Some Stereochemical Requirements of *Escherichia coli* Ribonucleic Acid Polymerase. Interaction with Conformationally Restricted Ribonucleoside 5′-Triphosphates: 8-Bromoguanosine, 8-Ketoguanosine, and 6-Methylcytidine Triphosphates*

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ABSTRACT: The polymerization and ribonucleoside 5′-triphosphate–inorganic pyrophosphate (NTP–PP_i) exchange reactions catalyzed by *Escherichia coli* RNA polymerase (transcriptase) have been studied with three new, conformationally restricted substrate analogs: BrGTP, OGTP, and 6MeCTP. The experimental results are as follows. (1) BrGTP and OGTP strongly inhibit the RNA polymerase reactions. They are competitive with GTP, their activity requires CMP residues in the template, and inhibition affects polymerization throughout the period of synthesis. 6MeCTP is a poor inhibitor, competitive with CTP. (2) BrGTP, OGTP, and 6MeCTP do not replace their natural counterparts in supporting RNA synthesis directed by calf thymus DNA, poly(C), poly[d(A-C):d(T-G)], or poly(dC). However, in the presence of GTP, BrGTP is polymerized at 1% of the rate of GTP in poly(dC)-directed reactions. With CT DNA template, both BrGTP and OGTP are incorporated at 2 and 4% of the rate of GTP, respectively, although only in the presence of GTP. The extent of polymerization of the analogs is independent of the degree of inhibition. 6MeCTP is not a substrate for the polymerase under the conditions tested; the same is true for BrGTP in reactions with poly[d(A-C):d(T-G)] template. (3) BrGTP is a substrate for the poly(dC)-directed NTP–PP_i exchange reaction but only in the presence of GTP. BrGTP is also a substrate in DNA-directed NTP–PP_i exchange, independent of the presence of GTP, but in the presence of the other three natural substrates. Exchange requires CMP residues in the template. From indirect evidence, the behavior of OGTP in the exchange reaction is the same as that of BrGTP. No evidence for exchange into 6MeCTP was obtained. These results are discussed in relation to the substrate conformation necessary for polymerization in the RNA polymerase reaction.

This paper describes some aspects of the interaction between *Escherichia coli* RNA polymerase and a group of three nucleotide analogs: the 5′-triphosphates of 8-bromoguanosine, 8-ketoguanosine, and 6-methylcytidine. Of these, BrGTP[1] has been studied in greatest detail. The analogs have the following properties in common: (i) all retain the normal H-bonding functional groups which appear to mediate base pairing in replication reactions; (ii) all contain bulky substituents on the aglycones at a position α to the glycosyl bond (purine C-8 or pyrimidine C-6). The substituents are remote from the normal H-bonding positions and thus do not directly impede the formation of base pairs (Kyogoku *et al.*, 1967). However, examination of molecular models and X-ray crystallographic studies of the parent nucleosides (Bugg and Thewalt, 1969; Tavale and Sobell, 1970) show that the space-filling ring substituents act to interfere with rotation at the glycosyl bond and thereby to restrict their conformation. The analogs are thus unusual in the sense that they tend to adopt the syn rather than anti conformation which exists in double-stranded polynucleotides with normal Watson–Crick geometry (Haeschemeyer and Rich, 1967; Sundaralingam, 1969; Saenger and Scheit, 1970).

The purpose of this investigation was to obtain additional information concerning the manner in which RNA polymerase interacts with and polymerizes substrate nucleotides (reviewed by Richardson, 1969). The results reveal that the conformationally restricted analogs bind to the enzyme–template complex in a manner apparently similar to their normal counterparts. In contrast to the normal substrates, the analogs behave as competitive inhibitors and are incorporated poorly. The findings are consistent with, but do not prove the assumption that the enzyme generates a double-helical intermediate during the synthetic process (Kapuler and Spiegelman, 1970).

Materials and Methods

Isolation and Assay of RNA Polymerase. Acid-insoluble radioactivity was measured by a filter technique using either Millipore (MF type HA, 0.45 μ) or glass fiber (GF/A, W. and R. Balston, England) filters with 5% cold trichloroacetic acid as precipitant (Kennell, 1967). RNA polymerase was isolated from *E. coli* B and assayed according to Chamberlin and Berg (1962). The enzyme was stored at −20° in a solution containing 50% glycerol and 1 mg/ml of BSA. The specific activities of the preparations used in these experiments were

* From the Rockefeller University, New York, New York 10021. *Received May 20, 1971.* This work was supported in part by a grant from the National Institutes of Health, U. S. Public Health Service (No. CA 08290-05).

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[1] Abbreviations used are: NTP, ribonucleoside 5′-triphosphate; BrGTP, 8-bromoguanosine 5′-triphosphate; OGTP, 8-oxoguanosine 5′-triphosphate; 6MeCTP, 6-methylcytidine 5′-triphosphate; CT DNA, calf thymus DNA; βME, 2-mercaptoethanol; TP, DP, MP, tri-, di-, and monophosphates; BSA, bovine serum albumin; PP_i, inorganic pyrophosphate; poly(G), poly(C), poly(dC), poly(dT), poly(dG:dC), poly(dA:dT), poly[d(A-C):d(T-G)], respectively, polyguanylic acid, polycytidylic acid, polydeoxycytidylic acid, polydeoxythymidylic acid, homopolymer mixture of polydeoxyguanylic and polydeoxycytidylic acids, homopolymer mixture of polydeoxyadenylic and polydeoxythymidylic acids, mixture of the alternating copolymers of deoxyadenylic–deoxycytidylic acids and deoxythymidylic–deoxyguanylic acids.

2000–3000 units/mg of protein with CT DNA as template. Unless otherwise specified, enzyme incubations were performed for 10 min at 37° with 3.0 units of enzyme in a final volume of 100 μl. Each reaction contained 4.0 μmoles of Tris·HCl (pH 7.9), 400 mμmoles of $MgCl_2$, 100 mμmoles of $MnCl_2$, 1.2 μmoles of βME, 12–25 mμmoles of template, and the appropriate NTP, generally 12.5–50 mμmoles each, depending on the specific radioactivity employed in a given experiment. Initial experiments were performed with Mg^{2+}:Mn^{2+} mixtures, but in subsequent experiments, to facilitate comparison of synthesis with NTP–PP_i exchange, the Mn^{2+} was omitted and replaced by an equal amount of Mg^{2+}.

The conditions of Fox *et al.* (1964) were used to study the synthesis of poly(G) with poly(C) as template. These incubations were conducted at 37° for 20 min with 3.0 units of enzyme in 125-μl reaction volumes.

PP_i–NTP Exchange Reaction. [^{32}P]PP_i (initial specific radioactivity of 290 mCi/mmole) was purchased from the New England Nuclear Corp. and the purity was verified by ion-exchange chromatography on Dowex 1 (Cl^-).

Exchange reactions directed by poly(dC) were performed in 100-μl volumes and contained 6.25 mμmoles of poly(dC), 4 μmoles of Tris·HCl (pH 7.9), 1 μmole of $MgCl_2$, 1.2 μmoles of βME, 200 mμmoles of $Na_4P_2O_7$ (specific activity as noted in each experiment), and 10.2 units of RNA polymerase. NTP was present as described in the individual experiments. Reactions were incubated at 37° for 15 min and terminated by the addition of 10 μmoles of nonradioactive $Na_4P_2O_7$, 1 mg of BSA, and 250 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation at low speed, and to 400 μl of supernatant was added 0.1 ml of an aqueous suspension (15% w/v) of Norit in 0.01 M $Na_4P_2O_7$. The adsorption of nucleotides was permitted to proceed for 30 min while the tubes were kept on ice. The charcoal was then harvested by centrifugation, transferred to a Whatman glass fiber filter (GF/A), washed with six 5-ml portions of 0.1 M sodium acetate buffer (pH 4.5) containing 0.01 M $Na_4P_2O_7$, dried with a heat lamp, and the radioactivity determined by standard liquid scintillation procedures.

Conditions for CT DNA directed exchange reactions were identical with those described above for reactions with poly(dC) except that 12.5 mμmoles of CT DNA replaced the poly(dC).

Synthesis and Characterization of Mononucleotides. A. 8-BROMOGUANOSINE 5′-MONO-, DI-, AND TRIPHOSPHATES. The 5′-mono-, di-, and triphosphates of 8-bromoguanosine were prepared starting from the corresponding guanosine compounds. One millimole of GMP, GDP, or GTP was dissolved in 50–100 ml of formamide and brominated by the addition of a 100- to 200-fold molar excess of bromine. The reaction was maintained at 4° (ice–water bath) for 15–30 min and the unreacted bromine consumed by the addition of aniline. The solution was made slightly alkaline with NH_4OH and the product isolated by addition of four volumes of cold absolute ethanol. The latter procedure was routinely successful also for the isolation of small quantities of brominated product (0.5–5 mg). The brominated guanosine nucleotides can be separated from the parent compounds by repeated chromatography on DEAE-cellulose (CO_3^{2-} form) but separation is accomplished much more efficiently on Dowex 1 (X2) Cl^-. The nucleotides are eluted with a gradient of LiCl (0–2.0 M) in 0.02 M HCl, GTP emerging between 0.5 and 0.6 M LiCl while BrGMP, BrGDP, and BrGTP are eluted at 0.8–1.2, 1.0–1.4, and 1.4–1.8 M LiCl, respectively, depending on the quantity of nucleotide adsorbed and the size of the column. For undefined reasons, it is sometimes difficult to separate BrGMP from BrGDP and BrGDP from BrGTP in this system although quantitative separation of all three from GTP is easily achieved. The purity of the BrGTP (and that of the other bromoguanine preparations) was assessed by the following procedure. An aliquot of nucleotide was depurinated at 100° on a steam bath for 20 min in 6.0 M HCl and the products analyzed by ascending paper chromatography on Whatman No. 1 paper in the solvent 1-butanol–98% formic acid–water (77:10:13, v/v) (Duval and Ebel, 1964). The R_F values in this system for 8-bromoguanine, adenine, and guanine are 0.32, 0.23, and 0.12, respectively. Analysis of depurinated BrGTP revealed a single spot with R_F 0.32 and no detectable guanine. The preparation of BrGTP used in the exchange and incorporation experiments contained less than 0.01% GTP, as judged by analytical chromatography on Dowex 1 (X2) Cl^-. The spectral properties of BrGTP at pH 2.0 are λ_{max} 261.5 mμ (ε 15,600) (Holmes and Robins, 1964) (260:280 = 1.34, 230:260 = 0.22). BrGTP was quantitatively degraded to 8-bromoguanosine by bacterial alkaline phosphatase, to BrGDP with hexokinase and glucose, and to BrGMP by snake venom phosphodiesterase. On paper electrophoresis in citrate buffer (0.05 M pH 3.5) BrGTP and BrGMP moved to the anode slightly more rapidly than the parent guanine nucleotides.

