Synthesis and Chemical Properties of Monomers and Polymers Containing 7-Methylguanine and an Investigation of Their Substrate or Template Properties for Bacterial Deoxyribonucleic Acid or Ribonucleic Acid Polymerases*

S. Hendler,† E. Fürer, and P. R. Srinivasan‡

ABSTRACT: As an approach to understanding the role of 7-methylguanine in nucleic acids, various monomers and polymers containing 7-methylguanine were synthesized and their chemical and biochemical properties were studied. The apparent pK_a values of the N-1 sites of the 7-methylguanine monomers fall in the range of 6.7–7.5, while the apparent pK_a of 7-methylguanine in poly-7-methylguanylic acid is above 9.8 and in 7-methyl-dGdC polymer, above 9.5. The stability to imidazole ring opening at pH 8.9 and 37° increases in the following order: 7-methylguanosine, 7-methyldeoxyguanosine, 3′–7-methyl-GMP, 2′–7-methyl-GMP, 5′–7-methyl-GMP, 7-methyl-GDP, and 7-methyl-GTP. 7-Methylguanine when present in either poly-7-methylguanylic acid or in 7-methyl-dGdC polymer is markedly resistant to ring opening under these conditions. These stability studies suggest that the 5′–7-methylguanine nucleotides probably exist in the anti conformation, while 2′-7-methyl-GMP exists in the syn conformation. The rate of release of 7-methylguanine from 7-methyl-dGTP is twice as great as its release from 7-methyl-dGdC polymer at neutral pH. Although the ionization of N-1 of 7-methylguanine as well as its susceptibility to hydroxide attack is markedly diminished when it is incorporated in a polymer, its release from 7-methyl-dGdC polymer is still significant at physiological pH. 7-Methyl-dGTP replaces only dGTP as a substrate for *Escherichia coli* DNA polymerase. The efficiency of replacement is dependent on the pH of the reaction as well as on the composition of the DNA template. It ranges from 18.9% at pH 7.3 to 1.6% at pH 8.8 with calf thymus DNA as template. At pH 8.3, the efficiency ranges from 7.5% with *Micrococcus lysodeikticus* DNA as the template to 12.6% with *Clostridium pasteurianum* as the template. As the A + T/G + C ratio of the template increases from 0.4 to 2.25, the extent of DNA synthesis increases from 15.4 to 40% (as compared to control values) when 7-methyl-dGTP replaces dGTP. The products of these reactions are always richer in A and T than the template. With dGdC as template, 7-methyl-dGTP is incorporated into the product *only* when dGTP is included in the reaction. Extensive methylation of calf thymus DNA abolishes its template activity for DNA polymerase. The efficiency of 7-methyl-GTP as a substrate for *M. lysodeikticus* RNA polymerase using polycytidylic acid as the template is 16% at pH 7.4 and decreases at higher pH values. Addition of GTP to the reaction mixture stimulates further incorporation of 7-methyl-GTP. Methylation of DNA destroys its template activity for RNA polymerase. The template activity of polycytidylic acid is significantly decreased when this polymer is present in the RNA polymerase reaction in a 1:1 mixture with polyguanylic acid. Most of the template activity of polycytidylic acid is retained when it is present in a 1:1 mixture with poly-7-methylguanylic acid. These results indicate that under these conditions polycytidylic acid and polyguanylic acid form a 1:1 complex while polycytidylic acid and poly-7-methylguanylic acid do not form such a complex.

Methyl groups as well as other alkyl groups can be introduced into nuclei acids and proteins with a variety of alkylating agents (Brookes and Lawley, 1964). Nucleic acids are particularly susceptible to attack by these compounds and the N-7 atom of guanine is the major site of alkylation (Lawley, 1966). The conversion of guanine into 7-alkylguanine in nucleic acids is considered to be an important event underlying the mutagenic, cytotoxic, carcinogenic, and carcinostatic activities of biological alkylating agents (Lawley, 1966).

Alkylation at N-7 of guanine in nucleic acids introduces a positive charge in the imidazole ring. The effects resulting from such a charge have been studied in 7,9-disubstituted guanine monomers. The pK_a of N-1 is about 2 units lower in these compounds than in the corresponding 9-monosubstituted compounds (Lawley and Brookes, 1963). Moreover, these compounds are more unstable with respect to hydrolysis of the glycosidic bond than the 9-monosubstituted compounds (Lawley and Brookes, 1963). Finally, 7-alkylguanosine compounds undergo fission of the imidazole ring in alkali (Haines *et al.*, 1962; Townsend and Robins, 1963). 9-Monosubstituted guanine derivatives are resistant to this reaction even when treated with hot strong alkali (Hurst and Kuksis, 1958; Jones *et al.*, 1966).

* From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York. *Received January 16, 1970.* This work was supported by grants from the National Science Foundation, and the National Institutes of Health, U. S. Public Health Service.

† Taken from a thesis submitted to Columbia University in partial fulfillment of the requirements for the Ph.D. degree by S. H. Present address: Department of Biology, University of California, San Diego, Calif.

‡ To whom to address correspondence.

The altered reactivity of monomeric guanine compounds following N-7 alkylation has led to several suggestions on the molecular mechanisms underlying the biological effects of alkylating agents. Lawley and Brookes (1961) have postulated that the ionized species of the alkylated guanine could pair erroneously with thymine. Such an anomalous pairing could result in a mutation in which a G-C base pair would be replaced by an A-T base pair during DNA replication. However, the actual extent of ionization of 7-alkylguanine in DNA is unknown and there is no evidence that the ionized species of a base is capable of participation in a base pair. The susceptibility of the 7-alkylguanine-sugar linkage to hydrolysis has also been implicated in the mutagenic as well as the toxic activity of alkylating agents (Bautz and Freese, 1960; Lawley and Brookes, 1968).

In the studies reported here we describe the preparation of various monomers and polymers containing 7-methylguanine and examine the chemical behavior of 7-methylguanine in these substances with regard to its ionization constant and stability. Furthermore, we report on the efficiency of 7-methylguanine nucleoside triphosphates as substrates for DNA and RNA nucleotidyl transferases as well as the template activity of polynucleotides containing 7-methylguanine in these reactions.

Experimental Section

Materials

We are indebted to Dr. Arthur Kornberg for a sample of *E. coli* DNA nucleotidyltransferase (DNA polymerase), fraction IV (550 units/mg of protein); Dr. Herman Shapiro for *Escherichia coli* W DNA as well as T4 DNA.

The following materials were obtained from commercial sources: Schwartz BioResearch, Inc.: deoxyguanosine, GDP, GTP, UTP, CTP, dGTP, [^{3}H]dCTP (2.68 Ci/mmole), [^{3}H]-dATP (5.66 Ci/mmole), [^{3}H]TTP (4.8 Ci/mmole), [^{14}C]-dATP (15.3 mCi/mmole), [^{14}C]dGTP (21.0 mCi/mmole), [^{14}C]CTP (20.6 mCi/mmole), [^{14}C]UTP (22.0 mCi/mmole), [^{14}C]ATP (38.0 mCi/mmole), and [^{14}C]GTP (30.0 mCi/mmole); Calbiochem: guanosine, TTP, dCTP, and ribonuclease T1; Boehringer Mannheim Corp.: 5′-GMP and ATP; Sigma Chemical Co.: 2′-GMP and 3′-GMP; Cyclo Chemical Co.: 7-methylguanine; Miles Laboratories: poly G, poly C, poly U, and *Micrococcus lysodeikticus* RNA nucleotidyltransferase (RNA polymerase); New England Nuclear Corp.: [*methyl*-^{14}C]dimethyl sulfate (1.2 mCi/mmole); Nuclear-Chicago: [*methyl*-^{3}H]dimethyl sulfate (90 mCi/mmole); Grain Processing Corp.: *E. coli* B cells (mid-log); Worthington Biochemical Corp.: calf thymus DNA, *Clostridium pasteurianum* DNA and bovine pancreas deoxyribonuclease. Dimethyl sulfate was obtained from Eastman Organic Chemicals and distilled before use.

Methods

Preparation of Monomers and Polymers Containing 7-Methylguanine. 7-METHYLGUANOSINE, AND 2′-DEOXY-7-METHYLGUANOSINE. These compounds were synthesized by the procedure of Jones and Robins (1963).

