with three concentrations of RNA polymerase. Parallel experiments in which one of the triphosphates was radioactive showed that the extent of incorporation was the same with all three enzyme concentrations. After isolation and dialysis of the product as described under "Methods," the ultracentrifugal patterns of the three samples were examined. As more enzyme is used the product becomes smaller (Table I). The values in Table I refer to the fastest moving component. In all ultracentrifuge runs some heterogeneity was seen in the sedimentation patterns of poly A:U. It is not certain to what extent the observed heterogeneity is due to possible breakdown of the products during preparation for analysis in the ultracentrifuge.

**Influence of Various Additions on Reaction**

**Polynucleotides**—We demonstrated previously that polydeoxycytidylate in sufficient concentration is a potent inhibitor of the reaction (9). In an extension of those experiments, the effects of d(pC)₄ and d(pC)₅ on unprimed poly A:U formation were tested. In the absence of GTP neither oligodeoxycytidylate inhibited the reaction. However, in the presence of GTP, d(pC)₄ exerted a significant inhibition, while d(pC)₅ still had no effect (Fig. 5). We have shown in other experiments that d(pC)₄ serves as a template for the enzyme to direct poly G formation but that d(pC)₅ is ineffective. The same result has been obtained previously by others (18, 19). It seems likely that inhibition by d(pC)₄ and GTP is related to their role as template and substrate. The inhibition produced by d(pC)₄ is much less than that caused by polydeoxycytidylate (9).

Polyribonucleotides also are effective inhibitors of the reaction. Dependence of the observed inhibition on concentration of added polynucleotide is illustrated for poly C in Fig. 6. Direct

**Table I**

<table>
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<th>Sedimentation coefficient</th>
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Fig. 4. Spectra of poly A:U at 25° and 65°. The spectrum of a poly A:U solution was determined at 25° before heating was started and again at 65°. The solvent was 0.1 M sodium phosphate buffer, pH 7.8, containing 10⁻³ M EDTA prepared as described by Chamberlin (16). The solid line gives the values at 25°; the broken line gives the values at 65°.

Fig. 5. Effect of d(pC)₄ and d(pC)₅ on unprimed poly A:U synthesis. The reaction mixtures and conditions were the same as those used in Fig. 1 except that ATP⁻¹⁴C (5 x 10⁵ cpm per μmole) was used, and 0.75 μmole of phosphoenolpyruvate, 20 μg of pyruvate kinase, and 10 μg of nucleoside diphosphokinase were included. Also included were 200 μmole of GTP, 130 μg of RNA polymerase (Preparation RC-50), and, where indicated, 190 μmole (as nucleoside equivalents) of d(pC)₄ and 231 μmole of d(pC)₅.

Fig. 6. Unprimed poly A:U synthesis as affected by polydeoxycytidylate acid. The reaction mixtures and conditions were the same as those in Fig. 1 except that 200 μmole of GTP were included, and 105 μg of RNA polymerase (Preparation 36-40NC) were used. Poly C was added as indicated (the values refer to monomer units).
experiments and those of others (1) show that, compared to poly C, poly I is a very poor template for formation of its comple-
mentary homopolymer. Poly I is relatively insoluble in the reaction mixtures. Our poly I has intermediate inhibitory activity (Fig. 7). The reason why poly I is a much better inhibitor than poly C is not clear. Poly I is relatively insoluble in the reaction mixtures. Our experiments and those of others (1) show that, compared to poly C, poly I is a very poor template for formation of its complementary homopolymer.

Discussion

There are several similarities between the unprimed synthesis of poly A:U catalyzed by RNA polymerase and the unprimed synthesis of deoxyadenylate:deoxycytidylate copolymer and deoxyguanylate:deoxycytidylate homopolymer catalyzed by DNA polymerase (13, 17). First, the reactions are observed after a lag period the length of which can be shortened through use of higher enzyme concentrations. Second, the reactions all require the presence of two complementary nucleoside triphosphates. Third, as is the case with unprimed polydeoxyguanyl-
ate:deoxycytidylate synthesis (13), there frequently is unequal incorporation of the two substrates during unprimed poly A:U synthesis. On the other hand, while DNA polymerase catalyzes unprimed reactions with each pair of complementary deoxy-
nucleoside triphosphates, with RNA polymerase no incorpora-
tion is observed when GTP and CTP are incubated together under a variety of conditions. Also, while the reaction catalyzed by RNA polymerase results in a product containing homopolymer strands of adenyllic acid and uridylic acid, the corresponding reaction with DNA polymerase results in a product of alternating base sequence. In addition, the reaction catalyzed by DNA polymerase readily takes place in the presence of Mg++, while that catalyzed by RNA polymerase requires the presence of Mn++ ions.