B. 8-KETOGUANOSINE 5′-TRIPHOSPHATE. The parent nucleoside, generously provided by Dr. R. K. Robins, was converted to the 5′-monophosphate by the method of Tener (1961) and then to the 5′-triphosphate according to Smith and Khorana (1958). The uv spectral characteristics of the final product were identical with those previously reported for the nuceloside (Holmes and Robins, 1965). The triphosphate is a substrate for yeast hexokinase and for phosphodiesterase from rattlesnake venom, and the monophosphate is readily hydrolyzed by snake venom 5′-nucleotidase.

C. 6-METHYLCYTIDINE 5′-TRIPHOSPHATE. The parent nucleoside, generously provided by Dr. R. K. Robins, was converted to the 5′-monophosphate with $POCl_3$ in acetone as previously described (Ward *et al.*, 1969). The 5′-monophosphate is poorly soluble under the conditions of Smith and Khorana (1958) which lead to the synthesis of polyphosphate, but a small yield of pure triphosphate is obtained if the mixture is agitated vigorously throughout the course of the reaction. On paper electrophoresis in citrate buffer (0.05 M, pH 3.5) the triphosphate migrated at a rate intermediate between those of CDP and CTP. The triphosphate was rapidly hydrolyzed by bacterial alkaline phosphatase. The uv spectral properties of 6MeCTP at neutral pH were as follows: λ_{max} 270 mμ and λ_{min} 252 mμ (ϵ_{max} 8800). Spectrophotometric titration of 6MeCTP demonstrated an acidic pK_a at pH 4.6 under conditions where the pK_a for CTP was 4.5.

All triphosphate preparations were subjected to hydrolytic procedures to verify the expected molar content of acid-labile and total phosphorus (Ames and Dubin, 1960).

Sources of Other Enzymes and Normal Substrates; Other Methods. Crude rattlesnake (*Crotalus adamanteus*) venom was purchased from Ross Allen Reptile Institute and micrococcal nuclease, pancreatic ribonuclease, pancreatic deoxyribonuclease, and snake venom phosphodiesterase from Worthington Biochemical Corp.

The usual ribo- and deoxyribonucleoside triphosphates were obtained from P-L Laboratories, and radioactive substrates from Schwarz BioResearch Co.

Uv absorption spectra were measured with a Cary Model 14 recording spectrophotometer. Radioactivity measurements

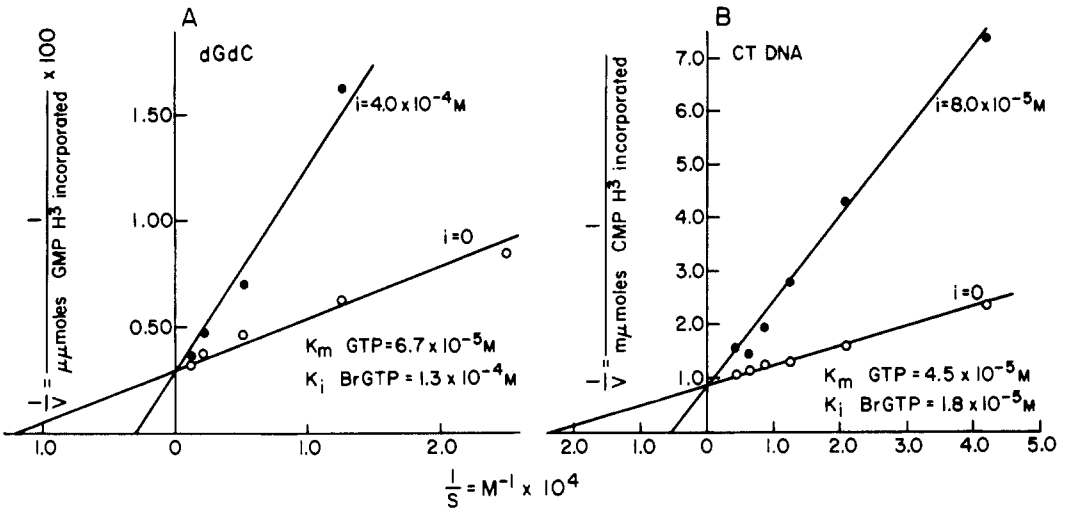

FIGURE 1: BrGTP as a competitive inhibitor for GTP in reaction directed by poly(dG:dC) and by CT DNA. (A) Standard Mg^{2+}:Mn^{2+} conditions were employed with 1.0 mμmole of poly(dG:dC) and 0.5 unit of enzyme per reaction. Increasing amounts of GTP were added to one series of reactions and to a second identical series, 50 mμmoles of BrGTP was added also. A constant amount of [^{3}H]GTP was added to each reaction. (B) CT DNA (22 mμmoles) was added under standard Mg^{2+}:Mn^{2+} reaction conditions. ATP, CTP, and UTP were present at 100 mμmoles/reaction. Increasing amounts of GTP were added to a series of reactions, prepared in duplicate, and to one of these series 10 mμmoles of BrGTP was added. The incorporation of [^{3}H] CTP (specific activity 3560 cpm/mμmole) was measured.

TABLE I: Specificity of Inhibition of RNA Synthesis by Bromoguanosine Nucleotides; Requirement for the 5′-Triphosphate of Bromoguanosine.[a]

Normal Substrates Plus	Ratio of Analog:GTP in Reaction	CT DNA Directed RNA Synthesis mμmole of [^{3}H]CMP Incorpd	% Inhibn	*E. coli* DNA Directed RNA Synthesis mμmole of [^{3}H]CMP Incorpd	% Inhibn	Poly(dC)-Primed Poly(G) Synthesis mμmoles of [^{3}H]CMP Incorpd	% Inhibn
		0.66		0.30		0.50	
BrGTP	2.8	0.43	35	0.14	53	0.31	38
	5.6					0.16	68
BrGDP	5.6	0.72	0	0.28	7	0.47	6
	11	0.56	15	0.24	20	0.35	30
BrGMP	3.6	0.65	2	0.32	0		
	11	0.66	0	0.36	0	0.53	0
OGTP	4.0	0.27	59	0.11	63	0.22	56

[a] CT DNA directed reaction: rate of synthesis was 67% of maximal. Each reaction was performed under standard Mg^{2+} conditions at 37° for 5 min and contained 7.2 units of enzyme, 12.5 mμmoles of DNA, and 5 mμmoles of GTP. [^{3}H]CTP specific radioactivity was 12,500 cpm/mμmole. *E. coli* DNA directed reaction: rate of synthesis was 51% of maximal. Conditions identical with CT DNA directed reaction with 13.2 mμmoles of DNA per reaction. Poly(dC)-directed reaction: rate of control reaction was 32% of maximal velocity. Each reaction was performed in 50 μl under standard Mg^{2+} conditions at 37° for 6 min and contained 3.4 units of enzyme, 9.7 mμmoles of poly(dC), and 5 mμmoles of GTP. [^{3}H]GTP specific radioactivity was 9100 cpm/mμmole.

were performed in a liquid scintillation system (Liquifluor, Pilot Chemical Co.) with a Packard Tri-Carb scintillation counter.

Poly(dC) and poly(dT) were obtained from poly(dGdC) and poly(dAdT) by centrifugation in alkaline Cs_2SO_4 gradients (Inman and Baldwin, 1964). The original complementary homopolymers were synthesized using *E. coli* DNA polymerase, fraction VII (Richardson *et al.*, 1964).

Results

Inhibition Experiments with BrGTP, OGTP, and 6MeCTP. Initial studies of the effects of BrGTP on RNA polymerase revealed that this nucleotide analog inhibits reactions in which GTP is required as a substrate. As seen in Tables I and II and in Figure 1, BrGTP inhibits RNA synthesis with calf thymus DNA, *E. coli* DNA, poly(dGdC), or poly(dC), but

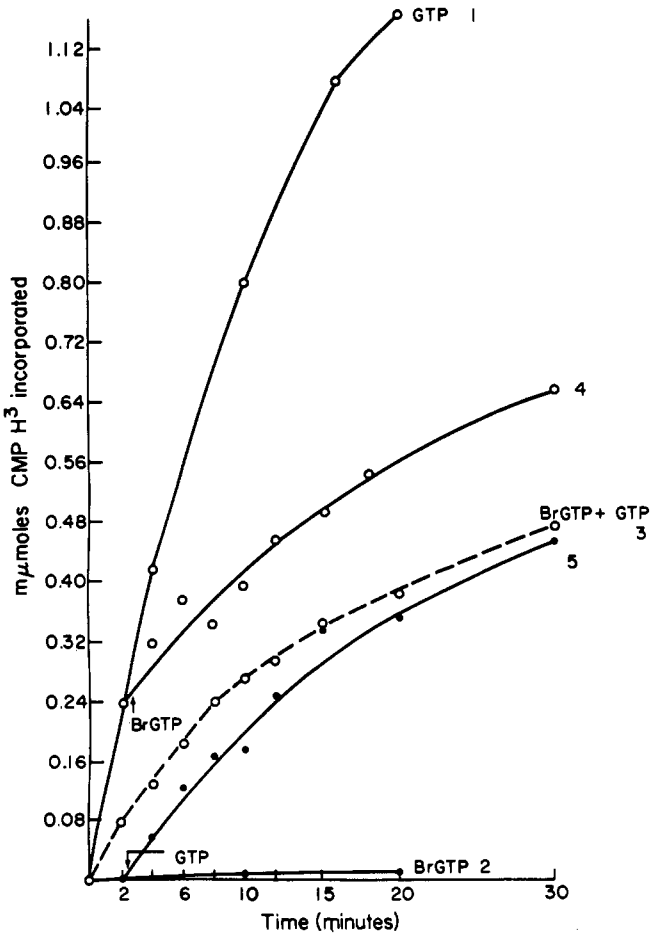

FIGURE 2: BrGTP does not selectively inhibit either chain initiation or chain propagation. Five reactions of 125 μl were prepared using standard Mg^{2+}:Mn^{2+} assay conditions with 22 mμmoles of CT DNA added to each reaction. The incorporation of [^{3}H]CTP (specific activity 26,600 cpm/mμmole) was followed by removing 10-μl aliquots at the times indicated on the graph. The addition of GTP and BrGTP was varied as follows.

