*AZOTOBACTER VINELANDII RIBONUCLEIC ACID POLYMERASE, IV. UNPRIMED SYNTHESIS OF RIC COPOLYMER**

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In the presence of a suitable template, RNA polymerase catalyzes the synthesis of polyribonucleotides having a base sequence complementary to the template.[1-6] RNA polymerase is also known to form polymers *de novo* in unprimed reactions; when incubated with ATP and UTP the synthesis of rA:rU homopolymers ensues after a lag period.[7-9]

This report describes a polymerization reaction catalyzed by the *Azotobacter vinelandii* RNA polymerase in which ITP and CTP in the absence of added template are polymerized to form a copolymer containing IMP and CMP in an alternating sequence.

Experimental Procedure.—RNA polymerase of *A. vinelandii* 0 was purified about 400-fold by a modification of the published procedure[10] and had a specific activity of 90. Two preparations were used in this report; the 280/260 ratios were preparation A, 1.63, and preparation B, 1.51. The results obtained did not vary with these enzyme preparations. *Escherichia coli* RNA polymerase was prepared by the method of Chamberlin and Berg[11] and had a specific activity of 2700.

Unlabeled ribonucleoside triphosphates were obtained from P. L. Biochemicals or Calbiochem; labeled nucleotides were products of Schwarz BioResearch. IMP^{32}-PP was prepared from $AMP^{32}PP$ by nitrous acid deamination.[12] The $IMP^{32}PP$ was purified by chromatography on Dow 1 Cl^- which removed unreacted ATP and lower phosphate derivatives.[13] After recovery of $IMP^{32}PP$ from the effluent as the Ba^{++} salt, the K^+ salt was prepared by treatment with Dow 50 K^+.

Results.—Both ITP and CTP must be present to obtain synthesis of polymer in the unprimed reaction. While the ineffectiveness of ATP or UTP in supporting CMP incorporation is to be expected, the failure of GTP to replace ITP, its Watson-Crick analogue, in the reaction is surprising and confirms the inability of Smith *et al.*[7] and Fox *et al.*[8] to obtain polymer synthesis with unprimed *E. coli* or *Micrococcus lysodeikticus* RNA polymerase incubated with CTP and GTP. Synthesis of rIC proceeded only in the presence of Mn^{++}; Mg^{++} was not effective (Table 1). The requirement of Mn^{++} for unprimed rIC synthesis is consonant with the Mn^{++} requirement for unprimed rA:rU synthesis[7, 9] and for polyribonucleotide-primed reactions in general.[4, 6]

TABLE 1

REQUIREMENTS FOR rIC SYNTHESIS

Components	H^3-CMP incorporated (mμmoles)
Complete system	105.0
No RNA polymerase	0.05
ITP omitted	0.05
ITP omitted, ATP added	0.05
ITP omitted, UTP added	0.05
ITP omitted, GTP added	0.05
ATP and UTP added	0.10
$MnSO_4$ omitted, $MgCl_2$ added	0.05

The complete system contained (in 0.5 ml): 25 μmoles Tris pH 8.0, 1 μmole $MnSO_4$, 10 μmoles mercaptoethylamine HCl, 300 mμmoles ITP, 300 mμmoles H^3-CTP (1.6×10^6 cpm/μmole), and 70 μg of RNA polymerase. Where indicated, ATP, UTP, and GTP were added at a concentration of 300 mμmoles and 5 μmoles $MgCl_2$ were added. Incubation was 2 hr at 37°.

Synthesis of rIC copolymer followed a lag period of from 15 to 30 minutes at 37° (Fig. 1). Following the lag, polymerization of ITP and CTP was rapid and

equal, and was accompanied by the formation of turbidity. After 90 minutes' incubation no further synthesis took place. When poly C was added to the reaction, synthesis of poly I and poly C began without a lag phase and also showed formation of turbidity. The turbidity seen in reaction leading to either rIC or rI:rC could be cleared by addition of EDTA to a concentration of 2.5×10^{-3} *M*. In the reactions primed by poly C there was no equivalence of IMP and CMP in the product (11.7 mμmoles IMP, 7.8 mμmoles CMP), while the rIC synthesized in the *de novo* reaction showed approximately equal amounts of IMP and CMP incorporated into an acid-insoluble form.

