Synthesis of 8-^{14}C-Labeled O^6-Methyldeoxyguanosine and Its Deoxynucleotide Copolymers†

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ABSTRACT: To study the nature and repair of the promutagenic DNA lesion O^6-methylguanine, we have synthesized 8-^{14}C-labeled O^6-methyldeoxyguanosine triphosphate and investigated the kinetics of its incorporation into the synthetic copolymers poly(dC,m^{6}dG) and poly(dT,m^{6}dG). Deoxy[8-^{14}C]guanosine was methylated with ethereal diazomethane and the products were separated by high-pressure liquid chromatography. O^6-Methyldeoxy[^{14}C]guanosine was converted to the 5′-monophosphate with carrot phosphotransferase and then to the 5′-triphosphate via the phosphorimidazolidate formed by the action of N,N'-carbonyldiimidazole. Although m^{6}dGTP was a poor substrate for *Escherichia coli* DNA polymerase I, copolymers could be synthesized from dCTP or dTTP and m^{6}dGTP with terminal deoxynucleotidyl transferase. The percent of m^{6}dG in the polymer increased linearly as the percentage of m^{6}dGTP in the polymerization mixture was increased to 20% of the total. The percent incorporation of m^{6}dGTP into poly(dT,m^{6}dG) was, however, higher than into poly(dC,m^{6}dG). Good yields of both polymers were readily obtained. The stability of O^6-methyldeoxyguanosine in poly(dT,m^{6}dG) was found to be pH dependent, and the half-life has been measured at four different pH values.

Chemical carcinogens are known to introduce a variety of lesions into DNA, and it is generally believed that such damage is related to the carcinogenic process. The significance of any particular DNA lesion would depend on its precise chemical nature and its rate of removal by specific repair enzymes, among other factors.

One such lesion, O^6-alkylguanine, is introduced by a variety of alkylating agents and was suggested as a promutagenic DNA modification by Loveless (1969). This hypothesis has been supported by studies of the informational content of O^6-methylguanine in synthetic polynucleotide templates. Such experiments with both RNA polymerase (Gerchman & Ludlum, 1973; Mehta & Ludlum, 1978) and DNA polymerase (Abbott & Saffhill, 1979) have shown that the presence of O^6-methylguanine leads to incorrect base incorporation.

Other studies have related the persistence of this lesion to tumor formation. Goth & Rajewsky (1974) showed that the lack of repair of O^6-ethylguanine in brain, compared to liver, was related to the induction of tumors by ethylnitrosourea in the central nervous system of newborn rats. Subsequent studies have supported the hypothesis that the persistence of O^6-alkylguanines may be related to the susceptibility of various tissues to tumor induction (Kleihues & Margison, 1974; Nicoll et al., 1975; Margison & Kleihues, 1975; Maitra & Frei, 1975; Frei & Lawley, 1976).

The recent synthesis by Mehta & Ludlum (1978) of O^6-methyldeoxyguanosine triphosphate and its subsequent incorporation into deoxynucleotide polymers have opened the way for the use of such templates in the analysis of the repair mechanism. Although repair of the O^6-methylguanine lesion by various enzyme systems has been reported (Kirtikar & Goldthwait, 1974; Pegg & Hui, 1978), no clear mechanism has emerged. The study of polymers methylated by ^{14}C-labeled carcinogens, such as N-methyl-N-nitrosourea, is complicated by the formation of numerous other ^{14}C-labeled methylation products (Lawley et al., 1973; O'Connor et al., 1973).

Accordingly, we report here the synthesis of ^{14}C-labeled O^6-methyldeoxyguanosine triphosphate and the kinetics of its incorporation into the synthetic copolymers poly(dC,m^{6}dG) and poly(dT,m^{6}dG), which may be used for repair studies or for studies of template coding properties. The stability of the O^6-methyldeoxyguanosine glycosidic linkage in poly(dT,m^{6}dG) has also been measured in buffers of different pH.