Reaction	NTP in Reaction before Addition of Enzyme: UTP, CTP, ATP and:	NTP Added 2.5 min after Addition of Enzyme
1	GTP, 60 mμmoles	
2	BrGTP,110 mμmoles	
3	GTP, 60 mμmoles + BrGTP, 110 mμmoles	
4	GTP, 60 mμmoles	BrGTP, 100 mμmoles
5	BrGTP, 120 mμmoles	GTP, 60 mμmoles

not with poly[d(A-T)] templates. This action of BrGTP therefore depends on the presence of complementary dC residues in the template. The data in Figure 1 show that BrGTP behaves formally as a competitive inhibitor of GTP in reactions directed by CT DNA or poly(dGdC). It is significant that the affinity of the enzyme for BrGTP, as revealed by the K_i of the analog, is no more than twofold different from the K_m of the natural substrate GTP. Maximum inhibition of RNA synthesis by the bromoguanine nucleotides requires a 5′-triphosphate grouping; the activity of BrGDP is 10- to 20-fold lower than that of BrGTP, whereas BrGMP is completely ineffective under the conditions tested (Tables I and II). Thus, the specificity of BrGTP with RNA polymerase–template complex is determined by the presence of complementary dC residues in the template and by the 5′-triphosphate grouping, just as in the case of GTP itself.

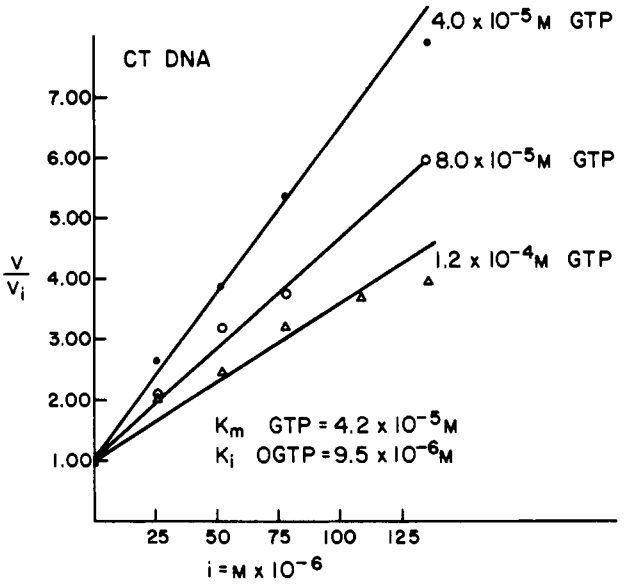

FIGURE 3: Competitive inhibition of CT DNA directed RNA synthesis by OGTP. The reactions were performed under standard Mg^{2+}:Mn^{2+} conditions with 22 mμmoles of CT DNA and 25 mμmoles each of UTP, ATP, and [^{3}H]CTP (8300 cpm/mμmole) per reaction. Three parallel series of reactions were prepared containing increasing quantities of OGTP as indicated in the figure. For each series, values on the ordinate were obtained by dividing the synthesis observed in the absence of inhibitor by the synthesis observed in its presence (Dixon and Webb, 1964).

Since RNA chains are frequently initiated by GTP and bear pppG as the 5′-terminal residue (Maitra and Hurwitz, 1965), it was of interest to test whether BrGTP might selectively interfere with the initiation process. As illustrated in Figure 2, the degree of inhibition produced by BrGTP is independent of the time of addition, showing that polymeriza-

TABLE II: Lack of Inhibition of Poly[d(A-T)]-Directed Poly[r(A-U)] Synthesis by BrGTP and Related Compounds.[a]

UTP, ATP, and	Ratio of Analog:UTP in Reaction	Poly[d(A-T)]-Directed Poly[r(A-U)] Synthesis mμmoles of [^{3}H]UMP Incorpd	% Inhibn
		1.83	
BrGTP	7.0	1.75	4
	23	1.58	13
BrGDP	6.3	1.75	4
	20	1.57	14
BrGMP	9.0	1.70	7
	24	1.60	12
OGTP	3.3	1.96	0
	16	1.53	16

[a] ATP and UTP were both rate limiting, each at 6×10^{-5} M. Each reaction was performed under standard Mg^{2+} conditions at 37° for 10 min and contained 3.4 units of enzyme and 4.8 mμmoles of poly[d(A-T)]. [^{3}H]UTP specific radioactivity was 50,000 cpm/mμmole.

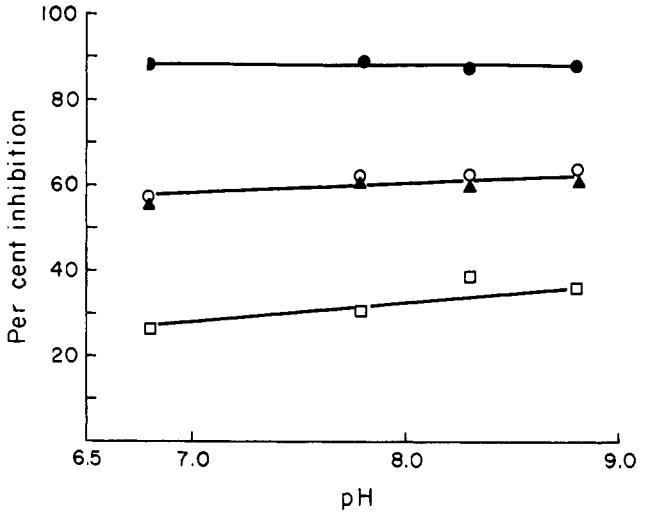

FIGURE 4: Inhibition of RNA polymerase by GTP and OGTP as a function of pH. At each pH, a series of reactions was performed under standard conditions with 18.6 units of enzyme, 12.5 mμmoles of CT DNA, 1 μmole of $MgCl_2$, and 5 mμmoles of GTP and [^{3}H]CTP (specific activity 22,500 cpm/mμmole). To individual reactions at each pH were then added 14 and 42 mμmoles of BrGTP and 16 and 40 mμmoles of OGTP. The rates of synthesis with 5 mμmoles of GTP were 60, 68, 61, and 59% of those with saturating amounts of GTP (100 mμmoles) at *in situ* reaction pH of 6.72, 7.88, 8.30, and 8.80, respectively. Synthesis in the absence of analogs at increasing pH values was 1.16, 2.22, 2.08, and 1.64 mμmoles of [^{3}H]CMP polymerized. At each pH, values of inhibition are calculated from the synthesis in the presence and absence of analog. Buffers used for the different pH values were potassium morpholinopropanesulfonate, 6.8; Tris·HCl, 7.9; potassium glycinate, 9.2; and Tris·HCl, 9.9; 5 μmoles each was added to the appropriate reactions conducted in 100-μl volumes. The pH of reactions containing these buffers was measured in reactions twice normal scale, equilibrated at 37°, using a Radiometer pH meter TTT 1c with scale-expander PHA 630T employing a single electrode system GK 2302B. (●) OGTP:GTP = 8.0; (○) BrGTP:GTP = 8.4; (▲) OGTP:GTP = 3.2; (□) BrGTP:GTP = 2.8.

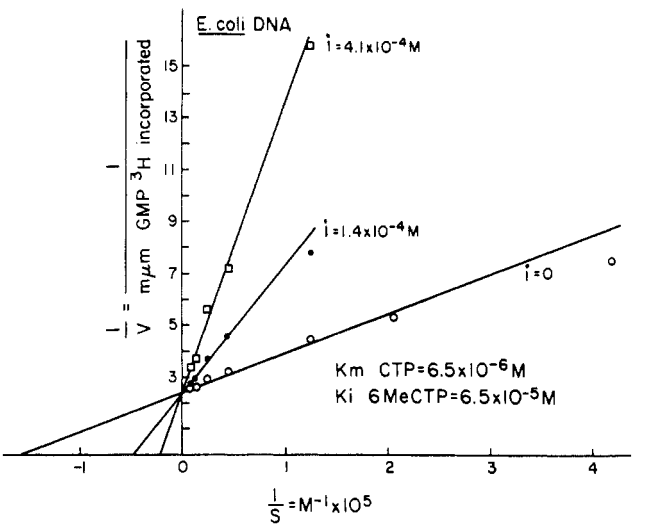

FIGURE 5: Inhibition of RNA polymerase by 6MeCTP. *E. coli* DNA (3.3 mμmoles) was added to each reaction together with 12.5 mμmoles each of UTP, ATP, and [^{3}H]GTP (specific activity 30,000 cpm/mμmole). Increasing amounts of CTP were added to a series of reactions, prepared in triplicate, and then 0, 17, or 51 mμmoles of 6MeCTP was added to each reaction of a given series. Standard Mg^{2+}:Mn^{2+} assay conditions were employed.

tion is inhibited by BrGTP throughout the course of RNA chain growth. These experiments do not define the extent to which BrGTP affects initiation, if it does so at all.