7-METHYLGUANOSINE 5′-MONOPHOSPHATE. The disodium salt of guanosine 5′-monophosphate (1 g, 2.46 mmoles) was dissolved in 8 ml of water and the pH was adjusted to 4.5 with 1 N HCl. Dimethyl sulfate (1.97 g, 15.6 mmoles) was added with continuous stirring over a period of 1 hr and the reaction was allowed to continue for an additional 3 hr. During the entire reaction the pH was maintained between 3.5 and 4.5 to prevent esterification of the phosphate group. The course of the reaction was followed by removing samples at various time intervals for analysis by chromatography in solvent system A (Table I). After 4 hr the initial spot of 5′-GMP completely disappeared and gave rise to a major ultraviolet fluorescent spot which had an R_F of 0.64 and a barely detectable ultraviolet fluorescent spot with an R_F of 0.52. The former spot was identified as 5′–7-methyl-GMP and the latter, the methyl ester of this compound. The reaction mixture was placed on a column of Dowex 1-Cl$^-$ (100–200 mesh, 14 × 1.8 cm) and eluted with H_2O. Fractions of 15 ml were collected and the fractions containing 5′–7-methyl-GMP were pooled (fractions 2–9) and evaporated under reduced pressure. The residue was dissolved in 10 ml of H_2O and the 5′–7-methyl-GMP precipitated by the addition of 10 volumes of ethanol and kept in the cold (4°) overnight. After centrifugation it was dried *in vacuo*. The solid was dissolved in H_2O and reprecipitated in the same manner to give a yield of 500 mg (48% of theory). Chromatography of the product gave one ultraviolet fluorescent spot (see Table I). Hydrolysis with acid (1 N HCl for 1 hr at 100°) yielded a substance which proved to be identical with 7-methylguanine by ultraviolet spectra (Table II) and by chromatographic and electrophoretic comparisons. Phosphate analyses showed that the compound contained 1 mole of phosphate/mole.

Lawley and Wallick (1957) have reported on the synthesis of 5′–7-methyl-GMP. However, the authors carried out the reaction at pH 7.0 and at this pH there is a considerable amount of esterification of the phosphate group.

7-METHYLGUANOSINE 2′- AND 3′-MONOPHOSPHATES. These compounds were prepared by methods similar to that described above for 5′–7-methyl-GMP. The properties of these compounds are presented in Tables I and II.

7-METHYLGUANOSINE 5′-DIPHOSPHATE. Guanosine 5′-diphosphate (100 mg, 0.205 mmole) was dissolved in 2 ml of water and the pH was adjusted to 4.5. Over a period of 1 hr dimethyl sulfate (252 mg, 2 mmoles) was added with constant stirring at room temperature and the reaction was allowed to continue for an additional 3 hr. The pH of the reaction was kept between 3.5 and 4.5. The reaction mixture was placed on a Dowex 1-Cl$^-$ column (100–200 mesh, 14 × 1.8 cm). The column was first eluted with 100 ml of water and then with 0.01 N HCl. Fractions of 5 ml were collected and the optical density of each fraction was measured at 260 mμ. The water effluent contained very little ultraviolet-absorbing material. Fractions 15–19 of the 0.01 N HCl effluent showed a small peak of ultraviolet absorbance and probably contained a small amount of the methyl ester of 7-methylguanosine diphosphate. Fractions 20–82 showed a large broad peak of ultraviolet absorbance and contained the major product of the reaction, 7-methylguanosine diphosphate. The latter fractions were pooled, neutralized with a saturated solution of LiOH, and evaporated under reduced pressure. The residue was dissolved in 6 ml of H_2O and the product was precipitated by the addition of 10 volumes of a mixture of acetone–ethanol (3:1, v/v), kept in the cold (4°) overnight, centrifuged, and dried *in vacuo*. The solid was redissolved in water and reprecipitated in the same

manner. The yield was 63 mg (58% of theory). The product gave one ultraviolet fluorescent spot on chromatography (Table I). Acid hydrolysis yielded a substance identical with 7-methylguanine and analysis for phosphate showed that the compound contained 1 mole of labile phosphate and 2 moles of total phosphate per mole. The ultraviolet absorption spectra of this compound, as well as of all the other compounds reported here, is presented in Table II. Phosphate was determined according to the method of Fiske and Subbarow (1925). The acid-labile phosphate was measured after hydrolysis in 1 N H_2SO_4 for 7 min at 100°.

7-METHYLGUANOSINE 5′-TRIPHOSPHATE. Guanosine 5′-triphosphate (60 mg, 0.1 mmole) was dissolved in 1 ml of water and the pH was adjusted to 5.0. Dimethyl sulfate (80 mg, 0.635 mmole) was added with constant stirring over a period of 1 hr. The reaction was allowed to continue for an additional 1.5 hr and the pH throughout the entire reaction was maintained between 3.5 and 4.5 by the addition of 1 N NaOH. At the end of the reaction period, the pH of the reaction mixture was adjusted to 7.0 and the mixture was placed on a column of Dowex 1-Cl^- (24 × 1 cm). The column was eluted with a linear gradient: 50 ml of H_2O to 50 ml of 0.04 N HCl containing 0.25 M LiCl; 6-ml fractions were collected and their absorbances was measured at 260 mμ. A peak of ultraviolet absorbance was noted in fractions 1 and 2 and this is probably the methyl ester of 7-methylguanosine triphosphate. Fractions 11–16 showed a broad peak of ultraviolet absorbance. They were pooled and the pH was adjusted to neutrality with a saturated solution of lithium hydroxide. It was taken to dryness by lyophilization; the residue was dissolved in 1.5 ml of water and precipitated with 20 volumes of an acetone–ethanol mixture (3:1, v/v), and left in the cold overnight. The precipitate was centrifuged, washed first with acetone–ethanol (3:1, v/v) and then with ether, and dried *in vacuo*. The solid was redissolved in 1.5 ml of H_2O, precipitated, and worked up as above. The yield of 7-methyl-GTP was 20 mg (35% of theory). Chromatography showed one ultraviolet fluorescent spot (Table I) and acid hydrolysis of the substance yielded a product identical with 7-methylguanine. Phosphate analysis showed that the compound contained 2 moles of labile phosphate and 3 moles of total phosphate per mole.

[*methyl*-^{14}C]-7-METHYLGUANOSINE TRIPHOSPHATE. This compound was prepared by a similar method to that used to prepare 7-methyl-GTP. The specific activity wa 0.6 mCi/mmole, exactly half that of the specific activity of [*methyl*-^{14}C]dimethyl sulfate used as the methylating agent.

7-METHYLDEOXYGUANOSINE 5′-TRIPHOSPHATE. The procedure for the synthesis of 7-methyldeoxyguanosine triphosphate was identical with that used for the synthesis of 7-methylguanosine triphosphate with the exception that the total reaction time was decreased to 1.5 hr. Methylation of dGTP at the 7 position renders the 7-methylguanine–deoxyribose bond unstable, resulting in the slow hydrolysis of the glycosidic linkage. The reaction time was reduced to keep this hydrolysis to a minimum. A 40% yield was obtained. Chromatography revealed one ultraviolet fluorescent spot (Table I) and acid hydrolysis of the substance yielded a product identical with 7-methylguanine. Phosphate analysis showed that the compound contained 2 moles of labile phosphate and 3 moles of total phosphate per mole.

[*methyl*-^{14}C]-7-METHYLDEOXYGUANOSINE TRIPHOSPHATE AND [*methyl*-^{3}H]-7-METHYLDEOXYGUANOSINE TRIPHOSPHATE. The procedure used for the synthesis of these compounds were similar to that described for the synthesis of 7-methyl-dGTP. The specific activity of the ^{14}C compound was 0.5 mCi/mmole and of the ^{3}H compound, 42.5 mCi/mmole.

POLY-7-METHYLGUANYLIC ACID. Poly-7-methylguanylic acid was prepared according to the procedure of Michelson and Pochon (1966). Alkaline hydrolysis of the polymer

TABLE I: R_f Values of 7-Methylguanine and Guanine Derivatives.[a,b]

Derivative	A	B	C	D	E
Guanine			0.37	0.67	1.0
7-Methylguanine	0.16		0.45	0.80	1.8
Guanosine			0.58		
7-Methylguanosine			0.41		
Guanosine 5′-monophosphate	0.48	0.25			
7-Methylguanosine 5′-monophosphate	0.64 (fl)[c]	0.19 (fl)			
Guanosine 3′-monophosphate	0.31	0.33			
7-Methylguanosine 3′-monophosphate	0.63 (fl)	0.19 (fl)			
Guanosine 2′-monophosphate	0.41	0.33			
7-Methylguanosine 2′-monophosphate	0.65 (fl)	0.19 (fl)			
7-Methylguanosine (2′,3′)-monophosphate (alkali treated)	0.63				
Guanosine diphosphate	0.53	0.17			
7-Methylguanosine diphosphate	0.72 (fl)	0.11 (fl)			
Guanosine triphosphate	0.57	0.12			
7-Methylguanosine triphosphate	0.78 (fl)	0.07 (fl)			
Deoxyguanosine triphosphate	0.50	0.17			
7-Methyldeoxyguanosine triphosphate	0.73 (fl)	0.10 (fl)			

[a] Chromatograms were developed on Whatman No. 1 paper in solvent systems A–D. Electrophoresis was performed on Whatman No. 3MM paper in solvent system E. [b] Solvent systems (ascending method): (A) saturated ammonium sulfate solution–isopropyl alcohol–0.1 M potassium phosphate buffer (pH 7.4) (79:2:19, v/v); (B) 1% ammonium sulfate solution–isopropyl alcohol (1:2, v/v); (C) methanol–water (7:3, v/v); solvent system (descending method); (D) isobutyric acid–ammonium hydroxide–water (66:1:33, v/v); solvent system (electrophoresis); (E) 10% acetic acid (pH 3.4). [c] fl refers to ultraviolet fluorescent spots, all the other spots are ultraviolet absorbing.