Experimental Section

Materials

Deoxyguanosine was purchased from ICN Pharmaceuticals and purified before use by high-pressure liquid chromatography on a preparative μBondapak C_{18} column. Deoxy[8-^{14}C]guanosine with a specific activity of 51 Ci/mol was purchased from Schwartz/Mann and similarly purified. Unlabeled deoxynucleoside triphosphates were obtained from P-L Biochemicals, while ^{3}H- and ^{14}C-labeled deoxynucleoside triphosphates were obtained from New England Nuclear. All nucleotides were purified before use by chromatography on DEAE-Sephadex A-25 with gradient elution of 0.05–1 M triethylammonium bicarbonate, pH 8.0. *Escherichia coli* DNA polymerase I was obtained from Boehringer Corp., S_1 nuclease was from Worthington, and deoxyribonuclease I was from Schwartz Bioresearch, Inc. Synthetic templates, poly(dA-dT), and poly(dC-dG) were obtained from Boehringer Corp., and poly(dT)·oligo(dA) and poly(dC)·oligo(dG) were from P-L Biochemicals. N-Methyl-N-nitrosotoluenesulfonamide for generating diazomethane was obtained from Aldrich Chemical Co. All other materials were standard reagent-grade chemicals.

Methods

O^6-Methyldeoxy[^{14}C]guanosine. Deoxy[^{14}C]guanosine was methylated with ethereal diazomethane as described for nonradioactive deoxyguanosine by Farmer et al. (1973). Diazomethane was prepared in ethereal solution (5 mmol in 50 mL of ether) by distillation of an alkaline solution of N-methyl-N-nitrosotoluenesulfonamide and added to a solution of deoxy[^{14}C]guanosine (50 μmol with a specific activity of 1 Ci/mol) in methanol (3.5 mL) at 0 °C. The mixture was

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allowed to stand at 0 °C for 1 h, and a small precipitate of 7-methyldeoxyguanosine was separated by centrifugation. Diazomethane was removed under vacuum, and the solution was reduced to dryness by rotary evaporation. The methylated deoxy[^{14}C]guanosine was redissolved in approximately 1.2 mL of methanol and was resolved by high-pressure liquid chromatography on a μBondapak C_{18} column (7.0-mm i.d. × 30 cm) from Waters Associates. After the column was equilibrated with 10% acetonitrile–water, the methylated deoxy-[^{14}C]guanosine was applied in approximately 2–3-mg quantities and eluted with 10% acetonitrile–water (1 mL/min). Three major peaks were seen, the last of which (elution time 40 min) was O^6-methyldeoxyguanosine in a yield of 25–30%.

The usual precautions were used in handling diazomethane. Thus, reactions with this compound were carried out in a hood behind an explosion shield, and its preparation was carried out in an apparatus with smooth rather than ground glass joints.

O^6-Methyldeoxy[^{14}C]guanosine 5′-Monophosphate. O^6-Methyldeoxy[^{14}C]guanosine was phosphorylated with carrot phosphotransferase purified according to the procedures of Strider et al. (1968) and Harvey et al. (1970). This step proved to be the most variable, and careful optimization of the reaction conditions was necessary to obtain a reasonable yield. The typical incubation mixture contained 2.0 mg of O^6-methyldeoxyguanosine, 0.5 mmol of *p*-nitrophenyl phosphate, 0.675 mmol of sodium acetate, pH 5.0, and carrot phosphotransferase in a total volume of 2.5 mL. Reactions were incubated from 2 to 5 h at 37 °C and then extracted 3 times with an equal volume of diethyl ether and applied to an AG-1 × 8 column (0.5 × 10 cm) in 0.05 M sodium acetate, pH 5.0. Elution with this buffer gave a peak of unreacted nucleoside; further washing with 0.03 M HCl gave a nucleotide peak which was neutralized with NH_4OH. This fraction was lyophilized and purified in a DEAE-Sephadex A-25 column (1 × 20 cm) as described earlier for nonradioactive m^{6}dGMP (Mehta & Ludlum, 1978). Yields for this step varied from 35 to 55%.