Since the action of BrGTP might have been due to the specific chemical properties of the bromine atom, it appeared desirable to study another guanine nucleotide analog, and OGTP was selected for this purpose. The keto group at C-8 of OGTP resembles the Br at C-8 in BrGTP in the sense that it produces a conformational restriction to rotation about the glycosyl bond, thereby constraining the molecule in the syn conformation (these effects can be expected with both tautomeric forms of the 8-keto group.

In its interactions with RNA polymerase OGTP qualitatively reproduces all the effects seen with BrGTP, and, on a molar basis, OGTP is a 2- to 3-fold more potent inhibitor. Thus, OGTP inhibits RNA synthesis directed by *E. coli* DNA, calf thymus DNA, and poly(dC) (Table I), while poly[d(A-T)]-directed reactions are virtually unaffected. As seen in Figure 3, OGTP like BrGTP, acts as a competitive inhibitor of GTP.

The interaction of an oxygen atom at C-8 of guanine gives rise to an acidic proton with pK_a = 8.6 (W. Guschlbauer, A. Ruet, and P. Fromageot, personal communication). However, the inhibitory ability of OGTP is not diminished in the pH range in which this proton is titrated (Figure 4). Thus, OGTP bearing a single negative charge on the base still interacts strongly with RNA polymerase. This characteristic of OGTP recalls the report (Ludlum, 1970) that 7-methylguanine, which carries a positive charge on the imidazole ring, functions as a template for RNA polymerase and forms base pairs with cytosine substrate residues. This is in contrast to other analogs, such as dXTP (Bessman *et al.*, 1958; Richardson *et al.*, 1963), 6azaUTP, and 5-fluoroUTP (Kahan and Hurwitz, 1962) in which the bases exist as anions at neutral pH; all of these interact poorly with DNA and RNA polymerases. The difference in behavior of these analogs can be reconciled if it is assumed that the negative charge in OGTP at pH 9.0 is localized on the imidazole ring, leaving the H-bonding positions on the pyrimidine ring of guanine unimpaired; in the anionic forms of dXTP, XTP, and 6azaUTP and 5-fluoroUTP it is likely that one of the potential H-bonding functional groups required for base pairing is at least partly charged.

The inhibitory activity of 6MeCTP is quantitatively lower than that of BrGTP or OGTP, but resembles the latter qualitatively in competing with CTP (Figure 5).

Substrate Properties of BrGTP, OGTP, and 6MeCTP. The potential substrate properties of BrGTP were studied in reactions primed by poly[d(A-C):d(T-G)], poly(C), and CT DNA (Table III). When BrGTP replaces GTP, the rate of synthesis with all three templates is reduced by 99.7%. In comparable experiments with poly(dC) as a template no incorporation of BrGTP into acid-insoluble polymer is detectable. However, under appropriate conditions the incorporation of BrGMP residues into internucleotide linkages does occur at low frequency. The data in Figures 6 and 7 demonstrate that BrGTP is utilized by RNA polymerase only in the presence of GTP and at about 1% of the rate of GTP in the same reaction. As shown by the chromatographic results in Figures 6 and 7, alkaline hydrolysis of the product formed in a reaction conducted with [α-^{32}P]GTP and BrGTP gives rise to a radioactive nucleotide that has the properties of 2′(3′)BrGMP. Since the labeled substrate was [α-^{32}P]GTP, radioactivity has been transferred from GMP to BrGMP residues during alkaline hydrolysis. These facts indicate that

TABLE III: RNA Synthesis with BrGTP or OGTP Replacing GTP, and with 6MeCTP Replacing CTP.[a]

Template	Substrates (mμmoles/Reaction)		
(1) Poly[d(A-C):d(T-G)]	A. GTP (100), UTP (100)	mμmoles of [^{3}H]GMP incorporated	0.84
	B. BrGTP (100), UTP (100)	mμmole of [^{14}C]BrGMP incorporated	0.002
(2) Poly(C)	A. GTP (100)	mμmole of [^{3}H]GMP incorporated	7.35
	B. BrGTP (100)	mμmole of [^{14}C]BrGMP incorporated	0.01
	C. GTP (100), BrGTP (100)	mμmole of [^{3}H]GMP incorporated	2.96
		mμmole of [^{3}H]CMP incorporated	
		1	*2*
(3) CT DNA	A. ATP (40), CTP (40), UTP (40), GTP (40)	0.38	
	B. ATP (25), CTP (25), UTP (25), GTP (40)		0.38
	C. ATP (40), CTP (40), UTP (40), BrGTP (50)	0.001	
	D. ATP (25), CTP (25), UTP (25), OGTP (40)		0.001
		mμmoles of [^{3}H]GMP incorporated	
(4) *E. coli* DNA	A. ATP (12.5), GTP (12.5), UTP (12.5), CTP (12.5)	0.37	
	B. ATP (12.5), GTP (12.5), UTP (12.5), 6MeCTP (14)	0.001	

[a] Reaction conditions: components per reaction–(1) poly[d(A-C):d(T-G)], 0.63 mμmole; [^{3}H]GTP, 5600 cpm/mμmole; [^{14}C]BrGTP, 6400 cpm/mμmole, Mg^{2+} and Mn^{2+}; (2) [^{3}H]GTP, 10,700 cpm/mμmole; [^{14}C]BrGTP, 12,800 cpm/mμmole, Mn^{2+}; (3) (a) [^{3}H]CTP, 24,300 cpm/mμmole, Mg^{2+}; (b) [^{3}H]CTP, 77,500 cpm/mμmole, Mg^{2+} and Mn^{2+}; (4) [^{3}H]GTP, 30,000 cpm/mμmole; 3.3 mμmoles of *E. coli* DNA; Mg^{2+} (identical results were obtained with Mg^{2+} and Mn^{2+}).

BrGMP residues are present at internal positions in the polymer product; thus, the inhibitory effect of the analog is probably not due solely to chain termination accompanying incorporation at the 3′ terminus of a growing polynucleotide.

Corroborative evidence suggesting that BrGMP is incorporated in the presence of GTP with certain templates has also been obtained by an indirect method. This experimental approach depends on the use of isotopic double labeling in reactions that require at least two nucleotide substrates (Levisohn and Spiegelman, 1969). For example, with CT DNA template, a reaction might be conducted with [α-^{32}P]GTP and [^{3}H]CTP as labeled compounds, and the ratio ^{32}P:^{3}H incorporated will be characteristic of the RNA product. Addition of a GTP analog, for example, BrGTP, will produce one of two effects, depending on whether BrGMP is polymerized or not. If the analog acts as a substrate (*i.e.*, if it is incorporated), then the ratio ^{32}P:^{3}H in the RNA product will decline, *i.e.*, there will be an isotopic dilution of ^{32}P for which the incorporation of ^{3}H serves as an internal standard. If the analog is not incorporated and merely inhibits the rate of synthesis, total incorporation of radioactivity will decrease without change in the ratio ^{32}P:^{3}H. By varying the ratio analog:normal substrate in the reaction the incorporation of analog can be detected reliably at a level of 0.5% of the total nucleotide polymerized. Under these conditions a plot of the ratio ^{32}P:^{3}H as a function of the ratio of the initial concentrations of analog:substrate yields a curve whose slope and shape can provide an accurate estimate of analog incorporation.

This method has been used with several templates and with BrGTP, OGTP, and 6MeCTP. As seen in Figure 8, BrGMP is not incorporated with poly[d(A-C):d(T-G)] template; however, BrGMP is incorporated at about 2% the rate of GMP in reactions directed by CT DNA (Figure 9). Identical results were obtained when the experiment was conducted with [^{3}H]UTP rather than [^{3}H]CTP.

Like BrGTP, OGTP is a substrate for RNA synthesis directed by calf thymus DNA only when GTP is also present in the reaction mixture. In the presence of GTP, OGTP is utilized by RNA polymerase and OGMP is incorporated into RNA at a level of about 4% compared with GMP (Figure 10). As with BrGMP, the relative incorporation of OGMP is independent of the ratio OGTP:GTP and of the degree of inhibition of the reaction.

With CT DNA template 6MeCTP is not incorporated into RNA either in the presence (Figure 11) or absence (Table III) of CTP.

One important reservation should be noted concerning the double-labeling isotope dilution experiments. The conclusion that the analogs are incorporated in place of the normal counterpart is based on changing ratios of ^{32}P:^{3}H in product RNA. This interpretation is valid only if the base composition of RNA synthesized is not altered throughout the range of changing proportions of normal nucleotide and analog. We have not verified that this is the case, and cannot rigorously exclude the possibility that different segments of template are being transcribed as the precursor ratio BrGTP:GTP increases. However, several facts indicate that this reservation does not seriously impair the significance of the preceding experiments. The most important of these is the finding that the apparent relative incorporation of BrGMP or OGTP is independent of the initial ratio of analog:GTP, or of the inhibition of RNA synthesis. If the effect of the analogs was to foster the synthesis of G-poor polynucleotides, the ^{32}P:^{3}H