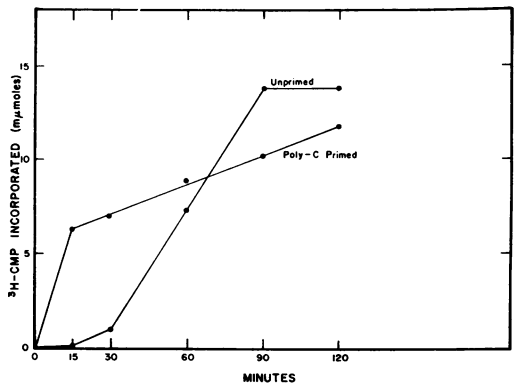

FIG. 1.—Time course of unprimed and poly C-primed H^3-CMP incorporation. The reaction contained in 0.5 ml: 25 μmoles Tris pH 8.0, 1 μmole $MnSO_4$, 10 μmoles mercaptoethylamine, 300 mμmoles H^3-CTP (1.5×10^6 cpm/μmole), 300 mμmoles ITP, and 70 μg of *A. vinelandii* RNA polymerase. Three μg of poly C were added for the primed reaction. The reactions were incubated at 37° for the times indicated. Each point represents the average of two 50-μl aliquots.

The polymer formed in the poly C-primed reaction was resistant to low levels of T_1 RNase or pancreatic RNase (Table 2); higher concentrations of pancreatic RNase selectively degraded the

TABLE 2

EFFECT OF NUCLEASES ON rIC COPOLYMER AND rI:rC HOMOPOLYMER

Polymer	Additions	Acid-Insoluble Nucleotide Remaining: IMP^{32} (mμmoles)	Acid-Insoluble Nucleotide Remaining: H^3-CMP (mμmoles)
(*i*) rIC copolymer	None	7.0	6.6
	T_1 RNase	0.05	0.05
	Pancreatic RNase	0.04	0.04
(*ii*) rI:rC homopolymer	None	8.0	6.7
	T_1 RNase	7.9	6.4
	Pancreatic RNase	7.9	3.6

Polymers were synthesized at 37° for 2 hr using the conditions listed in Fig. 1, and 0.5 ml of 5×10^{-3} *M* EDTA was added to terminate the reaction. Ten units of T_1 RNase or 0.05 μg of pancreatic RNase were added to 0.3-ml aliquots of rIC copolymer preparation. Either ten units of T_1 RNase or 1 μg of pancreatic RNase were added to 0.3-ml aliquots of rI:rC homopolymer (poly C-primed). The tubes were incubated for 60 min at 37° and 50-μl aliquots were assayed for acid-insoluble radioactivity.

poly C without affecting poly I as would be expected of the rI:rC homopolymer pair. The rIC copolymer was readily hydrolyzed by either T_1 RNase or pancreatic RNase at concentrations which were without effect on the rI:rC complex. The rates of release of H^3-CMP and IMP^{32} from the rIC copolymer by T_1 RNase were virtually identical (Fig. 2), indicating that the IMP and CMP are in the form of a copolymer.

To establish the alternating sequence of the copolymer, nearest-neighbor analysis was carried out. Polymers synthesized by RNA polymerase from *A. vinelandii* and *E. coli* in unprimed reactions after incubation with $IMP^{32}PP$ and H^3-CTP or $CMP^{32}PP$ and ITP were hydrolyzed with 0.3 *N* KOH to form 2′, 3′-CMP and 2′, 3′-IMP. As a control, reactions primed with poly C were also carried out to synthe-