O^6-Methyldeoxy[^{14}C]guanosine 5′-Triphosphate. The procedure for O^6-methyl-[^{14}C]dGTP was also similar to that used for unlabeled O^6-methyl-dGTP (Mehta & Ludlum, 1978). m^{6}dGMP (0.5 mg) was converted to the pyridinium salt by passage through a Dowex 50 X8 column (0.5 × 10 cm) in the pyridinium form and eluting with 20% aqueous pyridine. Water was removed by repeated evaporation with dry pyridine and dimethylformamide, and the pyridinium salt was converted to the tributylammonium salt by addition of 1 μL of tributylamine. This was further dried by the addition and evaporation of 100 μL of dimethylformamide. The tributylammonium salt was dissolved in 100 μL of dimethylformamide, a solution of freshly prepared *N*,*N*′-carbonyldiimidazole (1 mg) in 50 μL of dimethylformamide was added for the activation of nucleotide, and the mixture was allowed to react at room temperature for 5 h in a desiccator. Excess *N*,*N*′-carbonyldiimidazole was then removed by the addition of 1.5 μL of methanol. After 30 min, a solution of tributylammonium pyrophosphate (0.01 mmol in 50 μL of dimethylformamide) was added and the reaction was allowed to proceed overnight. Then the mixture was concentrated, redissolved in 0.05 M triethylammonium bicarbonate buffer, pH 8.0, and applied to a DEAE-Sephadex A-25 column (1.0 × 20 cm) equilibrated with the same buffer. Elution with a linear gradient of this buffer (0.05–1.0 M, 100 mL each) gave separate peaks of nucleoside and the mono-, di-, and triphosphates. Conversion to triphosphate was approximately 50–70%.

Synthesis of Copolymers. Random copolymers of dTMP with dGMP or m^{6}dGMP and of dCMP with dGMP or m^{6}dGMP were prepared with terminal deoxynucleotidyl transferase isolated according to the procedure of Yoneda & Bollum (1965). However, fine-grade phosphocellulose P-11 (Whatman) was found to give better recovery of enzyme from this small amount of calf thymus than P-1 phosphocellulose. The more recent method (Chang & Bollum, 1971), which produces a more purified enzyme, was found to be less suitable for small-scale preparations since the enzyme is unstable at low protein concentrations. Our enzyme, which was fraction VII of Yoneda & Bollum (1965), contained no "activated" DNA template dependent DNA polymerase activity but had a small amount of nonspecific nuclease activity.

For kinetic investigations, polymerization mixtures contained in 100 μL 20 μmol of potassium cacodylate buffer, pH 7.5, 0.1 μmol of purified deoxynucleoside triphosphates consisting of [^{3}H]dTTP or [^{3}H]dCTP and 0–0.2 μmol of [^{14}C]m^{6}dGTP, 0.1 μmol of $CoCl_2$, 1 μmol of $d(pT)_3$ as the initiator, and 0.65 unit[1] of purified terminal deoxynucleotidyltransferase. Reaction mixtures were incubated at 37 °C, and samples were removed at 3.5 and 24 h, spotted onto 2.3-cm GF/C glass fiber disks (Whatman), washed in 5% trichloroacetic acid, and counted in a toluene-based scintillation fluid. The nanomoles of each deoxynucleotide incorporated were calculated after correction for quenching and double-label counting. For the preparation of larger amounts of copolymers, the reaction was scaled up 20-fold.

Effect of pH on the Stability of the O^6-Methyldeoxyguanosine-Containing Polymer. To determine the stability of the glycosidic linkage of O^6-methyldeoxyguanosine in synthetic polymers, we prepared a poly(dT,m^{6}dG) which contained 2% of O^6-methyldeoxy[^{14}C]guanosine monophosphate. This copolymer was then incubated at 37 °C for various times in buffers of different pH. In separate experiments, poly(dT,[^{14}C]m^{6}dG) (10 nmol) and poly([^{3}H]dT) (10 nmol) were incubated in 100 μL of the following 0.1 M buffers: HCl–KCl, pH 1.8; glycine–HCl, pH 2.8; acetate–acetic acid, pH 4.2; K_2HPO_4–KH_2PO_4, pH 6.2. At the end of each incubation, a solution of carrier DNA (50 μg) and bovine serum albumin (100 μg) was added together with 850 μL of ice-cold 5% (w/v) trichloroacetic acid. After 5 min, the mixture was centrifuged and the radioactivity in the supernatant was determined by counting in a toluene-based scintillation fluid.