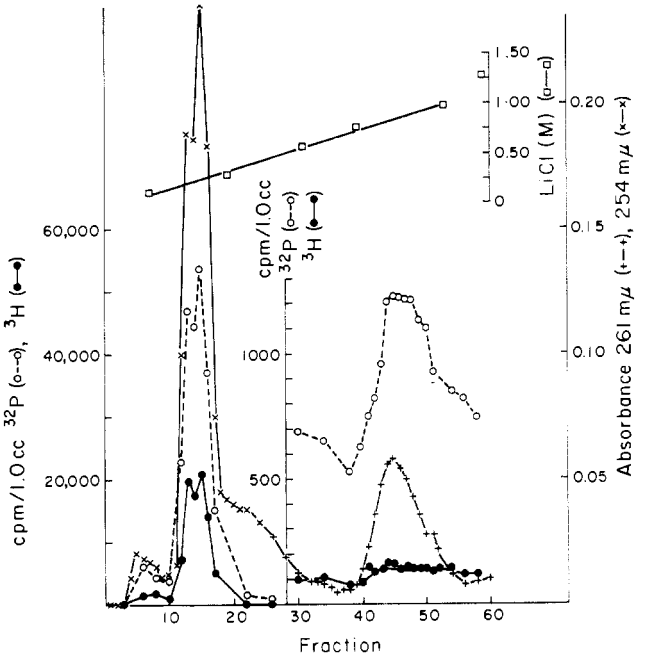

FIGURE 6: Poly(dC)-directed RNA synthesis with [α-^{32}P]GTP and BrGTP as substrates; label transfer in the product as demonstrated by column chromatography of the alkaline hydrolysate. One reaction twice normal scale was prepared under standard conditions (Table I) with 9.6 units of enzyme and incubated at 37° for 15 min. BrGTP (28 mmoles) and GTP (10.5 mmoles) were substrates. Both [^{3}H]GTP (final specific activity 500,000 cpm/mmole) and [α-^{32}P]GTP (specific activity 8.5 × 10^{5} cpm/mμmole) were included. Acid-insoluble polymer synthesis was determined on an aliquot removed at the end of the incubation. The reaction was ended by heating at 70° for 2 min followed by the addition of 200 mμmoles of poly(G), 160 mμmoles of poly(BrG), and excess bacterial alkaline phosphatase (Worthington Biochemical Corp.) and incubation at 37° for 4 hr. The reaction was than shaken with phenol to denature protein and the combined phenol and aqueous layers dialyzed against 1000 volumes of Tris·HCl, 0.01 M, 7.9, and 2 × 1000 volumes of the same buffer containing 1.0 M KCl. During the synthetic reaction, 1.35 mμmoles of [^{32}P]GMP and 1.42 mμmoles of [^{3}H]GMP were polymerized, and the recovery of acid-precipitable product after dialysis was 75%. The small precipitate of denatured protein was removed by centrifugation, the RNA was passed through a column 1.2 × 42 cm of Sephadex G-25 (coarse) and the material excluded from the column matrix was pooled and hydrolyzed with 0.3 M KOH for 18 hr at 37°. The KOH was neutralized with $HClO_4$, the solution cooled and centrifuged to remove $KClO_4$, and the neutral, salt-free supernatant adsorbed to a 0.9 × 5.0 cm Dowex 1 Cl column. The nucleotides were eluted with a 0–2.0 M LiCl gradient, 0.2 M in HCl, as illustrated in the figure. Fractions were monitored for radioactivity and uv absorbance by standard methods. The molarity of the eluting solution was determined conductimetrically.

ratio in the product (and therefore the apparent incorporation of analog) should change as the proportion of analog precursor is increased. Secondly, in the case of BrGTP, the incorporation of BrGMP at internal positions is demonstrated definitely by isolation of expected nucleotide following alkaline hydrolysis of the product. Moreover, with BrGTP the amount of incorporation indicated by double labeling is very close to that actually obtained by chemical analysis of the degraded product. Finally, if the action of the analogs led to transcription of preferred regions of template, the results obtained with 6MeCTP (which is not incorporated) would be expected to resemble those with BrGTP and OGTP.

NTP–PP_i Exchange Reaction with BrGTP, OGTP, and 6MeCTP. The RNA polymerase catalyzed exchange of PP_i

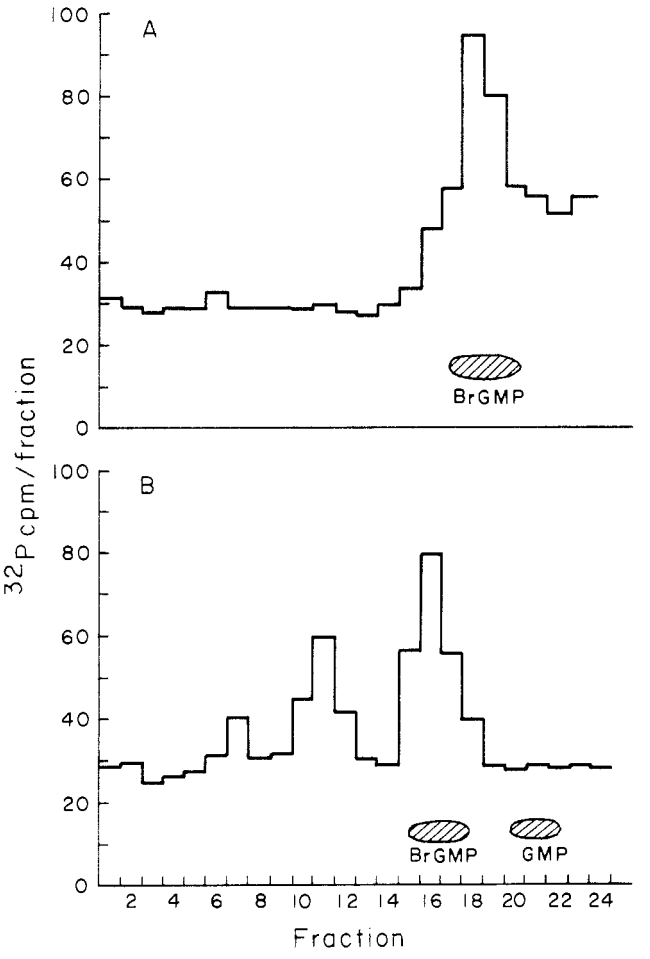

FIGURE 7: Characterization of [2′-(3′)-^{32}P]BrGMP. The material in fractions 42–49 (Figure 6) had an uv maximum at 261 mμ and an absorbancy ratio 254 mμ:261 mμ characteristic of BrGMP. The material remaining after radioactivity measurements was pooled and the nucleotides isolated by the following procedure. Washed Norit A (10–15 mg) was adjusted to pH 4 with acetic acid and incubated with 9 μmoles of 5′-BrGMP for 30 min at room temperature. The Norit was then washed twice with water and, together with 100 μl of glacial acetic acid, added to the pooled solution of fractions 42–49. Eighty-five per cent of the radioactivity was adsorbed to the charcoal in 5 min. The Norit was washed twice with water and the nucleotides eluted with two 0.5-ml portions of washed of absolute EtOH–water–concentrated NH_4OH (50:45:5, v/v). Ninety per cent of the adsorbed nucleotide was recovered in the eluate. Of the 2100 cpm of ^{32}P in fractions 42–49, 1600 was obtained, salt free, in this manner. The nucleotides eluted from Norit were concentrated by rotary evaporation and characterized by chromatography and electrophoresis, as illustrated in the figure. (A) Electrophoresis at pH 3.5: ^{32}P-labeled nucleotide (300 cpm) were spotted on Whatman No. 3MM paper together with 5′-BrGMP and electrophoresed for 3 hr in sodium citrate (pH 3.5,0.05 M). As shown in the figure, part A, 260 cpm was recovered in the electropherogram, 60% of them having the same mobility as 5′-BrGMP. (B) Chromatography: ^{32}P nucleotide (260 cpm) was spotted on DEAE paper with 5′-BrGMP and 5′-GMP markers and the chromatogram developed with 0.17 M triethylammonium bicarbonate for 36 hr in a descending system. Under these conditions, 5′-BrGMP and 5′-GMP were well separated and 60% of the radioactivity recovered in the chromatogram migrated with the BrGMP marker—none with the GMP marker. Eighty-five per cent of the radioactivity applied to the paper was recovered. The background was between 25 and 30 cpm. From these criteria, the adsorption to Norit, the chromatography on DEAE paper and Dowex 1, and the electrophoresis, we identify 60% of the ^{32}P-labeled material as 2′-(3′)-BrGMP produced from the alkaline hydrolysis of RNA containing BrGMP in internucleotide linkage next to [α-^{32}P]GMP.

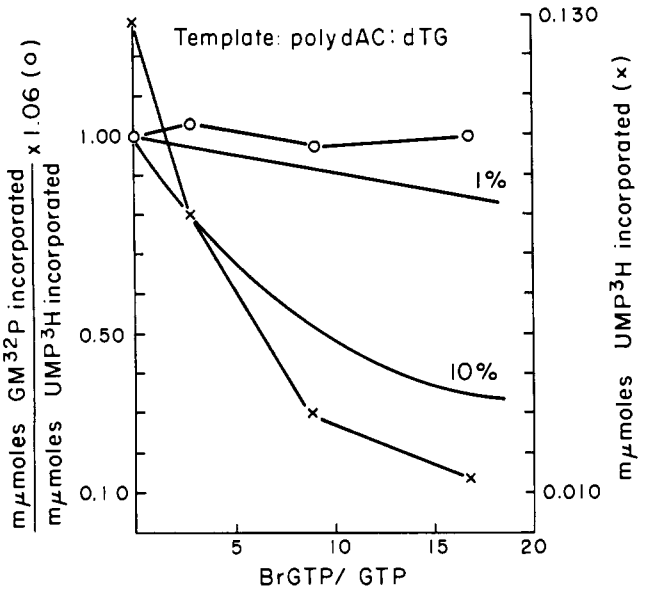

FIGURE 8: BrGTP is not a substrate in poly[d(A-C):d(T-G)]-directed reactions: double-label isotope dilution procedure. Template poly-[d(A-C):d(T-G)], 1.3 mμmoles, and 3.0 units of enzyme per reaction, Mg^{2+} conditions, incubated at 37° for 10 min; UTP (specific activity 105,400 cpm/mμmole, [α-^{32}P]GTP (specific activity 91,000 cpm/mμmole), UTP, 40 mμmoles, and 5 mμmoles of GTP per reaction; BrGTP added as indicated in the figure. The lines marked 1 and 10% correspond to the theoretical isotope dilution if BrGTP is incorporated at 1 and 10% the rate of GTP.

TABLE IV: PP_i–NTP Exchange Reaction: BrGTP and OGTP with Poly(dC) and Poly(dT) Templates.