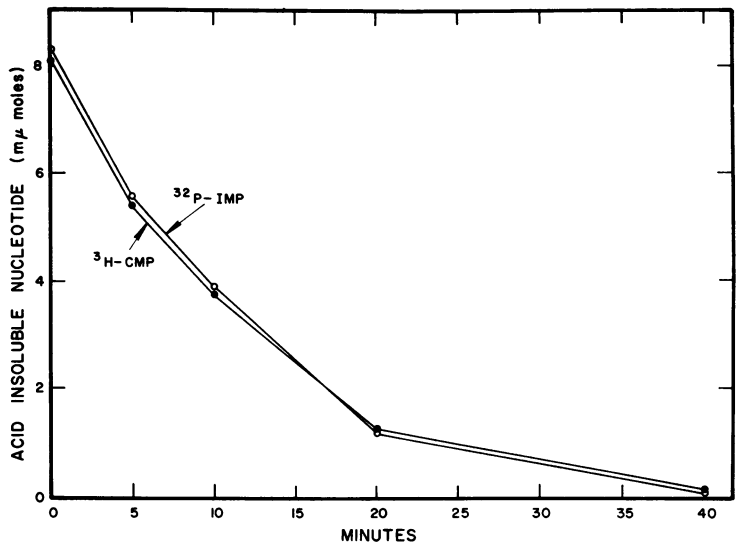

FIG. 2.—Time course of release of IMP and CMP from rIC by T_1 RNase. The rIC copolymer was synthesized in the unprimed reaction given in Table 2 and diluted with EDTA as indicated. Ten units of T_1 RNase were added to a 0.3-ml aliquot and the mixture was incubated at 37°. At the time indicated, 50-μl aliquots were removed and assayed for acid-insoluble H^3 and P^{32}.

size rI:rC homopolymer and the products were hydrolyzed as indicated in Table 3. Figure 3 shows the results obtained after hydrolysis of the product formed in the unprimed reaction with H^3-CTP and αP^{32}-ITP as the substrates. The H^3-CMP peak contained over 99 per cent of the P^{32} initially incorporated into the copolymer and only 0.5 per cent of the P^{32} was recovered as 2′,3′-IMP^{32}; therefore IMP was incorporated adjacent to CMP in the polymer. Table 3 summarizes the results of nearest-neighbor analyses obtained after hydrolysis of rIC and rI:rC polymers. Only IpI and CpC sequences were formed in the poly C primed reaction and IpC

TABLE 3

NEAREST-NEIGHBOR ANALYSES OF rIC COPOLYMER AND rI:rC HOMOPOLYMER

Reaction and source of enzyme	2,′3′ Ribonucleotide isolated	Labeled Triphosphate: $IMP^{32}PP$ Sequence	$IMP^{32}PP$ Cpm	$CMP^{32}PP$ Sequence	$CMP^{32}PP$ Cpm
Unprimed; *A. vinelandii*	Cp	CpI	101,400	CpC	1070
	Ip	IpI	480	IpC	170,410
Poly C-primed; *A. vinelandii*	Cp	CpI	370	CpC	134,970
	Ip	IpI	107,570	IpC	870
Unprimed; *E. coli*	Cp	CpI	39,000	CpC	200
	Ip	IpI	400	IpC	52,000

The reaction mixtures contained (in 0.5 ml): 25 μmoles Tris pH 8.0, 1 μmole $MnSO_4$, 10 μmoles mercaptoethylamine HCl, 70 μg of *A. vinelandii* RNA polymerase or 50 μg of *E. coli* RNA polymerase, and 300 mμmoles of the indicated ribonucleoside triphosphate. For analysis of the rIC copolymer synthesized *de novo* two reactions were carried out: (*i*) $IMP^{32}PP$ and H^3-CTP; (*ii*) $CMP^{32}PP$ and ITP. For the primed synthesis of rI:rC homopolymer, 3 μg of poly C were added to incubations set up as indicated in (*i*) and (*ii*). After 2 hr incubation at 37°, 0.1 ml of the reaction mixture was removed for determination of total incorporation. The remaining 0.4 ml was washed with TCA on a GF/C Whatman glass filter. The filter containing the labeled polymer was placed in a glass vial and 5 ml of 0.3 *N* KOH were added and the polymers were hydrolyzed overnight at 37°. Two μmoles each of 2,′3′-CMP and 2,′3′-IMP were added to the vials as markers; the solutions were removed from the vial and neutralized to phenol red end point with perchloric acid. The insoluble $KClO_4$ was centrifuged off. The nucleotides were resolved on Dow 1 formate using a convex gradient with 500 ml of H_2O in the mixing flask and 0.3 *M* ammonium formate, pH 4.5, in the reservoir. Ten-ml fractions were collected and 0.5 ml of each fraction was added to a solution containing 10 ml of toluene PPO-POPOP and 6 ml of ethylene glycol monomethylether, and counted in a Nuclear-Chicago scintillation counter set to determine H^3 and P^{32}.