Unprimed poly A:U synthesis has, in some cases, interfered with the use of RNA polymerase to copy templates in vitro (4–6). We have shown that this reaction does not take place if Mg++ is the only metal ion present in the reaction mixture. However, use of Mg++ is not feasible when polyribonucleotides are to serve as template for RNA polymerase, since this reaction requires Mn++. Under these conditions, the reaction can be inhibited or circumvented through the judicious use of enzyme concentration, template concentration, and length of incubation. Also, the reaction is inhibited by Mg++, in the presence of optimal Mn++ concentration. Clark and Jaouni recently advocated the use of Mn++ in place of Mg++ in experiments in vitro with RNA polymerase. Figure 7 shows the effect of poly C, poly I, and poly C:I on unprimed poly A:U synthesis. The reaction mixtures and conditions were the same as those used in Fig. 1 except that UTP-2,14C (6.8 \times 10^6 cpm per amole) was used, and 0.75 amole of phosphoenolpyruvate, 20 mg of pyruvate kinase, 200 mmoles of CTP, and 51 g of enzyme (Preparation 35NC-G) were included. Poly C (126 mmoles as nucleoside equivalents), poly I (91 mmoles), and poly C:I (105 mmoles) were added in different reaction mixtures. Poly C:I was prepared by mixing equimolar amounts of poly C and poly I.

When CTP is included in the reaction mixture, a lengthening of the lag period is observed (Fig. 8). When both CTP and GTP are added, the lag period is lengthened even more; however, in each case, all the added UTP is ultimately polymerized. Thus, several components which are normally added to reaction mixtures for studies of RNA polymerase in vitro produce an inhibition of unprimed poly A:U synthesis. These include the template, GTP, CTP, and Mg++. Spermidine also is frequently included (21, 22), and it also would inhibit the reaction.

Other experiments showed that addition of 10 \mu g of RNase inhibited the reaction 94 to 98% when it was followed by incor-
poration of radioactive UTP; DNase had no effect.

In some early experiments in which a nucleoside triphosphate-
regenerating system was included in the reaction mixture, an increase in the lag period was noted. This regenerating system consisted of phosphoenolpyruvate, pyruvate kinase, and nucleo-
side diphosphokinase. We subsequently found that the length-
ened lag period was due to the ammonium sulfate solution in which pyruvate kinase was suspended. Upon removal of ammonium sulfate from the pyruvate kinase solution, no increase in length of the lag period was observed. Furthermore, addition of ammonium sulfate to the concentration provided by addition of the original pyruvate kinase solution lengthened the lag period to the same extent.

Comparisons of poly C and polydeoxycytidylate have shown that poly C is not nearly as effective an inhibitor as its deoxyribose analogue. Poly I is a very potent inhibitor compared to poly C, while poly C:I made by mixing equimolar amounts of poly C and poly I has intermediate inhibitory activity (Fig. 7). The reason why poly I is a much better inhibitor than poly C is not clear. Poly I is relatively insoluble in the reaction mixtures. Our experiments and those of others (1) show that, compared to poly C, poly I is a very poor template for formation of its complementary homopolymer.

Other Additions—Spermidine has been reported to dissociate RNA polymerase:RNA complexes (20). Therefore, it was thought that this polycation might inhibit unprimed poly A:U synthesis. Experiments done in the presence of 2 mm spermidine showed that the lag period was increased by about 20 min, but that the reaction when started had the same rate as did a control reaction.

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polymerase (19). This would prevent possible complications due to polynucleotide phosphorylase, which requires Mg++ (23), and those due to poly A polymerase (24), which is much more active in the presence of Mg++ than Mn++. These enzymes sometimes are present as impurities in preparations of RNA polymerase (4), although in our experience this is a very infrequent occurrence. Therefore, we prefer to use Mg++ whenever possible with studies involving RNA polymerase, particularly when longer periods of incubation are utilized. Also, it has been noted by others that when Mg++ is replaced by Mn++ both DNA polymerase and RNA polymerase catalyze reactions which presumably are not physiological in nature (25). On the other hand, the unprimed syntheses catalyzed by DNA polymerase take place in the presence of Mg++. The experimental conditions utilized in most studies in vitro with RNA polymerase are not likely to lead to interference due to unprimed poly A:U synthesis (assuming, of course, that the enzyme preparation is free from poly A polymerase). The presence of a template, GTP, CTP, Mg++, and the use of a relatively short incubation period all would tend to minimize such interference. In addition, the amount of enzyme utilized in most experiments in vitro is small enough that the lag period would be several hours in length.

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