Substrates (mμmoles/Reaction)	mμmoles of [^{32}P]PP_i Incorpd into NTP/100 μl of Reaction: Poly(dC) Template	Poly(dT) Template
ATP (80)		2.83
GTP (80)	1.24	0.016
GTP (20)	0.38	
BrGTP (84)	<0.001	<0.001
OGTP (80)	<0.001	
GTP (20), BrGTP (84)	0.82	<0.001
GTP (20), OGTP (80)	0.79	
GTP (80), no template	<0.001	

[a] Poly(dC)-directed reaction: reactions were performed for 15 min at 37° with 4.8 units of enzyme/reaction. Background in controls lacking enzyme was 2750 cpm and the specific radioactivity of the $Na_4P_2O_7$ was 70,000 cpm/μmole. Poly(dT)-directed reaction: reactions were performed as described for poly(dC) except that 8.5 mμmoles of poly(dT) replaced poly(dC) as template. Each reaction received 4.8 units of enzyme and $Na_4P_2O_7$, specific radioactivity 78,000 cpm/mμmole.

with the β,γ positions of NTP was studied to gain further insight into the mechanism by which the enzyme interacts with these conformationally restricted substrates. In a reaction with poly(dC) template, no exchange with BrGTP or OGTP

TABLE V: Pyrophosphate–NTP Exchange Reaction: BrGTP, OGTP, and 6MeCTP with CT DNA Template.[a]

Substrates (mμmoles/Reaction)	mμmoles of [^{32}P]PP_i Incorpd into NTP/100 μl of Reaction: 1	2	3
UTP (80)			0.067
CTP (80)			0.068
6MeCTP (68)			<0.001
UTP (80), CTP (80)	0.81		0.67
UTP (80), CTP (20)			0.41
UTP (80), 6MeCTP (68)			0.046
UTP (80), CTP (20), 6MeCTP (68)			0.35
UTP (80), CTP (80), GTP (100)		2.32	
UTP (80), CTP (80), GTP (80)	2.83		
UTP (80), CTP (80), GTP (5)		1.50	
UTP (80), CTP (80), BrGTP (84)	0.50		
UTP (80), CTP (80), OGTP (80)	0.52		
UTP (80), CTP (80), GTP (5), BrGTP (84)		1.14	
UTP (80), CTP (80), GTP (5), OGTP (80)		1.11	
UTP (80), CTP (80), ATP (80)	2.30		
UTP (80), CTP (80), ATP (80), GTP (100)		2.20	
UTP (80), CTP (80), ATP (80), GTP (80)	2.24		
UTP (80), CTP (80), ATP (80), GTP (40)	1.94		
UTP (80), CTP (80), ATP (80), GTP (5)		1.98	
UTP (80), CTP (80), ATP (80), BrGTP (84)	2.01		
UTP (80), CTP (80), ATP (80), OGTP (80)	1.43		
UTP (80), CTP (80), ATP (80), GTP (40), BrGTP (42)	1.76		
UTP (80), CTP (80), ATP (80), GTP (5), BrGTP (84)		1.54	
UTP (80), CTP (80), ATP (80), GTP (40), OGTP (40)	1.80		
UTP (80), CTP (80), ATP (80), GTP (5), OGTP (80)		1.50	

[a] Reactions were performed under standard conditions for 15 min at 37° with 4.8 units of enzyme/reaction. Specific radioactivity of $Na_4P_2O_7$ was 70,000, 39,000, and 74,000 cpm per mμmole in series 1, 2, and 3, respectively.

alone was observed under conditions where extensive exchange into GTP takes place (Table IV). However, in a CT DNA primed reaction with rate-limiting concentrations of GTP the addition of a fourfold excess of BrGTP or OGTP produces a substantial stimulation of exchange (Table V) suggesting that the two GTP analogs are themselves substrates for the exchange reaction, provided some GTP is present. The exchange reaction with the analogs, in common with inhibition and polymerization, is template dependent and template specific. Thus, no exchange into GTP occurs in the absence of poly(dC), and exchange stimulated by BrGTP and

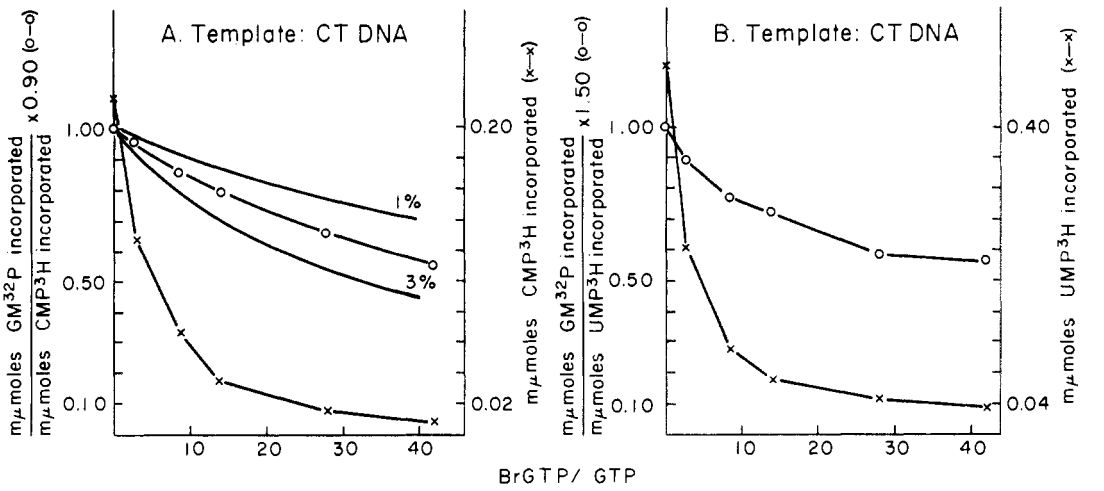

FIGURE 9: BrGTP is a substrate in CT DNA directed reactions: double-label isotope dilution procedure. (A) Template CT DNA, standard Mg^{2+} conditions, 3.0 units of enzyme/reaction; [^{3}H]CTP (specific activity 26,200 cpm/mμmole), [α-^{32}P]GTP (specific activity 26,800 cpm/mμmole), 40 mμmoles each ATP, CTP, and UTP, and 5 mμmoles of GTP per reaction; BrGTP added as indicated in the figure. (B) Template CT DNA as in part A but with UTP as second isotope (specific activity 33,000 cpm/mμmole). The lines marked 1 and 3% correspond to the theoretical isotope dilution if BrGTP is polymerized at 1 and 3% the rate of GTP.

OGTP is not observed when poly(dT) replaces poly(dC) (Table IV).

To demonstrate that the kinetic stimulation of exchange observed when BrGTP is added to rate-limiting GTP actually reflects incorporation of [^{32}P]PP_i into the β,γ positions of BrGTP, the nucleotides in a large-scale reaction were isolated, identified, and characterized as shown in Figures 12 and 13. These results show that [^{32}P]PP_i is indeed introduced into the β,γ positions of BrGTP in this reaction with poly(dC) template.

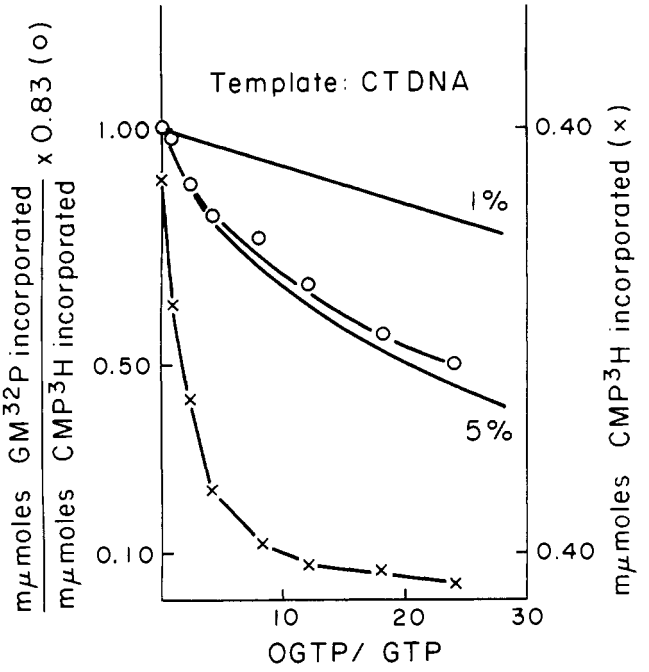

FIGURE 10: OGTP is a substrate in CT DNA primed reactions: double-label isotope dilution procedure. Template CT DNA, standard conditions as in Figure 9A; [^{3}H]CTP (specific activity 26,200 cpm/mμmole) and [α-^{32}P]GTP (specific activity 23,100 cpm/mμmole); OGTP added as indicated in the figure. The lines marked 1 and 5% correspond to the theoretical isotope dilution if OGTP is polymerized at 1 and 5% the rate of GTP.

A similar exchange of PP_i with BrGTP occurs in reactions directed by CT DNA (Figures 14 and 15). Under these conditions, extensive exchange occurs into both GTP and BrGTP; however, the exchange with BrGTP is independent of the presence of GTP and is in fact increased when GTP is omitted from the reaction and no acid-insoluble polymer is formed.