TABLE II: Ultraviolet Absorption Spectra of 7-Methylguanine Derivatives.[a]

Derivative	pH 2				pH 7.4				pH 12			
	λ_{max} (mμ)	ε	λ_{min} (mμ)	ε	λ_{max} (mμ)	ε	λ_{min} (mμ)	ε	λ_{max} (mμ)	ε	λ_{min} (mμ)	ε
7-Methylguanine	249	11,400	226	4200	247	6,500	234	5770	278	8,000	257	4050
	265–275 (s)	7,500			282	8,000	261	4610				
7-Methylguanosine	257	10,900	230	2400	258	7,400	242	5200	265	10,900	245	5900
	273–283 (s)	7,500			282	8,100	267	7000				
7-Methylguanosine 2′-monophosphate	257	12,600	230	2670	258	9,600	239	5400	268	9,600	245	5800
	273–283 (s)	9,000			280	9,000	271	8600				
7-Methylguanosine 3′-monophosphate	257	13,200	230	2700	258	9,800	240	5800	266	9,900	245	5800
	273–283 (s)	9,400			282	9,600	270	8700				
7-Methylguanosine 5′-monophosphate	257	12,000	230	2850	258	10,300	236	5300	268	8,300	244	5300
	273–283 (s)	8,200			280	8,600	271	8300				
7-Methylguanosine diphosphate	257	11,000	230	2650	258	8,900	236	4600	272	7,000	242	4450
	273–283 (s)	7,400			280	7,300	271	7200				
7-Methylguanosine triphosphate	257	11,700	230	2820	258	9,800	236	5200	281	8,550	243	5280
	273–283 (s)	8,000			280	8,000	271	7900	257–262 (s)	6,550		
7-Methyldeoxyguanosine triphosphate	257	10,600	230	2680	258	8,900	236	4700	281	7,900	243	4800
	273–283 (s)	7,200			280	7,250	271	7150	257–262 (s)	6,000		
Poly-7-methylguanylic acid	260		233		260		233		269		243	
	278–285 (s)				278–285 (s)							
7-Methyl-dGdC polymer	258		234		258		234		268		243	

[a] Spectra were taken immediately after preparation of solutions, in a Cary 14 spectrophotometer.

TABLE III: pK_a's of 7-Methylguanine Derivatives.

Compound	pK_a
Guanosine	3.3, 9.4
7-Methylguanosine	6.7
7-Methylguanosine 3′-monophosphate	6.9
7-Methylguanosine 2′-monophosphate	7.0
7-Methylguanosine 5′-monophosphate	7.1
7-Methylguanosine diphosphate	7.2
7-Methylguanosine triphosphate	7.5
7-Methyldeoxyguanosine triphosphate	7.5
Poly-7-methylguanylic acid	>9.8
7-Methyl-dGdC polymer	>9.5

TABLE IV: Hydrolysis of 7-Methyldeoxyguanosine Triphosphate to Yield 7-Methylguanine at Different pH's at 37°.

Incubn Period (hr)	Release of 7-Methylguanine (%)		
	pH 6.5	pH 7.4	pH 8.5
1	5.5	3.0	0
3	12.0	6.5	0
5	21.0	11.0	0
30		30.0	4.0

[a] Experimental details are given in the text.

(0.3 N KOH for 18 hr at 37°) gave three ultraviolet-absorbing spots on chromatography (solvent system A, Table I). Two of the spots were identical with guanosine 2′-monophosphate and guanosine 3′-monophosphate, while the third was identical in its mobility with the spot obtained from an alkali-treated mixture of 7-methylguanosine (2′,3′)-monophosphate. The spots were cut out and eluted with 0.05 N HCl. Analysis of the ultraviolet spectral data showed that the polymer contained 70% 7-methylguanylic acid and 30% guanylic acid. Acid hydrolysis of the polymer (0.1 N HCl for 1 hr at 100°) gave two ultraviolet-absorbing spots on chromatography (solvent system C, Table I), one spot identical in behavior with 7-methylguanine and the second to guanine. Analysis of the spots, obtained by cutting and eluting the spots in 0.05 N HCl, gave 70% 7-methylguanine and 30% guanine. No additional methylation was obtained when the polymer was subjected to further treatment with dimethyl sulfate under the same conditions. Furthermore, attempts to increase the extent of methylation by adding polycytidylic acid to the reaction mixture proved to be unsuccessful.

7-METHYL-dGdC POLYMER. The preparation of dGdC polymer has been described by Radding *et al.* (1962). Dimethyl sulfate (53.5 mg, 40 μl) was slowly added in 10-μl portions to a 2-ml solution of dGdC polymer (optical density at 260 mμ, 5.5; S, 29.25) in 0.02 M potassium phosphate buffer (pH 7.4) over a period of 1 hr. The pH of the solution was maintained between 7 and 8 by the addition of 0.2 N NaOH. The reaction was allowed to continue for another 30 min at room temperature and for several hours at 0° until the changes in pH were negligible. The reaction mixture was then dialyzed exhaustively against 0.02 M potassium phosphate buffer (pH 7.4). Acid hydrolysis of the polymer and chromatography of the products gave 65% 7-methylguanine and 35% cytosine.

[*methyl*-^{14}C]-7-METHYL-dGdC. [*methyl*-^{14}C]-7-Methyl-dGdC was prepared in the same manner as the unlabeled substance using [*methyl*-^{14}C]dimethyl sulfate (1.2 mCi/mmole). After dialysis the preparation had an activity of 1.3×10^5 cpm/ml (optical density at 260 mμ, 3.5) as measured in a Nuclear-Chicago low-background counter with a 25% efficiency for ^{14}C.

Ultraviolet Spectra. These were determined in a Cary recording spectrophotometer Model 14. Maximum and minimum wavelengths are reported.

pK_a Determinations. Apparent pK_a determinations were made on the 7-methylguanine-containing substances by taking ultraviolet spectra at different pH values. The absorbance at 258 mμ was then plotted against pH. This procedure has been described by Shugar and Fox (1952). The different pH values were obtained with the following buffers of 0.02 ionic strength: acetate buffer at pH 5, phosphate buffer at pH's 6.4–7.4, Tris-chloride buffer at pH's 7.4–8.8 and glycine buffer at pH's 9.0–10.4. The pK_a's of the polymers were examined in the above system of buffers as well as in the above buffers containing 0.15 M NaCl.

Imadazole Ring Opening. The stability of the 7-methylguanine derivatives to alkaline ring fission was determined by incubating the compounds at 37°, pH 8.9 and measuring their ultraviolet spectra at various time intervals. This procedure has been described by Lawley and Brookes (1963) for 7-methylguanosine.

Hydrolysis of the Glycosidic Linkage. To determine the stability of the 7-methyl-dGdC polymer to glycosidic fission, 0.04 ml of [*methyl*-^{14}C]-7-methyl-dGdC polymer (optical density at 260 mμ, 3.5) was incubated at 37° in 0.1 ml of 0.1 M buffer of different pH values for varying periods. The incubations were terminated by chilling the samples in ice and analyzed for release of 7-methylguanine by chromatography using solvent system C (Table I). A few micrograms of 7-methylguanine were included as carrier to aid in the localization of the radioactive spot. The polymer remains at the origin in the system. The spots were cut out and counted in a Nuclear-Chicago low-background counter.

The stability of 7-methyldeoxyguanosine triphosphate was determined by incubating the compound at 37° at various H^+ ion concentrations. Samples were removed from the incubation mixture at different times, spotted on chromatography paper, and developed in solvent system A (Table I). The 7-methylguanine and 7-methyldeoxyguanosine triphosphate spots were cut out, eluted, and analyzed by ultraviolet spectra.

Incorporation of Labeled Nucleotides into DNA. Escherichia coli DNA nucleotidyl transferase (DNA polymerase) fraction IV or VII prepared according to Richardson *et al.* (1964) was used in these experiments. The preparation of the d(A-T) copolymer has been described by Schachman *et al.* (1960), while the method of Radding *et al.* (1962) was used for the synthesis of the dGdC polymer. DNase-treated calf thymus DNA ("activated" calf thymus DNA) and heat-denatured DNAs were prepared by following the procedures of Aposhian and Kornberg (1962). Methylated calf thymus DNA was prepared by reacting calf thymus DNA with dimethyl sulfate according to the protocol of Pochon and Michelson (1967). Analysis of the methylated DNA by chromatography of a formic acid hydrolysate of the product (Fujimoto *et al.*, 1965) showed that 95% of the quanine was converted into 7-methylguanine and 10% of the adenine was converted into 3-methyladenine. *Micrococcus lysodeikticus* DNA was isolated by the method of Marmur (1961).