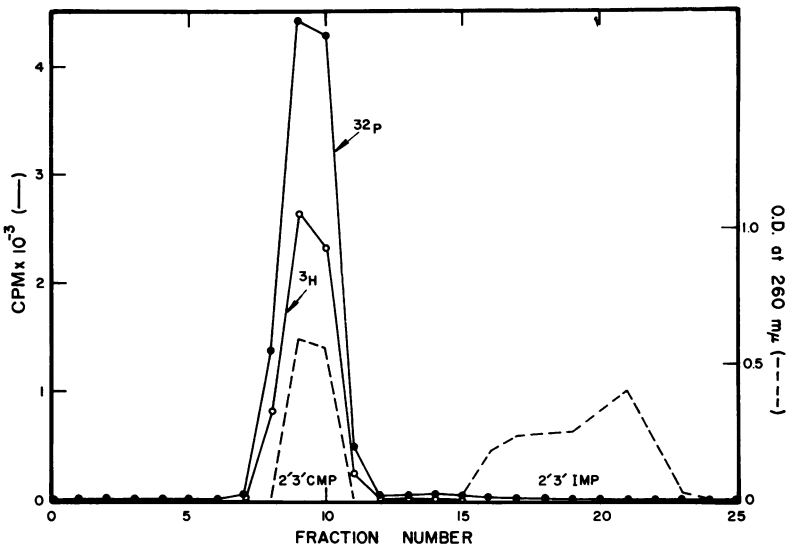

FIG. 3.—Formation of 2',3' H^3-CMP^{32} after alkaline hydrolysis of rIC copolymer synthesized from αP^{32}-ITP and H^3-CTP. The synthesis of rIC and subsequent treatment were as described in Table 3. H^3 and P^{32} were determined in a 0.5-ml aliquot from each tube in the chromatogram.

and CpI almost exclusively in the unprimed reaction. Therefore the polymer synthesized by RNA polymerase from either *A. vinelandii* or *E. coli* incubated with ITP and CTP in the absence of primer was the alternating rIC copolymer.

When rIC or rC:rI were hydrolyzed with 1 *N* HCl at 100° for one hour, the products were 2',3'-CMP, hypoxanthine, and 2',3'-ribose phosphate. If rIC is labeled with αP^{32}-ITP, all the P^{32} should be in 2',3'-CMP. However, in the poly C-directed synthesis of rI:rC, the P^{32} initially incorporated as IMP should be degraded by acid to 2',3'-ribose phosphate. It is possible to carry out nearest-neighbor analyses relatively rapidly by combining acid hydrolysis with charcoal adsorption. The results shown in Table 4 are in agreement with those obtained by alkaline hydrolysis and chromatography.

Incubation of all four of the relevant ribonucleoside triphosphates (ITP^{32}, CTP, H^3-UTP, ATP) with the enzyme system resulted only in the synthesis of rA:rU with

TABLE 4

DETERMINATION OF NEAREST-NEIGHBOR SEQUENCES BY ACID HYDROLYSIS AND CHARCOAL ADSORPTION

Polymer	Composition of precursor mix	Acid-stable P^{32} bound to charcoal (% of total P^{32})
rIC	αP^{32}-ITP + CTP	97
	αP^{32}-CTP + ITP	2
rI:rC	αP^{32}-ITP + CTP	2
	αP^{32}-CTP + ITP	94

rIC copolymer and poly C-primed rI:rC homopolymer were synthesized as indicated in the legend to Table 3. The polymers were isolated by addition of 0.1 ml of 0.1 *M* Na pyrophosphate and 5 ml of 5% TCA to 0.3 ml of the reaction. After centrifugation at 10,000 rpm at 5°, the pellets were washed four times with 5 ml of 5% TCA. Five ml of 1 *N* HCl was added to each tube, and the polymers were hydrolyzed for 1 hr at 100°. A 0.5-ml aliquot was placed directly on a stainless-steel planchet containing 0.5 ml of 5 *N* NH_4OH and was then dried. To 1 ml of the hydrolysate were added 0.1 ml of 6 *N* KOH and 0.2 ml of a charcoal suspension (100 mg/ml). The suspension was filtered onto a Whatman glass filter (GF/C) after mixing occasionally for 5 min and the charcoal was washed with 3 × 5 ml of H_2O. The filters were placed charcoal side down on a planchet dried, and the P^{32} was determined in a low-background flow counter.