Incorporation of O^6-Methyldeoxyguanosine Triphosphate by DNA Polymerase. Various templates were used with *E. coli* DNA polymerase I to test for m^{6}dGTP incorporation into the double-stranded polymer. The assay (total volume 100 μL) contained 5 μmol of Tris-HCl, pH 7.5, 0.2 μmol of $MgCl_2$, 10 nmol each of the appropriate deoxynucleotide triphosphate, including one with a ^{3}H label, 0–10 nmol of [^{14}C]m^{6}dGTP, 0.6 unit of *E. coli* DNA polymerase I, and template. Templates used were DNase I activated calf thymus DNA (Aposhian & Kornberg, 1962) (20 nmol), poly(dA-dT) (15 nmol), poly(dC-dG) (15 nmol), poly(dT)·oligo(dA) (10 nmol), and poly(dC)·oligo(dG) (10 nmol). In each case, the reaction was stopped after 2 h of incubation at 37 °C, aliquots were spotted onto GF/C glass fiber disks, washed in 5% (w/v) trichloroacetic acid, and dried, and the radioactivity was determined by counting in a toluene-based scintillation fluid.

[1] One unit is defined as the incorporation of 1 nmol of dATP into acid-precipitable product with $d(pT)_3$ initiator in 1 h at 37 °C in a polymerization mixture containing, per milliliter, 200 μmol of sodium cacodylate, pH 7.5, 8 μmol of $MgCl_2$, 10 nmol of $d(pT)_3$, and 1 μmol of [^{14}C]dATP.

Assay mixtures were prepared as above with either 10 nmol each of dATP, dTTP, dCTP, and m^{6}dGTP or 10 nmol each of dATP, dTTP, and dCTP and 5 nmol each of dGTP and m^{6}dGTP to test for the formation of m^{6}dGMP during incubation with *E. coli* DNA polymerase I (which could occur if m^{6}dGTP were incorporated and then excised). Activated calf thymus DNA (20 μg) was used as the template in both cases. One aliquot was removed before the addition of DNA polymerase, and one was removed after a 2-h incubation. Aliquots were diluted with 0.05 M triethylammonium bicarbonate buffer, pH 8.0, and applied to a DEAE-Sephadex A-25 column (1.0 × 20 cm) equilibrated with the same buffer. Elution with a linear gradient (0.05–1 M, 100 mL each) separated mono- and triphosphates. Fractions were collected and the radioactivity was measured in a hydroflour scintillation fluid. Authentic marker compounds were used to identify the peaks of O^6-methyldeoxyguanosine mono- and triphosphates.

Results and Discussion

Monomer Synthesis. The synthesis of O^6-methyldeoxyguanosine via the 6-chloro-3′,5′-diacetyldeoxyguanosine derivative (Mehta & Ludlum, 1978) was found to be unsuitable for the small-scale reaction necessary when using deoxy-[^{14}C]guanosine because of the difficult chlorination step. However, a good yield (25–30%) of O^6-methyldeoxyguanosine was obtained by direct reaction of deoxyguanosine with ethereal diazomethane. Good separation of the O^6-methylated product from the other methylated products (7-methyl-, 1-methyl-, ring open 7-methyl-, and possibly some disubstituted deoxyguanosines) was obtained on a μBondapak C_{18} column. Purification of the deoxyguanosine before reaction with diazomethane removed any contaminating guanosine. Conversion of the nucleoside to mono- and triphosphate at such small levels required careful optimization of the reaction conditions and manipulation of the intermediates.

Conversion to the monophosphate with crude carrot phosphotransferase was found to reach a maximum and then to decline, presumably due to contaminating phosphatases which destroyed the product. Nevertheless, yields from 35 to 55% could be obtained.

Conversion to the triphosphate gave good yields (50–70%) as long as the intermediates were kept free of moisture.