The assay for DNA polymerase has been described by Richardson *et al.* (1964). Incubation mixtures contained either glycine buffer or potassium phosphate buffer, magnesium chloride, 2-mercaptoethanol, deoxyribonucleoside triphosphates, the appropriate DNA template, and DNA polymerase in the quantities indicated in the legends to the tables. DNA synthesis was measured by the incorporation of labeled deoxyribonucleoside triphosphates into an acid-insoluble form. After incubation, the assay mixture (0.3 ml) was cooled in ice, 0.1 ml of a solution of calf thymus DNA (1 mg/ml) was thoroughly mixed with the sample, and 0.4 ml of ice-cold 10% trichloroacetic acid was added to precipitate the DNA. Thirty seconds later, 2 ml of ice-cold water was added and the precipitates were collected on a nitrocellulose filter (Schleicher & Schuell, B6, 0.45 μ) and washed twice with 2.5 ml of cold water. The filter was then placed in a counting vial and 0.1 ml of 0.2 N NaOH was added followed by 10 ml of Bray's scintillation mixture for aqueous solutions (Bray, 1960). Counting was carried out in a Nuclear-Chicago liquid scintillation counter. Appropriate controls without DNA and without enzyme were included in every experiment.

The composition of the enzymic DNA products were determined according to the following method: separate

TABLE V: Hydrolysis of 7-Methyl-dGdC Polymer to Yield 7-Methylguanine at Various pH's at 37°.[a]

Incubn Period (hr)	Release of 7-Methylguanine (%)			
	pH 5.0	pH 6.5	pH 7.4	pH 9.0
0	1.5	1.5	1.5	1.5
2.3	9	6		
5.0	16	12	10	9
7.5	23	15	13	11
24	41	33	31	21

[a] Experimental details are given in the text.

TABLE VI: Rate of Imidazole Ring Fission of 7-Methylguanine Derivatives at pH 8.9 and 37°.[a]

Derivative	Half-Life (hr)	k_1 (hr^{-1})
7-Methylguanosine	6.1	0.114
7-Methyldeoxyguanosine	9.8	0.071
7-Methylguanosine 3′-monophosphate	19.8	0.035
7-Methylguanosine 2′-monophosphate	25.6	0.027
7-Methylguanosine 5′-monophosphate	40.6	0.017
7-Methylguanosine diphosphate	46.0	0.015
7-Methylguanosine triphosphate	53.5	0.013

[a] The first-order rate constants were determined by plotting log ($OD_\infty - OD_t$) *vs.* time, where OD_t denotes the absorbance of a solution at 270 mμ and time, t, and OD_∞ refers to the absorbance of a solution at 270 mμ that had been adjusted to pH 13, allowed to stand at 25° for 30 min and finally readjusted to pH 8.9. Appropriate corrections were made in the latter case for the volume change during the pH adjustment.

reaction mixtures containing either [^{14}C]dGTP or [^{14}C]-7-methyl-dGTP and one of other three dNTPs (dATP, dCTP, and TTP) used in the reaction labeled with ^{3}H were incubated for 30 min at 37°. The incorporation of label into an acid-insoluble product was assayed as described above. Appropriate corrections were made in order to obtain the actual counts per minute of ^{14}C and ^{3}H, and these values were used to determine the amounts of the dNTPs incorporated. It was found that in each of the three separate reaction mixtures the amount of [^{14}C]dGTP or [^{14}C]-7-methyl-dGTP incorporated remained fairly constant. Therefore, it was assumed that the amount of product in each of the reaction mixtures was the same. The composition of the product was then obtained from the amounts of labeled dNTPs incorporated in the separate reaction mixtures. The total amount of DNA product was obtained by adding the four dNTPs incorporated.

Incorporation of Labeled Nucleotides into RNA. M. lysodeikticus RNA nucleotidyl transferase (RNA polymerase) was used in these experiments and assayed by the method of Nakamoto *et al.* (1964). Incubation mixtures for the synthetic ribopolynucleotide primed reactions contained Tris-HCl buffer, $MnCl_2$, nucleoside triphosphates, polynucleotides, and RNA polymerase in the quantities indicated in the legends to the tables. Poly-7-methyl-G was prepared and analyzed in the manner described above. Incorporation of the labeled nucleotides into acid-precipitable material was measured after incubation for 60 min at 30°.

Results and Discussion

Chemical Studies. PREPARATION OF 7-METHYLGUANINE DERIVATIVES. Griffin and Reese (1963) have shown that methylation of adenosine 5′-monophosphate with dimethyl sulfate occurs primarily at the N-1 position and hardly at all at the phosphate group if the pH of the reaction is maintained around 4.5. Advantage was taken of these findings to synthesize the various nucleotide derivatives of 7-methylguanine. When nucleotide derivatives containing guanine are allowed to react with dimethyl sulfate, methylation occurs at the N-7 position of guanine, if the pH of the reaction is maintained between 3.5 and 4.5. The isolated products are free of any methyl esters. Likewise, methylation of polyguanylic acid and of the dGdC polymer occurs at the N-7 atom of guanine.

pK_a DETERMINATIONS. Spectrophotometric pK_a determinations of 7-methylguanine in its various monomeric forms (Table III) show that the apparent pK_a values of the N-1 sites of these compounds fall in the range of 6.7–7.5. Reported values of the corresponding guanine derivatives are in the range of 9.2–9.6 (Shapiro, 1968). Thus, methylation of these guanine compounds lowers the pK_a of N-1 by about two units. The absence of a pK_a in the region of 3 for the 7-methylguanine derivatives is also to be noted. 9-Monosubstituted guanine derivatives accept a proton in acidic solution to form a cation with pK_a values in the range of 2–3.5.

The spectra of poly-7-methyl-G at different pH values are identical with the pH 2 spectrum up to pH 9.8. Above pH 11.0, however, an examination of the spectra indicates that considerable imidazole ring opening has occurred. Michelson and Pochon (1966) have recorded comparable findings. Similarly, spectral shifts are observed in the case of 7-methyl-dGdC polymer above pH 9.5 with considerable ring opening above pH 11.0. Therefore, apparent pK_a values for 7-methylguanylic acid in these polymers cannot be determined with accuracy but the spectral shifts suggest that it is greater than 9.8 in poly-7-methyl-G and greater than 9.5 in the 7-methyl-dGdC polymer. These determinations were made in 0.15 M NaCl. A similar picture was obtained when the spectrophotometric titrations were performed in 0.02 M NaCl. This increase in the apparent pK_a of 7-methylguanylic acid in the polymeric state is similar to the increase that has been found when guanylic acid is incorporated into poly G (Pochon and Michelson, 1965) and the corresponding higher apparent pK_a of deoxyguanylic acid in DNA (Beaven *et al.*, 1955).

The difference in the pK_a values between the monomeric and polymeric state suggests the presence of forces which stabilize the protonated form in polynucleotide structures. Thus, the increase of the pK_a of 7-methylguanine in poly-7-

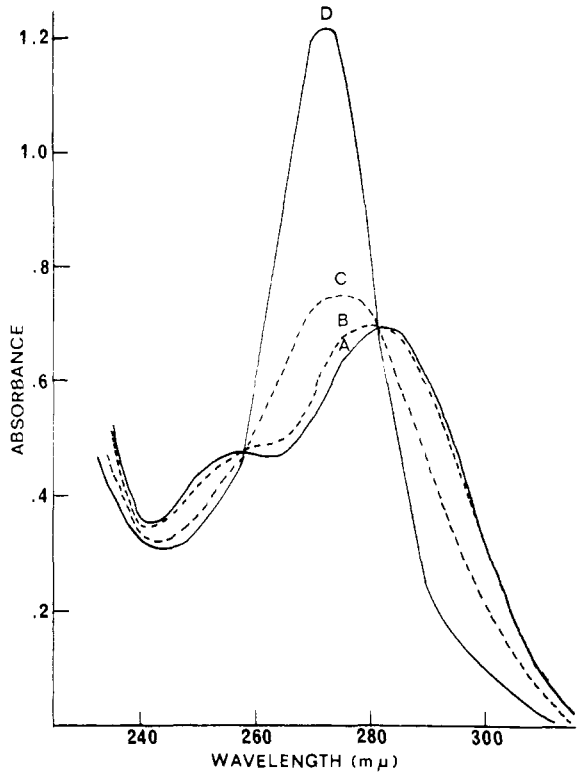

FIGURE 1: Spectroscopic determination of the rate of alkaline ring fission of 7-methylguanosine 5′-monophosphate (0.04 mg/ml) at pH 8.9 and 37°. Spectra at zero time (A), after 4 hr (B), 24 hr (C), and ∞ (D) are shown.