Discussion

The preceding data show that the conformationally restricted nucleotide analogs all interact specifically with RNA polymerase. In the case of BrGTP, the evidence for inter-

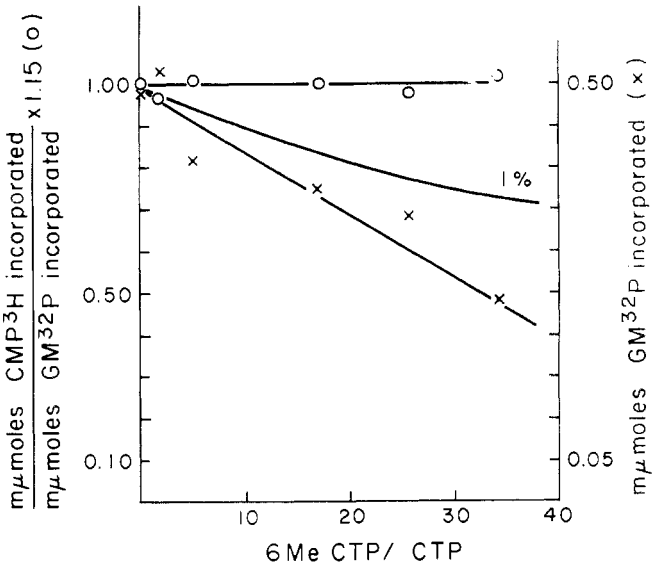

FIGURE 11: 6MeCTP is not a substrate in CT DNA directed reactions; double-label isotope dilution procedure. Template CT DNA, standard conditions, [^{3}H]CTP (specific activity 91,100 cpm/mμmole), and [α-^{32}P]GTP (specific activity 46,000 cpm/mμmole); 40 mμmoles of ATP, UTP, and GTP and 2 mμmoles of CTP added to each reaction; 6MeCTP present as indicated in the figure. The line marked 1% corresponds to the theoretical isotope dilution were 6MeCTP incorporated at 1% the rate of CTP.

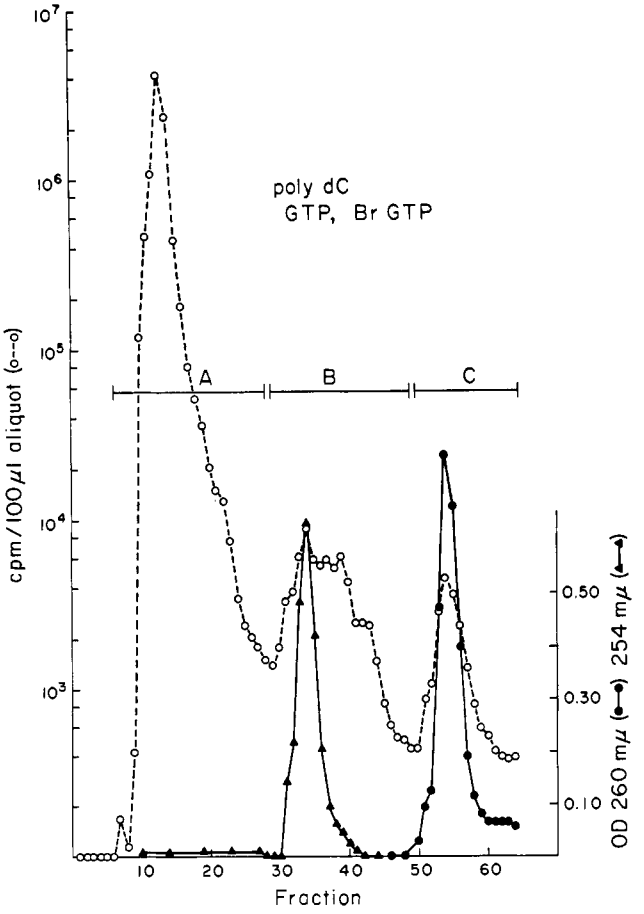

FIGURE 12: Isolation of [β,γ-^{32}P]BrGTP as one product of the poly-(dC)-directed exchange reaction. One reaction, five-times normal scale (500 μl) was incubated at 37° for 15 min; it contained poly-(dC), 31.5 mμmoles; Tris·HCl (pH 7.9), 20 μmoles; GTP, 25 mμmoles; BrGTP, 420 mμmoles; $MgCl_2$, 5 μmoles; βME, 6 mμmoles; $Na_4P_2O_7$ (specific activity 118,000 cpm/mμmole), 1.0 mμmole; and 24 units of enzyme. The reaction was terminated by adding 5 mg of BSA, 4 mμmoles of GTP, and 30 μl of 100% trichloroacetic acid. The precipitate was removed by centrifugation and the supernatant adjusted to pH 7–8 with NaOH, diluted to 20 ml with H_2O, and adsorbed to a column (0.9 × 3.0 cm) of Dowex 1-X2 (Cl). The column was washed sequentially with water (10 ml), 0.02 M LiCl + 0.02 M HCl (30 ml) (A), 0.5 M LiCl + 0.02 M HCl (30 ml) (B), and 2.0 M LiCl + 0.02 M HCl (30 ml) (C). Fractions of 5.0 ml (1–6) or 1.5 ml (7–64) were collected and assayed for radioactivity and uv absorbance. The uv-absorbing peaks were identified as GTP (fractions 33–35) and BrGTP (fractions 53–56) spectrophotometrically. The [β,γ-^{32}P]BrGTP (pooled fractions 53–56) was characterized by subsequent procedures (Figure 13). Eighty-five per cent of the material in fractions 53–56 was [β,γ-^{32}P]BrGTP. In the exchange reaction 3.26 and 1.49 mμmoles of [^{32}P]P_2O_7 were exchanged into GTP and BrGTP, respectively.

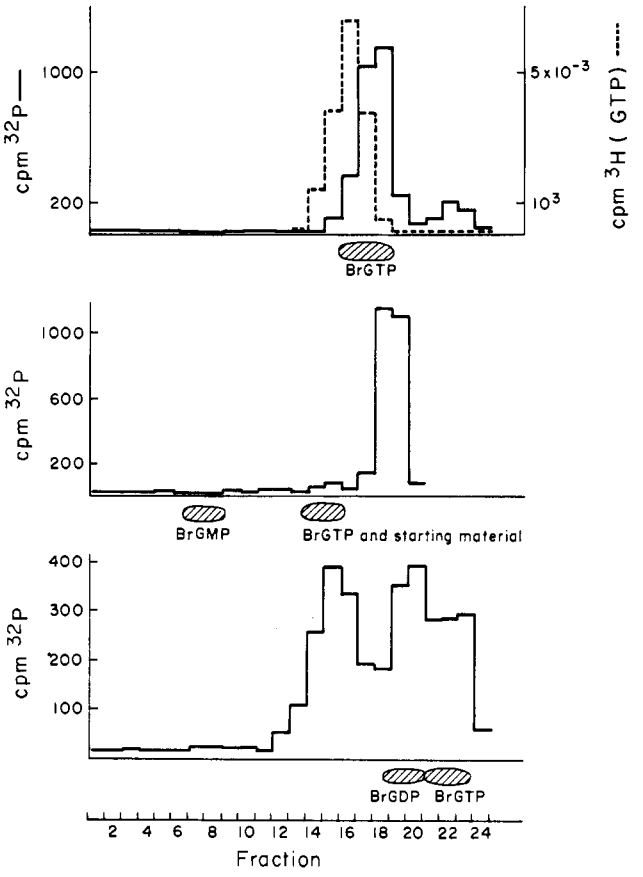

FIGURE 13: Characterization of [β,γ-^{32}P]BrGTP. (1) Electrophoresis at pH 3.5, sodium citrate, 0.05 M, 2 hr, 2000 V, Whatman No. 1 paper, [^{3}H]GTP and BrGTP markers. (2) Pretreatment of an aliquot of fractions 53–56 (Figure 12) with snake venom phosphodiesterase (Worthington Biochemical Corp.). [β,γ-^{32}P]BrGTP (4500 cpm, 0.038 mμmole) was incubated with 10 μg of enzyme in 0.02 M Tris·HCl (pH 7.9) and 0.02 M $MgCl_2$ for 5 hr at 37°. The products were electrophoresed in sodium citrate, 0.05 M, pH 3.5, for 1.75 hr at 2000 V with BrGMP, BrGDP, and BrGTP as optical density markers and [^{32}P]PP_i and [β,γ-^{32}P]BrGTP as radioactivity markers in Whatman No. 3MM paper. [^{32}P]PP_i and [β,γ-^{32}P]BrGTP were found in fractions 18–19 and 14–15, respectively. (3) Pretreatment of an aliquot of fractions 53–56 (Figure 12) with hexokinase (Boehringer and Soehne Co.). [β,γ-^{32}P]BrGTP (4500 cpm, 0.038 mμmole) was incubated with 5 μg of enzyme in Tris·HCl (pH 7.9, 0.02 M), 0.02 M $MgCl_2$, and 0.1 M dextrose for 4 hr at 37°. The products were electrophoresed, with appropriate markers, for 3 hr on Whatman No. 1 paper, as described above. Radioactivity in the electropherograms was measured on 2-cm sections of the paper by liquid scintillation procedures.

action at the nucleotide polymerizing site of the enzyme is direct: thus (1) like the normal substrates, the analog requires a complementary template residue and binds effectively only as the 5′-triphosphate. Indeed, the affinity of the enzyme, as shown by the K_i for BrGTP, is essentially the same as that implied by the K_m for the normal substrate GTP. (2) BrGMP is incorporated at low frequency and can be recovered from hydrolysates of product RNA. (3) BrGTP participates in the enzyme-catalyzed exchange reaction with [^{32}P]PP_i.