no evidence of rIC synthesis. It is possible to adjust the system so that only rIC will be synthesized by taking advantage of the differential effect of KCl concentration on rA:rU and rIC synthesis (Fig. 4). As shown in Figure 4, rA:rU synthesis

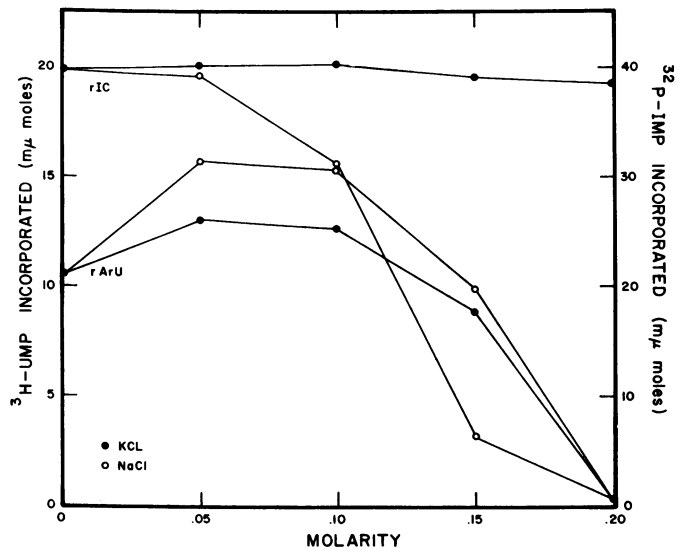

FIG. 4.—Effects of KCl and NaCl on the unprimed synthesis of rIC copolymer and rA:rU homopolymers. The reactions contained in 0.1 ml: 5 μmoles Tris, pH 8.0, 0.2 μmole $MnSO_4$, 2 μmoles mercaptoethylamine, and 19 μg of RNA polymerase. Sixty mμmoles each of αP^{32}-ITP and CTP were added to determine rIC synthesis. Sixty mμmoles each of ATP and H^3-UTP (1.7×10^6 cpm/μmole) were added to determine rA:rU synthesis. The reactions were incubated at 37° for 2 hr after addition of KCl or NaCl to the indicated concentration.

was almost completely inhibited by 0.2 *M* KCl, while rIC synthesis was not affected. The synthesis of both rIC and rA:rU were inhibited by increasing the NaCl concentration. The effect of 0.2 *M* KCl on unprimed synthesis of polymers when the reaction mixture contained RNA polymerase and four ribonucleoside triphosphates (H^3-UTP, αP^{32}-ITP, ATP, and CTP) is shown in Figure 5. When the reaction was carried out in the absence of KCl, only rA:rU was synthesized and no rIC was formed. There was extensive synthesis of rIC in the presence of 0.2 *M* KCl. Only a small amount of H^3-UMP was incorporated into acid-insoluble form in the presence of 0.2 *M* KCl: 0.09 mμmole of H^3-UMP compared to 14 mμmoles of IMP^{32}.

GTP would not replace ITP in the reaction and was in fact an inhibitor of rIC synthesis. The presence of low concentrations of GTP lengthened the lag phase and subsequently slowed the rate of rIC synthesis (Fig. 6). When H^3-GTP was incubated with αP^{32}-CTP and ITP, H^3-GMP appeared in the rIC in an amount corresponding approximately to the ratio of ITP to GTP present in the reaction mixture. Nearest-neighbor analysis showed that the GMP occupied positions in the polynucleotide normally taken by IMP, and about 60 per cent of the H^3-GMP incorporated was found at nucleosidic termini of the chains. It appeared as H^3-

guanosine after alkaline hydrolysis. This may be related to the mechanism of GTP inhibition of rIC synthesis, since it is possible that GMP may readily add to CMP residues while the subsequent addition of CMP to GMP residues may be slow.