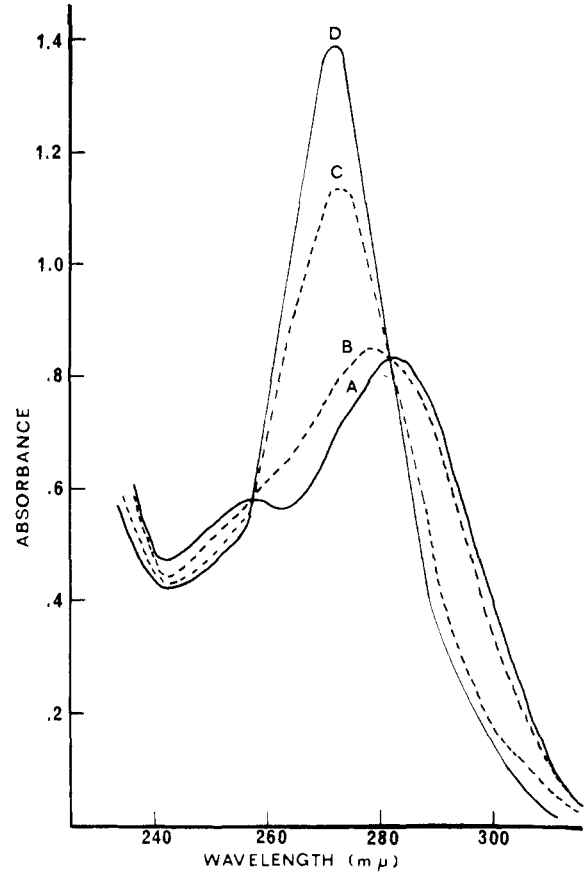

FIGURE 2: Spectroscopic determination of the rate of alkaline ring fission of 7-methylguanosine 3′-monophosphate (0.04 mg/ml at pH 8.9 and 37°. Spectra at zero time (A), after 4 hr (B), 24 hr (C), and ∞ (D) are shown.

methyl-G as well as in the 7-methyl-dGdC polymer can be interpreted in several ways. Hydrogen bonding between the bases could lead to the suppression of the ionization of 7-methylguanine. Or, the positive charge on the imidazole ring could be partially neutralized as a result of the stacking of the bases in these structures. Such a reduced positive charge would be less effective in withdrawing electrons from N-1 and consequently cause an increase in the pK_a. Finally, the conformation of these polymers may be such that the N-1 regions of the purines are present in hydrophobic regions, and this too could result in suppressing the ionization of N-1.

SCISSION OF THE GLYCOSIDIC BOND. The stability of 7-methyl-dGTP to glycosidic scission at pH 6.5, 7.4, and 8.5 is illustrated in Table IV. At pH 8.5 and 37° this compound is quite stable for 5 hr. However, at lower pH values the glycosidic bond is slowly cleaved to yield 7-methylguanine. After 30 hr at pH 7.4, 30% of the 7-methyl-dGTP is converted into 7-methylguanine. A study of the stability of 7-methyldeoxyguanosine at pH 7.4 and 37° revealed that 50% of this compound is converted into 7-methylguanine after 5 hr. The rate of hydrolysis of 7-methyl-dGMP at 37° and at pH 6.9 has been investigated by Lawley and Brookes (1963) who found a half-life of 16.4 hr. Thus, 7-methyl-dGTP is relatively more stable to glycosidic scission than 7-methyl-GMP which in turn is more stable than 7-methyldeoxyguanosine.

Whereas, the rate of depurination of 7-methyl-dGdC polymer remains nearly constant between pH 6.5 and 9.0 (Table V), the rate of depurination of 7-methyl-dGTP significantly decreases in going from pH 6.5 to 8.5 (Table IV). The apparent pK_a of 7-methylguanine in the polymer is greater than 9.5, while the apparent pK_a of 7-methyl-dGTP is 7.5 (Table III). Therefore, at pH 9.0, the protonated species of 7-methylguanine predominates in the polymer and at pH 8.5 it is the ionized form which predominates in the monomer. Ionization at N-1 apparently confers stability to the 7-methylguanine–deoxyribose bond and in order to compare the rates of 7-methylguanine release from 7-methyl-dGTP and 7-methyl-dGdC polymer the extent of ionization of the base has to be taken into consideration. At pH 7.4 about 50% of the 7-methyl-dGTP exists in the protonated form and essentially all of the 7-methylguanine is in the protonated form in the polymer. As only the protonated form of 7-methylguanine is susceptible to glycosidic cleavage, the rate of depurination of 7-methyl-dGTP is twice as great as the corresponding rate of 7-methyl-dGdC polymer at pH 7.4.

The 7-methylguanine–ribose linkage in 7-methylguanine monomers as well as in poly-7-methylguanylic acid was found to be very stable to hydrolysis at neutral pH and 37°.

RATE OF IMIDAZOLE RING OPENING. To explore the stability of the various 7-methylguanine derivatives to hydroxide attack, the rates of imidazole ring opening at pH 8.9 and 37° were determined by following the shifts in their ultraviolet

TABLE VII: Incorporation of 7-Methyl-dGTP into DNA.[a]

Deoxynucleoside Triphosphates	^{3}H Incorpd (cpm)	mμmoles of dATP or TTP Incorpd	^{14}C Incorpd (cpm)	mμmoles of 7-Methyl-dGTP Incorpd
1. [^{3}H]dATP, TTP, dCTP, dGTP, [^{14}C]-7-methyl-dGTP	11,330	2.08	13	0.02
2. [^{3}H]dATP, —, dCTP, dGTP, [^{14}C]-7-methyl-dGTP	313	0.06	<10	<0.01
3. dATP, [^{3}H]TTP, —, dGTP, [^{14}C]-7-methyl-dGTP	680	0.06	<10	<0.01
4. —, [^{3}H]TTP, dCTP, dGTP, [^{14}C]-7-methyl-dGTP	680	0.06	<10	<0.01
5. dATP, [^{3}H]TTP, dCTP, —, [^{14}C]-7-methyl-dGTP	8,980	0.74	130	0.18

[a] The reaction mixtures, 0.30 ml, contained: 20 μmoles of glycine buffer, pH 8.8 (final pH of reaction mixture, 8.3); 2 μmoles of $MgCl_2$; 0.3 μmole of 2-mercaptoethanol; 13 μg of activated calf thymus DNA; 0.8 unit of DNA polymerase; 10 mμmoles each of dATP, TTP, dCTP; and 20 mμmoles of [^{14}C]-7-methyl-dGTP (specific activity 7.15×10^5 cpm/μmole). The ^{3}H label was contained in either [^{3}H]dATP (specific activity 5.45×10^6 cpm/μmole) or [^{3}H]TTP (1.22×10^7 cpm/μmole). The mixtures were incubated at 37° for 30 min, stopped with acid, and assayed as described in the text.

spectra with time. The rates of these changes for 5′- and 3′–7-methyl-GMP (Figures 1 and 2) as well as for the other 7-methylguanine derivatives follow first-order kinetics. The first-order rate constants and half-lives for these derivatives presented in Table VI reveal that the 7-methylguanine nucleotides are significantly more stable than the nucleosides to hydroxide attack. Moreover, the stability increases as the number of phosphate groups are increased. Among the various monophosphates the stability decreases in the following order: 5′ > 2′ > 3′. In contrast, poly-7-methyl-G and 7-methyl-dGdC polymer are remarkably resistant to ring opening at pH 8.9 and 37°. It appears, therefore, that the C-8 atom of 7-methylguanine is much less electron deficient when the base is incorporated in a polymer.[1] However, at pH 12, 7-methylguanylic acid behaves similarly in the polymeric state and in the monomeric state.

The stability of the 7-methylguanine nucleotides increases with the number of phosphate groups, *i.e.*, with the increase in negative charge. However, if the amount of negative charge in the molecule were the only variable involved, then one would expect that the rates of imidazole fission of the three monophosphate isomers would be identical. This is not the case. The order of stability of these isomers, *i.e.*, 5′ > 2′ > 3′, suggests that the phosphate groups in these compounds are present at different distances from the C-8 atom. The closer the phosphate group is to C-8, the more effectively its negative field can protect this position from hydroxide attack.

It can be shown with Corey–Pauling–Koltun space-filling models that the phosphate substituents of the 5′–7-methylguanine nucleotides are in very close proximity to C-8 when these compounds are in the anti conformation with respect to the sugar-base torsion angle. They are furthest removed from C-8 when the compounds are in the syn conformation. The phosphate group of the 3′ isomer is closest to C-8 when this compound is likewise in the anti conformation. The phosphate group of 2′–7-methyl-GMP is far removed from C-8 when this compound is in the anti conformation. Since the 2′ isomer is even more stable to ring opening than the 3′ isomer, it is likely that its phosphate group is in the neighborhood of C-8 and this would occur only if this compound were in the syn conformation. Thus, it is reasonable to suggest that the 5′–7-methylguanine nucleotides exist in the anti conformation, while 2′–7-methyl-GMP probably exists in the syn conformation. These conclusions are in agreement with the results of the previous investigations on the conformation of purine nucleotides in solution (Schweitzer *et al.*, 1968; Danyluk and Hruska, 1968).

Biochemical Studies. ENZYMATIC INCORPORATION OF 7-METHYL-dGTP INTO DNA USING NATURAL DNA TEMPLATES. An inspection of Table VII reveals that 7-methyl-dGTP can substitute for dGTP in the DNA polymerase reaction using calf thymus DNA as the template. However, the analog does not substitute for any of the other deoxynucleoside triphosphates. Furthermore, these results suggest that the efficiency of participation of 7-methyl-dGTP in DNA synthesis is considerably lower than that of dGTP and that dGTP is the preferred substrate when present in the reaction along with its analog.