The monomers were carefully checked for purity at each step of the synthesis. Deoxy[^{14}C]guanosine appeared as a single, symmetrical peak on the μBondapak C_{18} preparative column and as a single spot (R_f 0.85) on paper chromatography (3MM paper with 7:3 CH_3CN–0.1 M ammonium acetate). The nucleotides eluted from the DEAE columns as symmetrical peaks and appeared as single spots on PEI paper in 0.3 M KH_2PO_4 (pH 4.5). Spectroscopic properties of the ^{14}C-labeled monomers were checked at all stages of purification and were found to be identical with those reported previously for the unlabeled monomers (Mehta & Ludlum, 1978).

Polymer Synthesis. Polymerization of dTTP and dCTP to high molecular weight polymer was achieved with terminal deoxynucleotidyltransferase. O^6-Methyl-dGTP has previously been shown to be a substrate for this enzyme (Mehta & Ludlum, 1978) and is capable of copolymerizing with both dCTP and dTTP. The effect of increasing amounts of m^{6}dGTP in the polymerization mixture on the synthesis of poly(dC,m^{6}dG) and poly(dT,m^{6}dG) was studied at two different times, 3.5 and 24 h. For comparison, the effect of dGTP on the synthesis of poly(dC,dG) and poly(dT,dG) was also determined.

Parts A and B of Figure 1 show the change in total synthesis of poly(dC,dG) and poly(dC,m^{6}dG) as the percentage of purine in the polymerization mixture was increased from 0 to 20%. At 3.5 h dGTP decreased the yield of polymer at high dGTP concentrations (Figure 1A), whereas m^{6}dGTP decreased the total polymer synthesis at all concentrations (Figure 1B). However, by 24 h, copolymer yields were as good as those of the homopolymer (Figure 1). In fact, when the purine concentration was less than 10% of the total, a significant increase in copolymer synthesis was observed. Since the composition of the double-labeled polymer was about the same at 3.5 and 24 h (Figure 3), this increase at 24 h must represent a slow, steady polymerization.

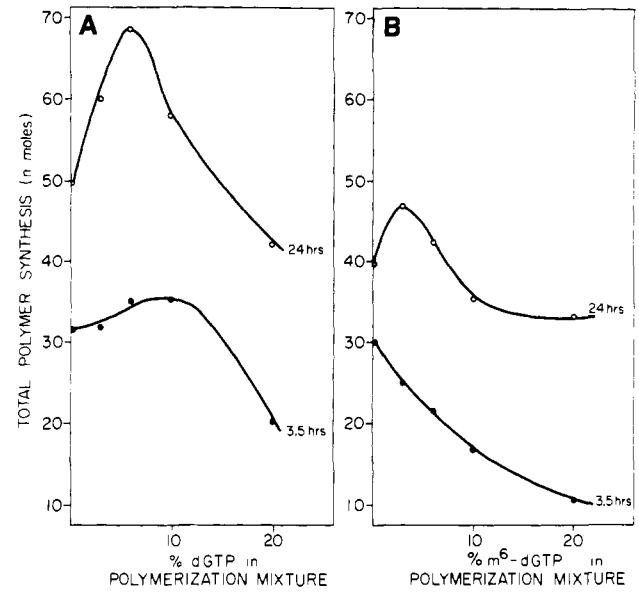

FIGURE 1: Effect of monomer composition on copolymer yield for polymerization of dCTP and dGTP (A) and of dCTP and m^{6}dGTP (B). Total deoxynucleoside triphosphate concentration was 1 mM; aliquots were removed to measure acid-insoluble radioactivity at 3.5 (●) and 24 h (○).

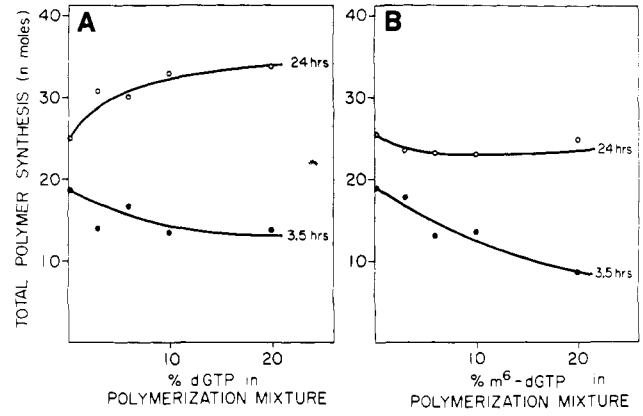

FIGURE 2: Effect of monomer composition on copolymer yield for polymerizations of dTTP and dGTP (A) and of dTTP and m^{6}dGTP (B). Total deoxynucleoside triphosphate concentration was 1 mM; aliquots were removed to measure acid-insoluble radioactivity at 3.5 (●) and 24 h (○).