Discussion.—The synthesis of rIC copolymer by RNA polymerase and of dAT[14] copolymer by DNA polymerase are related reactions. Both polymers are formed in unprimed reactions following a lag phase and both polymers contain the nucleo-

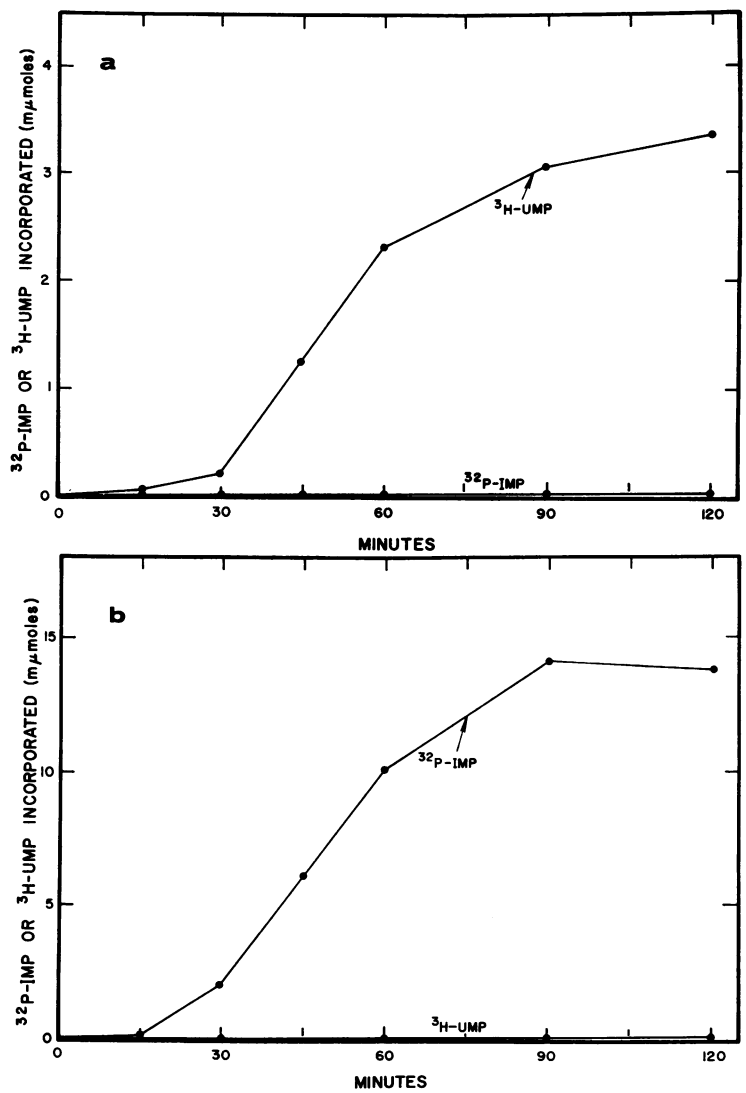

FIG. 5.—Selective synthesis of rA:rU homopolymer or rIC copolymer in the presence of four ribonucleoside triphosphate precursors. The reactions contained in 0.5 ml: 25 μmoles Tris pH 8, 1 μmole $MnSO_4$, 10 μmoles mercaptoethylamine, 300 mμmoles each ATP, CTP, αP^{32}-ITP, and H^3-UTP, and 70 μg *A. vinelandii* RNA polymerase. (*a*) No KCl; (*b*) 0.2 *M* KCl. The reactions were incubated at 37° for the times indicated, following which 50-μl aliquots were removed for assay.

tides in exactly alternating sequences. In each case the mechanism for bringing about perfect alternation is obscure. The Watson-Crick pairs of nucleoside triphosphates are both polymerized by RNA polymerase and DNA polymerase,