In terms of RNA synthesis, BrGTP could affect chain initiation, propagation, or termination, either singly or in combination. Several observations suggest that the action of BrGTP is not due solely to either inhibition or initiation or to premature chain termination. These include (1) the fact that the inhibitory effects of a given concentration of the analog are quantitatively the same irrespective of the time of addition to the reaction; (2) the failure of BrGTP to support chain growth in four-nucleotide reactions with CT DNA template under conditions that allow a substantial number of chains to be initiated by ATP. It appears also unlikely that BrGTP inhibits RNA synthesis predominantly by causing chain termination (as seen with 3′-dATP) (H. Shigeura and G. Charles, 1965) since BrGMP is incorporated at low but detectable frequency into internucleotide linkages in RNA, and (3) the pyrophosphate exchange reaction is stimulated by BrGTP but is inhibited by 3′-dATP (P. Fromageot, personal

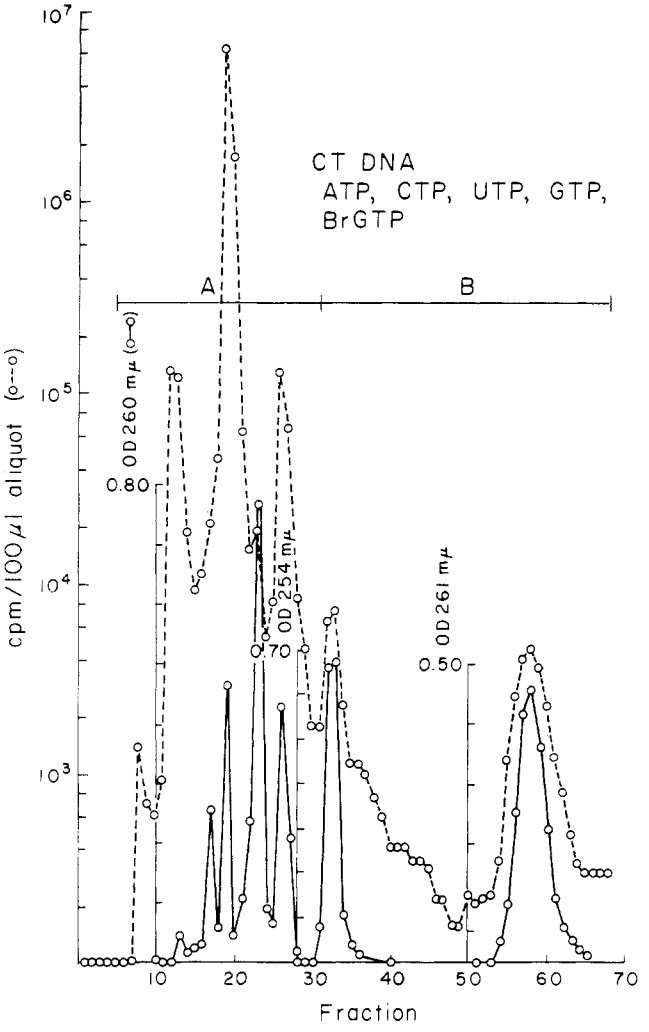

FIGURE 14: Chromatography of the products of CT DNA directed NTP-PP_i exchange reaction with ATP, CTP, UTP, and BrGTP as substrates. Two reactions, each five-times normal scale, were prepared and incubated at 37° for 15 min. Both contained Tris·HCl (pH 7.9, 20 μmoles), 63 nmoles of CT DNA, 400 nmoles each of CTP, UTP, and ATP, 5 μmoles of $MgCl_2$, 6 nmoles of βME, 1 μmole of $Na_4P_2O_7$ (specific activity 300,000 cpm/nmole in no. 1 (Figure 14) and 250,000 cpm/nmole in no. 2 (Figure 15)) and 24 units of enzyme In addition, reaction 1 contained 420 nmoles of BrGTP and reaction 2 contained 420 nmoles of BrGTP and 20 nmoles of GTP. Reactions were terminated as described for the poly(dC) reaction (Figure 12) except that no carrier GTP was added to reaction 1. The acid-soluble products from both reactions were chromatographed on Dowex 1-X2 (Cl) columns (0.9 × 5.5 cm) using two successive gradients to elute the nucleotides. The first gradient had limiting solutions of 0.02 M HCl (30 ml) − 0.02 M HCl + 0.5 M LiCl (30 ml) (A) and was followed by a second gradient of 0.02 M HCl + 0.5 M LiCl (30 ml) − 0.02 M HCl + 2.0 M LiCl (30 ml) (B). For reaction 1, fractions of 2.5 ml were collected; for reaction 2 the fractions were 5.0 ml (1–8), 2.5 ml (9–27), and 1.5 ml (28–68). Recovery of radioactive material applied to the columns was 98–100%. Absorbance and radioactivity were assayed in each fraction by standard procedures and spectral ratios were used to identify the different nucleotide peaks. The sequence of major optical density peaks, from right to left, is BrGTP, GTP, ATP, UTP, and CTP.

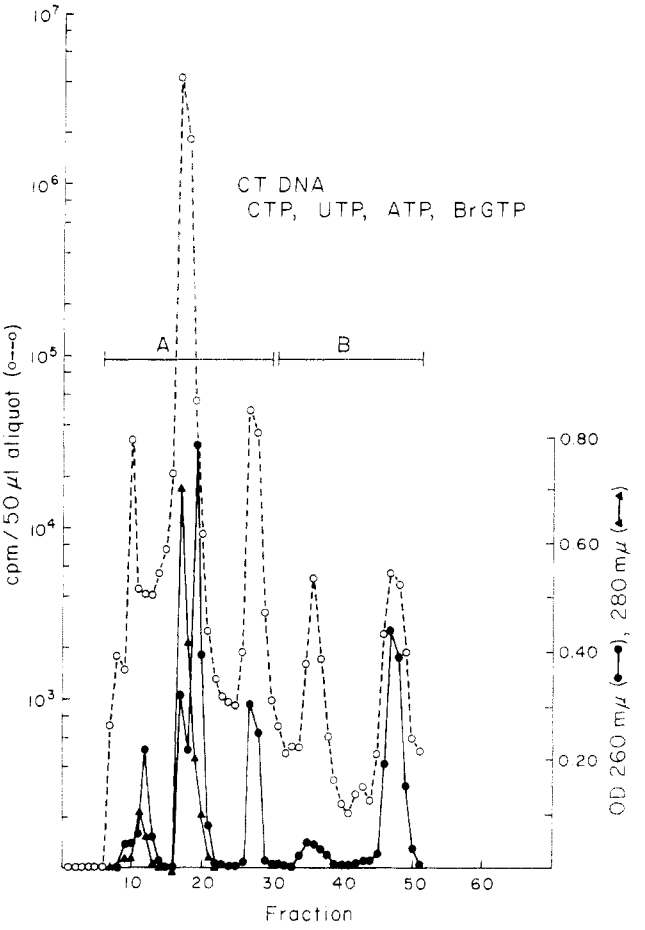

FIGURE 15: Chromatography of the products of CT DNA directed NTP-PP_i-exchange reaction with ATP, CTP, UTP, GTP, and BrGTP as substrates. The conditions for the reaction and chromatography are described in the legend to Figure 14.

communication). We therefore conclude that the conformationally restricted analogs inhibit RNA synthesis by binding at the active site during chain elongation, in a manner comparable to that of the normal substrates; the analogs differ from the normal substrates in that their interaction with the ternary complex is only very rarely followed by the formation of a phosphodiester bond. Such unproductive occupancy of the enzyme site by the analogs would reduce its accessibility for normal substrates and thereby also the rate of RNA synthesis, in the same manner envisioned for competitive inhibitors of other enzymes.

The observation that GTP is needed to permit both the incorporation of BrGMP into RNA and the exchange of pyrophosphate BrGTP primed by poly(dc) is probably best explained by assuming that BrGTP cannot initiate chains, an interpretation consistent with previous findings that the substrate specificity for chain initiation is much more exacting than for elongation (Darlix *et al.*, 1971). That BrGTP is not a substrate when poly[d(A-C):d(T-G)] is template, even in the presence of GTP can be considered to reflect a dependence, for incorporation, upon the sequence of bases in the RNA product; *i.e.*, a nearest-neighbor effect (Goldberg and Rabinowitz 1961; Slapikoff and Berg, 1967).

The behavior of OGTP, although characterized by somewhat less direct evidence, is qualitatively identical with that of BrGTP. The data indicate that BrGTP and OGTP do not differ qualitatively from GTP with respect to enzyme affinity, specificity for the complementary template nucleotide, or the exchange reaction with PP_i. This suggests that they too interact with RNA polymerase at the site that normally catalyzes nucleotide polymerization. In view of the limited evidence available for 6MeCTP, the only conclusions that can safely be

drawn for this compound are that it is not a substrate for incorporation, and is probably a competitive inhibitor for CTP.

In view of the similarities between the analogs and the natural substrates in their interaction with RNA polymerase, why are the analogs so poorly incorporated into RNA, and why do they act as competitive inhibitors rather than as substrates? While no single interpretation follows uniquely from the present experiments, we find the following considerations to be the most attractive. We may assume that nucleotide polymerization resembles other complex enzymatic processes in the sense that the formation of a phosphodiester bond is merely the last in a series of discrete enzymatic steps (Ishihama and Hurwitz, 1969). If this were the case, the nucleotide analogs could be visualized as fulfilling the structural requirements for the first but not for the last steps in the reaction sequence. The structural abnormality common to the three triphosphate analogs (Kapuler and Spiegelman, 1970; Michelson *et al.*, 1970) suggests a plausible basis for such behavior. The initial binding of nucleotides to the enzyme can be imagined to require a conformation at the glycosyl bond that could be assumed by both normal substrates and analogs (perhaps syn or intermediate between syn and anti); in each case, base pairing with template residues would be unimpeded. All evidence points to the likelihood that the residues in product RNA (*i.e.*, after polymerization) exist in the anti conformation (Bremer and Konrad, 1964; Chamberlin and Berg, 1964; Sinsheimer and Lawrence, 1964). Thus, a change in conformation at the glycosyl bond of the substrate could be coupled to one or more of the later steps in enzyme action, perhaps to the formation of the diester bond.[2] Such a conformational change would be consistent with the poor reversibility of diester-bond synthesis as reflected by the difficulty in pyrophosphorolysis of product; it could also account for the differences in utilization between the normal substrates and the analogs. The restrictions inherent in the structure of the analogs could allow them to function in many of the early steps that can be envisioned in enzyme action, but would prevent them from assuming the anti conformation needed for perhaps the last step—the formation of the phosphodiester bond.

Acknowledgments

We thank Drs. Harry Wood and R. Engle, CCNSC, National Cancer Institute, Bethesda, Md. 20014, and R. K. Robins, International Chemical and Nuclear Corporation, Irvine, Calif., for general supplies of nucleoside analogs, and A. Cerami and D. C. Ward and P. Leininger for collaboration in the preparation of some of the enzyme fractions.

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[2] Experiments with these analogs in the polynucleotide phosphorylase reaction are also consistent with this notion (Kapuler *et al.*, 1970).