As shown in Figure 2, the inhibitory effects of purine nucleotides on total synthesis at 3.5 h were again seen during copolymerization of dTTP with dGTP and m^{6}dGTP at 3.5 h, but the yield improved after 24 h. The higher yield of copolymer obtained with dCTP compared to dTTP is in agreement with the kinetic data of Kato et al. (1967).

In previous work (Mehta & Ludlum, 1978), the presence of m^{6}dGTP in the polymerization mixtures caused a significant decrease in total polymer synthesis even after a 24-h incubation. This result may have been due to the use of less pure

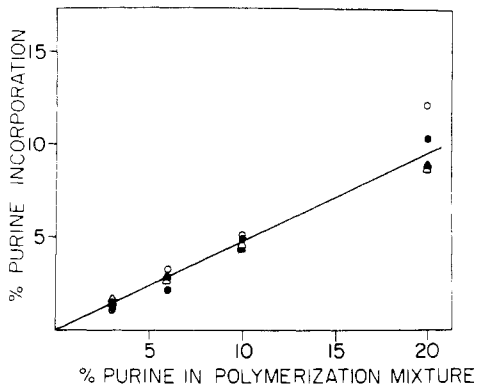

FIGURE 3: Dependence of copolymer composition on substrate composition for poly(dC,dG) and poly(dC,m^{6}dG). The poly(dC,dG) composition is shown at 3.5 (Δ) and 24 h (▲), and the poly(dC,m^{6}dG) composition is shown at 3.5 (○) and 24 h (●).

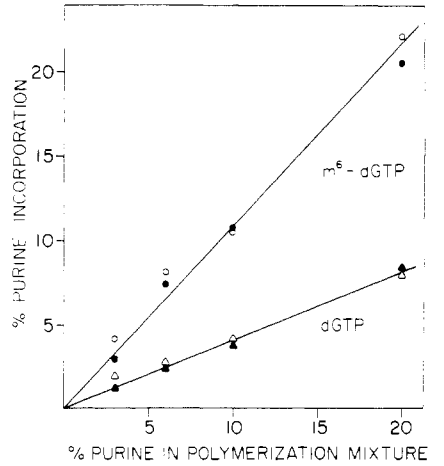

FIGURE 4: Dependence of copolymer composition on substrate composition for poly(dT,dG) and poly(dT,m^{6}dG). The poly(dT,dG) composition is shown at 3.5 (Δ) and 24 h (▲), and the poly(dT,m^{6}dG) composition is shown at 3.5 (○) and 24 h (●).

enzyme [purified to step IV, Yoneda & Bollum (1965)] which could contain nuclease or phosphatase activity. The present results suggest that although m^{6}dGTP causes a decrease in the rate of DNA synthesis, good yields of copolymer can be achieved with purified enzyme by incubation for a longer time period.

The relationship between polymer composition and substrate concentration was studied for all four copolymers. Figure 3 shows the incorporation of dGTP and m^{6}dGTP into copolymers with dCTP as the percent purine in the polymerization mixture increased. Similar results were obtained with both purines, but the percentage of the purine in the polymer was less than half of the percentage in the polymerization mixture.

Figure 4 shows the percent incorporation of dGTP and m^{6}dGTP into copolymers with dTTP. Although the composition of the polymer is linearly dependent on the composition of the polymerization mixture in each case, more m^{6}dGTP is incorporated than dGTP at a given concentration. For the polymer poly(dT,m^{6}dG), the composition is nearly stoichiometric. Again, the differences for copolymerization of dCTP with dGTP or m^{6}dGTP between this result and previous data (Mehta & Ludlum, 1978; Figure 2) may be a function of enzyme purity.