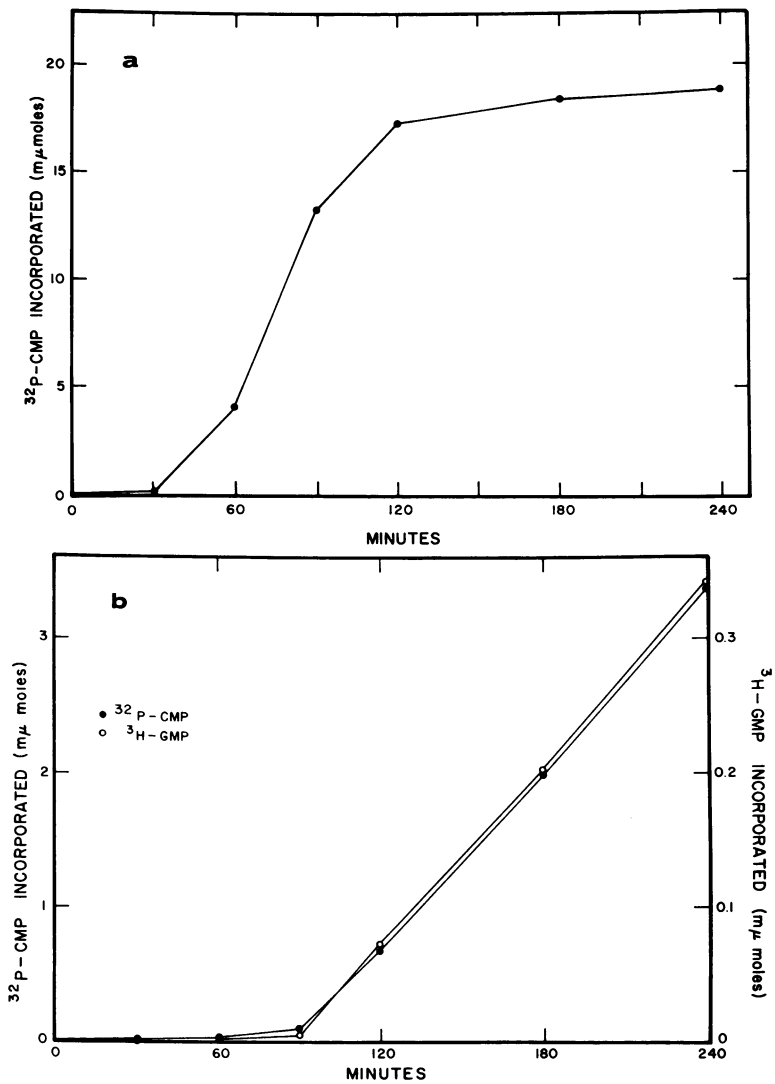

FIG. 6.—Effect of GTP on unprimed synthesis of rIC copolymer. The reactions contained in 0.5 ml: 25 μmoles Tris pH 8, 1 μmole $MnSO_4$, 10 μmoles mercaptoethylamine, 300 mμmoles each ITP and αP^{32}-CTP (2×10^6 cpm/μmole), and 70 μg RNA polymerase. (*a*) No GTP added; (*b*) 20 mμmoles of H^3-GTP (2.4×10^7 cpm/μmole) added. The reactions were incubated at 37° for the times indicated and 50-μl aliquots were then removed for assay.

respectively, following a characteristic lag. However, DNA polymerase synthesizes dAT copolymer and dG:dC[15] homopolymers, while RNA polymerase synthesizes rA:rU[7-9] homopolymers and rIC copolymer in unprimed reactions.

The inability of RNA polymerase to utilize GTP in the unprimed reaction is unexpected since it is the normal substrate in RNA synthesis and is readily polymerized to form poly G in the poly C-directed reaction.[3, 4, 6] The addition of 10^{-5} *M* GTP to the reactions containing CTP and ITP led to a lengthening of the lag phase and to a slowing of the rate of rIC synthesis. The presence of GTP was without effect on the course of reactions leading to the synthesis of rA:rU.

Summary.—Following a short lag period, ITP and CTP were polymerized by RNA polymerase in an unprimed reaction to form a copolymer. Nearest-neighbor analyses showed that IMP and CMP were incorporated in the copolymer in an alternating sequence. The rIC copolymer was hydrolyzed by either pancreatic or T_1 ribonuclease. There was no detectable polymerization when GTP and CTP were incubated with RNA polymerase, and addition of GTP to ITP and CTP resulted in an inhibition of rIC copolymer synthesis.

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Abbreviations: I, inosine; rA:rU, double-stranded complex of polyadenylic acid (poly A) and polyuridylic acid (poly U); rIC, copolymer of inosinic and cytidylic acids; rI:rC, double-stranded complex of polyinosinic acid (poly I) and polycytidylic acid (poly C); dG:dC, double-stranded complex of poly dG and poly dC; dAT, copolymer of deoxyadenylic and deoxythymidylic acids; RNase, ribonuclease; AMP, CMP, GMP, and IMP, 5′-phosphates of adenosine, cytidine, guanosine, and inosine; UMP, uridine monophosphate; ATP, CTP, GTP, and ITP, 5′-triphosphates (pyro) of adenosine, cytidine, guanosine, and inosine; UTP, uridine triphosphate (pyro); EDTA, ethylenediaminetetraacetic acid; Tris, tris-(hydroxymethyl)-aminomethane. TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene.

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