The factors affecting the incorporation of 7-methyl-dGTP into DNA, the kinetics of the reaction and the influence of pH and of various templates were studied. At pH 8.3, the rate of incorporation of both 7-methyl-dGTP and dGTP was found to be linear for the first 20 min. The relative extent of incorporation of 7-methyl-dGTP was about 9% that of dGTP. Lineweaver–Burk plots revealed the apparent K_m for the incorporation of 7-methyl-dGTP as well as dGTP to be identical, at pH 8.3, and to have the value 13 μM. The V_{max} for the 7-methyl-dGTP reaction was about 9% of the value obtained with dGTP.

The amount of incorporation of 7-methyl-dGTP into

[1] Nuclear magnetic resonance studies of 7-methylguanosine in D_2O have shown that the proton at C-8 is shifted downfield to a considerable degree when compared to H-8 of 1-methylguanosine (J. A. Glasel, S. Hendler, and P. R. Srinivasan, unpublished data; Broom, 1965). Townsend and Robins (1963) have likewise shown that the H-8 of 7-methylguanosine in dimethyl sulfoxide is shifted downfield. This shift is a reflection of the decreased electron density of C-8 in the compound. It is also interesting to point out that the rate of exchange of H-8 in D_2O is extremely rapid with a half-life of less than 2 min.

TABLE VIII: Effect of pH on Incorporation of [^{14}C]-7-Methyl-dGTP into DNA.[a]

pH	Labeled Substrate in the Incubn Mixture	dGTP or 7-Methyl-dGTP Incorpd (mμmoles)	7-Methyl-dGTP Incorpd (% of Control)
7.3	[^{14}C]dGTP	0.70	
7.3	[^{14}C]-7-Methyl-dGTP	0.13	18.6
8.3	[^{14}C]dGTP	1.22	
8.3	[^{14}C]-7-Methyl-dGTP	0.12	9.8
8.8	[^{14}C]dGTP	1.25	
8.8	[^{14}C]-7-Methyl-dGTP	0.02	1.6

[a] The reaction mixtures (0.30 ml) contained: 2 μmoles of $MgCl_2$; 0.3 μmole of 2-mercaptoethanol; 13 μg of activated calf thymus DNA; 0.6 unit of DNA polymerase; 10 mμmoles each of dATP, TTP, and dCTP; and either 20 mμmoles of [^{14}C]dGTP (specific activity 8.60 × 10^5 cpm/μmole) or 20 mμmoles of [^{14}C]-7-methyl-dGTP (specific activity 6.00 × 10^5 cpm/μmole). For pH 7.3, Tris-HCl buffer was used; for pH 8.3 and 8.8, glycine buffer was used. The reaction mixtures were incubated at 37° for 30 min, and assayed as described in the text.

DNA decreases as the pH of the reaction is increased (Table VIII). This is paralleled by an increase in the amount of the ionized form of the analog since its apparent pK_a is 7.5. One can, therefore, conclude that the ionized species is incapable of replacing dGTP as a substrate in the polymerase reaction. It is conceivable that the negative charge in the hydrogen-bonding region of the analog precludes its bonding to the template. In this light, it is interesting to examine an observation made by Bessman *et al.* (1958), on the inability of deoxyxanthosine triphosphate to act as a substrate for DNA polymerase. The pK_a of this nucleotide is about 6.0 (Ogston, 1935) and under their reaction conditions (pH 7.4) most of the compound will be present in its ionized form. Based on the results obtained with 7-methyl-dGTP, it seems likely that the ionized form of deoxyxanthosine triphosphate is incapable of base pairing with the template.

If 7-methyl-dGTP is incorporated slowly as a result of weak bonding between the substrate and the template, then the incorporation of this analog may be dependent on the base composition of the template. This idea was explored by using templates differing in their A + T/G + C ratio and the results are presented in Table IX. In each case 7-methyl-dGTP replaced only dGTP in the enzymic reaction and it is clear that the extent of analog incorporation does vary with the A + T/G + C ratio of the template. The lowest incorporation is found in the case of *M. lysodeikticus* DNA which is richest in C (A + T/G + C = 0.40), while the highest incorporation is observed when *Clostridium pasteurianum* DNA which is poorest in C (A + T/G + C = 2.25) is used as the template. Thus, the efficiency of replacement of dGTP by 7-methyl-dGTP appears to increase as the frequency of its bonding to the template decreases.

TABLE IX: Efficiency of Replacement of dGTP by 7-Methyl-dGTP and Extent of DNA Synthesis with Various DNAs as Templates.

DNA Templates	A + T/G + C	Replacement (%)	Extent of DNA Synthesis (%)
M. lysodeikticus	0.4	7.5 (1.34)[a]	15.4 (3.65)[b]
E. coli	1.0	7.65 (2.36)	15.6 (9.41)
Calf thymus	1.3	9.65 (1.87)	27.2 (8.51)
T4	1.9	12.20 (0.28)	34.3 (1.69)
Cl. pasteurianum	2.25	12.6 (0.93)	40.0 (5.98)

[a] mμmoles of dGTP incorporated into DNA in control experiments. [b] mμmoles of DNA formed in the presence of natural deoxyribonucleoside triphosphates in appropriate control.

This difference in efficiency with various DNA templates may be expected to result in different extents of DNA synthesis. The extent of DNA synthesized when 7-methyl-dGTP replaces dGTP increases from 15.4 to 40.0% as the A + T/G + C ratio of the template increases from 0.40 (*M. lysodiekticus*) to 2.25 (*Cl. pasteurianum*) (Table IX). The greater the efficiency of analog replacement, the greater the extent of DNA synthesized.

The composition of the DNA synthesized in the presence of 7-methyl-dGTP as well as dGTP was determined by the use of a double-labeling technique which is described in the Experimental Section. The results using various DNAs as templates are summarized in Table X. In the presence of dGTP the A/G, C/G, and T/G as well as the A +T/G + C ratios of the product are identical with these same ratios in the template. This is true irrespective of the template used. However, when 7-methyl-dGTP replaces dGTP, it is found that the corresponding A/7-methyl-G, C/7-methyl-G, T/7-methyl-G and A + T/7-methyl-(G + C) ratios are all significantly higher than those of the template. Again, this is true for all five templates used. These results suggest that the regions of the DNA template that are being copied by the enzyme when 7-methyl-dGTP is the substrate are different from those regions copied when dGTP is the substrate. It appears that the C-rich regions of the templates are being avoided while the regions rich in A, T, and G are being preferentially copied. This appears to be an additional consequence of the low efficiency of bonding of 7-methyl-dGTP to the template.

INCORPORATION OF 7-METHYL-dGTP WITH d(A-T) AS TEMPLATE. With d(A-T) copolymer as template, the incorporation of [^{14}C]-7-methyl-dGTP was investigated at 37° and at pH 6.5, 7.4, 7.9, and 9.2 in the presence of either dATP or dATP and TTP. No significant incorporation of [^{14}C]-7-methyl-dGTP was observed at any of these pH's, although when dATP and TTP were both present the formation of d(A-T) polymer could be demonstrated by the incorporation of [^{3}H]TTP. Since the specific activity of [^{14}C]-7-methyl-dGTP was not very high, [^{3}H]-7-methyl-dGTP was synthesized using [*methyl*-^{3}H]dimethyl sulfate which was commercially available at a much higher specific activity than that

TABLE X: Base Ratios of DNA Synthesized in the Presence of Various DNA Templates on Replacement of dGTP by 7-Methyl-dGTP.[a]

DNA Templates	A/7-Methyl-G	C/7-Methyl-G	T/7-Methyl-G	A + T/7-Methyl-(G + C)
M. lysodeikticus	0.99 (0.34)	2.66 (0.99)	1.03 (0.36)	0.56 (0.35)
E. coli	2.26 (0.97)	2.50 (0.97)	2.50 (1.04)	1.36 (1.10)
Calf thymus	4.50 (1.25)	2.68 (0.99)	4.50 (1.30)	2.36 (1.29)
T4	6.35 (1.96)	3.50 (1.04)	6.75 (2.08)	2.86 (2.07)
Cl. pasteurianum	9.30 (2.20)	3.33 (0.89)	9.30 (2.38)	3.96 (2.36)

[a] The values in parentheses represent the corresponding experimentally determined base ratios when the reaction is carried out in the presence of dGTP.

TABLE XI: Incorporation of 7-Methyl-dGTP with dGdC as Primer.[a]

Deoxynucleoside triphosphate	^{3}H Incorpd (cpm)	^{14}C Incorpd (cpm)	$\mu\mu$moles of 7-Methyl-dGTP Incorpd	$\mu\mu$moles of dGTP Incorpd
[^{14}C]dGTP, dCTP		6700		18,600
[^{3}H]-7-Methyl-dGTP, dCTP	100		8	
[^{3}H]-7-Methyl-dGTP, [^{14}C]dGTP, dCTP	31,200	5800	2400	16,100

[a] The reaction mixtures, 0.30 ml, contained: 20 μmoles of potassium phosphate buffer, pH 7.4; 2 μmoles of $MgCl_2$; 0.3 μmole of mercaptoethanol; dGdC polymer (0.3 optical density unit); 5 units of DNA polymerase fraction VII; and 30 mμmoles of each of the indicated deoxynucleoside triphosphates. The specific activities of [^{14}C]dGTP and [^{3}H]-7-methyl-dGTP were 3.60×10^5 and 1.30×10^7 cpm per μmole, respectively. After incubation at 37° for 3 hr, the reaction was assayed as described in the text.

TABLE XII: Incorporation of [^{14}C]dATP with DNA Polymerase and Methylated Calf Thymus DNA as Template.[a]

Template	cpm Incorpd	mμmoles of dATP Incorpd
1. Native calf thymus DNA	1845	1.08
2. Heat-denatured calf thymus DNA	4400	2.58
3. DNase-activated calf thymus DNA	4334	2.55
4. Methylated calf thymus DNA	93	0.05
5. Calf thymus treated as in 4 but without dimethyl sulfate	1800	1.06
6. Methylated calf thymus DNA + activated calf thymus DNA	4350	2.56

[a] The reaction conditions were similar to those described in Table VII. 13 μg of the appropriate DNA and 0.85 enzyme unit were present in the reaction. The specific activity of [^{14}C]-dATP was 1.70×10^6 cpm/μmole.

of [*methyl*-^{14}C]dimethyl sulfate. When [^{3}H]-7-methyl-dGTP is present in the reaction along with [^{14}C]dATP and TTP, the frequency of [^{3}H]-7-methyl-dGTP incorporation was less than 1 molecule/3400 molecules of [^{14}C]dATP incorporated. These results as well as those previously discussed indicate that 7-methyl-dGTP substitutes only for dGTP.

INCORPORATION OF 7-METHYL-dGTP USING dGdC AS TEMPLATE. All attempts to form a 7-methyl-dGdC polymer with 7-methyl-dGTP and -dCTP either *de novo* or with dGdC as primer failed. Prolonged incubations up to 30 hr at 37° as well as at pH 7.4, 7.9, and 9.2 were tried without success. However, if dGTP was included in the reaction mixture along with 7-methyl-dGTP and -dCTP, 7-methyl-dGTP was incorporated to the extent of 13% of the total amount of 7-methyl-dGTP and -dGTP incorporated (Table XI).

The preferential utilization of dGTP can be rationalized on the basis of the relatively poorer bonding capacity of 7-methyl-dGTP with C in the template. It appears that dGTP must be present to initiate the synthesis and only when the synthesis is in progress can 7-methyl-dGTP get incorporated.

TEMPLATE PROPERTIES OF METHYLATED DNA. Calf thymus DNA was methylated with dimethyl sulfate and analysis

TABLE XIII: Incorporation of 7-Methyl-GTP Using Various Ribohomopolymers as Templates and Template Activity of Various Ribohomopolymers and Ribohomopolymer Mixtures with RNA Polymerase.[a]

Ribonucleoside Triphosphates	Primer	^{14}C Incorpn (cpm)	mμmoles of [^{14}C]-NTP Incorpd
[^{14}C]GTP	Poly C	34,100	42.0
[^{14}C]-7-Methyl-GTP	Poly C	4,700	6.5
[^{14}C]GTP, CTP	Poly C + poly G	1,460	1.8
GTP, [^{14}C]CTP	Poly C + poly G	<10	<0.014
[^{14}C]GTP, CTP	Poly C + poly 7-methyl G	27,200	33.6
GTP, [^{14}C]CTP	Poly C + poly 7-methyl G	<10	<0.014
[^{14}C]CTP	Poly G	<10	<0.014
[^{14}C]CTP	Poly 7-methyl G	<10	<0.014
[^{14}C]UTP	Poly 7-methyl G	<10	<0.015
[^{14}C]ATP	Poly U	2,150	2.30
[^{14}C]-7-Methyl-GTP	Poly U	<10	<0.014
[^{14}C]ATP, 7-methyl-GTP	Poly U	2,100	2.25
ATP, [^{14}C]-7-methyl-GTP	Poly U	<10	<0.014
[^{14}C]ATP,	Poly U + poly 7-methyl G	2,080	2.22
[^{14}C]ATP	Poly U + poly G	2,050	2.2

[a] The reaction mixtures contained in 0.30 ml: 30 μmoles of Tris-HCl buffer, pH 7.4; 1 μmole of $MnCl_2$; 20 μg of each of the indicated ribohomopolymers; 10 units of RNA polymerase; and 0.2 μmole of each of the appropriate ribonucleoside triphosphates. The labeled substrates were [^{14}C]-7-methyl-GTP (7.25×10^5 cpm/μmole); [^{14}C]UTP (6.80×10^5 cpm/μmole); [^{14}C]CTP (7.06×10^5 cpm/μmole); [^{14}C]ATP (9.45×10^5 cpm/μmole); and [^{14}C]dGTP (8.10×10^5 cpm/μmole). The reaction mixtures were incubated for 60 min and assayed for incorporation as described in the text. The mixtures containing more than one polynucleotide were preincubated for 24 hr at 37° without enzyme to ensure complex formation where possible.

showed that about 95% of the guanine residues had been converted into 7-methylguanine. The template activity of this polymer is presented in Table XII. The methylated template is completely inactive in this reaction. Calf thymus DNA that was treated in a similar manner but without dimethyl sulfate is found to have the same activity as native DNA. Therefore, the inactivation of this DNA is evidently due to its extensive methylation. It may be argued that methylation results in loss of template activity because this reaction denatures the DNA. However, heat-denatured calf thymus DNA is as active a template as DNase-treated DNA. It can also be seen that methylated DNA does not inhibit the enzyme.

STUDIES WITH RNA POLYMERASE. Table XIII shows that when 7-methyl-GTP replaces GTP in the RNA polymerase reaction, with poly C as the template, the extent of replacement is 16%. It is interesting that while 7-methyl-dGTP cannot serve as a substrate with dCTP for the DNA polymerase reaction when dGdC is used as the template, 7-methyl-GTP does have this ability with RNA polymerase and poly C as the template. Poly U does not direct the incorporation of 7-methyl-GTP either in the presence or absence of ATP.

Although poly C is a very good template for RNA polymerase, it loses practically all of its template activity when it is present in a 1:1 mixture with poly G. Furthermore, poly G shows no template activity either by itself or in the mixture. One possible explanation for these results is that poly G may inhibit the enzyme and thus what is being observed is not due to the loss of template activity of poly C, but due to the inactivation of the enzyme. However, it can be seen that a 1:1 mixture of poly G and poly U still shows 100% poly U template activity. Therefore, one is led to conclude that the template activity of poly C is lost because it forms a complex with poly G which is inactive as a template. The ultraviolet absorption–temperature profiles of these polynucleotides also suggest that poly C and G form a stable 1:1 complex (S. Hendler and P. R. Srinivasan, unpublished data). Similar findings for the case of *E. coli* RNA polymerase have been reported by Hirschbein *et al.* (1967). In contrast, a 1:1 mixture of poly-7-methyl-G and poly C still retains about 80% of the template activity of poly C. Moreover, poly-7-methyl-G, like poly G is inactive by itself or in the mixture. Poly-7-methyl-G also does not inhibit the enzyme as evidenced by the complete template activity of poly U when present in a 1:1 mixture with poly-7-methyl-G. It, therefore, appears that there is only a small degree of association between the polymers, poly-7-methyl-G and poly C. This is also in agreement with the melting profiles of these polymers (S. Hendler and P. R. Srinivasan, unpublished data).

The effect of pH on the incorporation of 7-methyl-GTP is illustrated in Table XIV. As the pH increases, 7-methyl-GTP becomes less efficient as a substrate, a result similar to that obtained with 7-methyl-dGTP and DNA polymerase and can be explained in a like manner. Thus, the ionized form of 7-methyl-GTP ($pK_a = 7.5$) seems incapable of incorporation into a product.

TABLE XIV: Effect of pH on the Incorporation of 7-Methyl-GTP with Poly C as the Template.[a]

pH	Incorpn of 7-Methyl-GTP (% of Control)
7.0	16.0 (29.4)
7.4	16.0 (34.8)
8.0	10.0 (33.9)
8.4	4.8 (19.4)

[a] The conditions were similar to those described in Table XIII. The values in parentheses are the amounts (in millimicromoles) of [^{14}C]GTP incorporated in the control reactions. For reaction at pH 7.0, 30 μmoles of Tris-maleate buffer was used. Tris-HCl buffers were used in the other experiments.

Table XV reveals that GTP is the preferred substrate when present in the reaction along with 7-methyl-GTP. When the concentration of 7-methyl-GTP is greater than that of GTP, the latter compound has the ability to stimulate the incorporation of 7-methyl-GTP. When the incorporation of 7-methyl-dGTP under the influence of dGdC as template was considered it was shown that although this analog was not able to initiate polymer synthesis by itself, the presence of dGTP stimulated its incorporation. The present finding is analogous to this effect. It is possible that this stimulation is due to a stacking phenomenon. Thus, the stacking forces between GTP and 7-methyl-GTP as well as the corresponding deoxy compounds may be very strong and as the natural substrate gets incorporated, so does the analog, even though the analog may not bond as well with the template.

When methylated calf thymus DNA was used as the template for RNA polymerase, the template activity of the DNA was completely destroyed, a result identical with that found in the case of DNA polymerase.

TABLE XV: Effect of Varying Concentration of GTP and 7-Methyl-GTP on Their Incorporation with Poly C as Template.[a]

Ribonucleoside Triphosphates	mμmoles of GTP Incorpd	mμmoles of 7-Methyl-GTP Incorpd
[^{14}C]GTP (0.3)	38.8	
[^{14}C]GTP (0.2), 7-methyl-GTP (0.1)	32.0	
GTP (0.2), [^{14}C]-7-methyl-GTP (0.1)		5.6
[^{14}C]GTP (0.1), 7-methyl-GTP (0.2)	29.0	
GTP (0.1), [^{14}C]-7-methyl-GTP (0.2)		10.4
[^{14}C]-7-Methyl-GTP (0.3)		6.8

[a] The reaction conditions are similar to those described in Table XIII. The concentration of GTP and 7-methyl-GTP present in the reaction mixtures are given in micromoles.

In conclusion, the results presented here clearly demonstrate that 7-methyl-dGTP replaces *only* dGTP as a substrate for DNA polymerase albeit with low efficiency. Moreover, the ionized form of 7-methyl-dGTP is incapable of replacing dGTP in the polymerase reaction. Similar conclusions can also be drawn from the studies with 7-methyl-GTP and RNA polymerase. These findings in conjunction with the chemical properties of 7-methyl-dGdC polymer reported in this paper suggest that depurination rather than anamolous base pairing may play a major role in mutagenesis by alkylating agents.

References

Aposhian, H. V., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 519.

Bautz, E., and Freese, E. (1960), *Proc. Nat. Acad. Sci. U. S. 46*, 1585.

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, p 493.

Bessman, M. J., Lehman, I. R., Adler, J., Zimmerman, S. B., Simms, E. S., and Kornberg, A. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 633.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brookes, P., and Lawley, P. D. (1964), *Brit. Med. Bull. 20*, 91.

Broom, A. D. (1965), Ph.D. Dissertation, Arizona State University.

Danyluk, S. S., and Hruska, F. E. (1968), *Biochemistry 7*, 1038.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Fujimoto, D., Srinivasan, P. R., and Borek, E. (1965), *Biochemistry 4*, 2849.

Griffin, B. E., and Reese, C. B. (1963), *Biochim. Biophys. Acta 68*, 185.

Haines, J. A., Reese, C. B., and Todd, A. R. (1962), *J. Chem. Soc.*, 5281.

Hirschbein, L., Dubert, J. M., and Babinet, C. (1967), *Eur. J. Biochem. 1*, 135.

Hurst, R. O., and Kuksis, A. (1958), *Can. J. Biochem. Physiol. 36*, 931.

Jones, A. S., Mian, A. M., and Walker, R. T. (1966), *J. Chem. Soc. C*, 692.

Jones, J. W., and Robins, R. K. (1963), *J. Amer. Chem. Soc. 85*, 193.

Lawley, P. D. (1966), *Progr. Nucl. Acid Res. Mol. Biol. 5*, 89.

Lawley, P. D., and Brookes, P. (1961), *Nature 192*, 1081.

Lawley, P. D., and Brookes, P. (1963), *Biochem. J. 89*, 127.

Lawley, P. D., and Brookes, D. (1968), *Biochem. J. 109*, 433.

Lawley, P. D., and Wallick, C. A. (1957), *Chem. Ind. (London)*, 633.

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Michelson, A. M., and Pochon, F. (1966), *Biochim. Biophys. Acta 114*, 469.

Nakamoto, T., Fox, C. F., and Weiss, S. B. (1964), *J. Biol. Chem. 239*, 167.

Ogston, A. G. (1935), *J. Chem. Soc.*, 1376.

Pochon, F., and Michelson, A. M. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 1425.

Pochon, F., and Michelson, A. M. (1967), *Biochim. Biophys. Acta 149*, 99.

Radding, C., Josse, J., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 2869.

Richardson, C. C., Schildkraut, C. L., and Aposhian, H. V. (1964), *J. Biol. Chem. 239*, 222.
Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A. (1960), *J. Biol. Chem. 235*, 3242.
Schweitzer, M. P., Broom, A. D., Ts'o, P. O. P., and Hollis, D. P. (1968), *J. Amer. Chem. Soc. 90*, 1042.
Shapiro, R. (1968), *Progr. Nucl. Acid Res. Mol. Biol. 8*, 73.
Shugar, D., and Fox, J. J. (1952), *Biochim. Biophys. Acta 9*, 199.
Townsend, L. B., and Robins, R. K. (1963), *J. Amer. Chem. Soc. 85*, 242.

Absolute Configuration of the Carboxyethyl (Lactyl) Side Chain of Muramic Acid [2-Amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose]*

Alain Veyrières† and Roger W. Jeanloz‡

ABSTRACT: The absolute configuration of the lactyl side chain of muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] has been confirmed as D by two procedures.

In the first, alkaline degradation of the *N*-acetyl derivative gave a lactic acid which had an optical rotation identical with that of authentic D-(−)-lactic acid. A definite proof of the configuration was obtained by degradation of 2-amino-2-deoxy-3-*O*-[(*R*)-1-(hydroxymethyl)ethyl]-α-D-glucopyranose (obtained by reduction of the side chain of muramic acid) with ninhydrin, followed by sodium borohydride reduction, periodate oxidation, and sodium borohydride reduction to (*R*)-1-(hydroxymethyl)ethyl 2-glyceryl ether, characterized by a crystalline tri-*p*-nitrobenzoate. (*S*)-1-(Hydroxymethyl)-ethyl 2-glyceryl ether, characterized by a crystalline tri-*p*-nitrobenzoate, was obtained from L-rhamnose by addition of nitromethane to give 1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-hepitol. This compound was cyclized to give 2,6-anhydro-1,7-dideoxy-1-nitro-L-*glycero*-L-*galacto*-heptitol, which was catalytically reduced to the 1-amino-1-deoxy derivative. Treatment with sodium nitrite gave 2,6-anhydro-7-deoxy-L-*glycero*-L-*galacto*-heptitol which was oxidized with periodic acid. The resulting product was reduced with sodium borohydride to the *S* ether. Comparison of the *R* and *S* ethers and of their crystalline tri-*p*-nitrobenzoates established the D configuration of the lactyl side chain.

Muramic acid [2-amino-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] is one of the two carbohydrate components of the insoluble peptidoglycan backbone found in all bacterial cell walls (Salton, 1964). Degradation of the bacterial cell wall with egg-white lysozyme involves the hydrolysis of the glycoside linkage of the 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-β-D-glucopyranosyl residue to the O-4 of the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue (Salton, 1964). Similarly, egg-white lysozyme is able to split off the β-D-1→4 linkage of the 2-acetamido-2-deoxy-β-D-glucopyranosyl units of chitin, but the rate of the latter reaction is lower than that of the hydrolysis of the peptidoglycan backbone (Chipman and Sharon, 1969). Since the spatial structure of egg-white lysozyme has been elucidated by Phillips and coworkers (Blake *et al.*, 1965, 1967a,b; Johnson and Phillips, 1965; Phillips, 1966, 1967) and the site of enzyme–substrate interaction has been determined (Chipman and Sharon, 1969), exact knowledge of the configuration of the natural bacterial substrate would provide an interesting contribution to an understanding of enzyme activity.

Whereas the configuration of the 2-amino-2-deoxy-D-glucose moiety of muramic acid is well established on the basis of degradation and synthesis (Blix and Jeanloz, 1969), the exact configuration of the lactyl side chain is less firmly ascertained. Strange and Kent (1959) obtained two isomers by condensation of a racemic 2-halogenopropionic acid with a protected 2-acetamido-2-deoxy-D-glucose compound and assumed that the isomer corresponding to the natural product and having the highest optical rotation had a carboxyethyl (lactyl) side chain with the D configuration, since ethers of D-(−)-lactic acid were known to have an optical rotation higher than that of the corresponding ethers of L-(+)-lactic acid. The additivity of the optical rotations of two compounds linked through an ether bond has, however,

* From the Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts 02114. *Received April 10, 1970.* This is Publication LV of the series Amino Sugars and No. 516 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital. This investigation has been supported by research grants from the National Institute of Allergy and Immunology, National Institutes of Health, U. S. Public Health Service (Grant AI-06692), and from the National Science Foundation (Grant GB-5031). It was presented at the 158th National Meeting of the American Chemical Society, New York, N. Y., Sept 1969.

† Fellow from the Centre National de la Recherche Scientifique (France).
‡ To whom inquiries should be sent.