Stability of O^6-Methyldeoxyguanosine in Poly(dT,O^6-methyl-dG). Figure 5 shows the rate of release of O^6-methyldeoxy[^{14}C]guanosine from the polymer at different H$^+$ ion concentrations at 37 °C. The control poly([^{3}H]dT) was

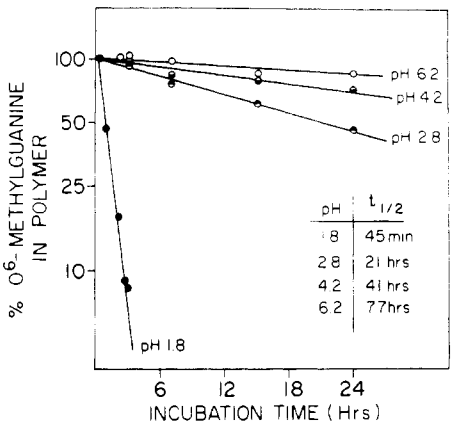

FIGURE 5: Stability of O^6-methylguanine in poly(dT,m^{6}dG) at various pH values. Release of O^6-methylguanine is plotted vs. incubation time at 37 °C. Insert shows the calculated half-time for O^6-methylguanine in each of the buffer solutions.

stable in all four buffer solutions, but poly(dT,m^{6}dG) was markedly unstable at lower pH values. Judging from these data, however, polymers which contained O^6-methyldeoxyguanosine would be quite stable at neutral pH as long as the solution was well buffered and there were no local regions of low pH.

Incorporation of O^6-Methyldeoxyguanosine Triphosphate by DNA Polymerase. Although the data presented above show that m^{6}dGTP is a good substrate for terminal deoxynucleotidyl transferase, it was a poor substrate for *E. coli* DNA polymerase I. No definite incorporation could be shown to occur under any of the conditions listed under Methods. Thus, its rate of incorporation was less than one-hundredth the rate of incorporation of normal deoxynucleoside triphosphates. When activated calf thymus DNA was used as the template, m^{6}dGTP, present in equimolar or higher concentrations than the normal deoxynucleoside triphosphates, produced a 30% inhibition of DNA synthesis. This may indicate some competition for the normal triphosphate binding sites on the enzyme.

E. coli DNA polymerase I contains a 3′→5′-exonuclease which serves a "proofreading" function, deleting any nucleotide which has been wrongly inserted by the polymerase (Kornberg, 1969). If m^{6}dGTP were incorporated and then excised by this exonuclease, it should appear as the corresponding monophosphate in the incubation mixture. However, no conversion of m^{6}dGTP to m^{6}dGMP could be detected in assays where m^{6}dGTP was present at concentrations equal to dGTP or instead of dGTP. Thus, the failure of m^{6}dGTP to appear in a product strand cannot be attributed to an exonuclease activity.

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Effects of Fibrinopeptide Cleavage on the Plasmic Degradation Pathways of Human Cross-Linked Fibrin†

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ABSTRACT: The presence of fibrinopeptide B in human fibrin has a significant effect on plasmic degradation pathways of cross-linked clots. Two types of fibrin were obtained from fibrinogen by incubation either with thrombin, to remove both fibrinopeptides A and B, or with batroxobin, to cleave fibrinopeptide A only. Fibrins obtained after various incubation times were characterized by the determination of the NH_2-terminal amino acids, the content of fibrinopeptides, and the extent of cross-linking. The fibrins were digested by plasmin and were analyzed by polyacrylamide gel electrophoresis. The presence and concentration of the (DD)E complex, as well as fragments E_1 and E_2, in the digests were dependent upon the loss of fibrinopeptide B from cross-linked fibrin. These degradation products, and also fragment DD, appear to be useful molecular markers of fibrinolysis.

Human fibrinogen is converted to fibrin through a limited proteolytic cleavage by thrombin, releasing two molecules of the fibrinopeptides A and B from a dimeric fibrinogen molecule (Bailey et al., 1951; Bettelheim & Bailey, 1952; Lorand, 1951, 1952; Blombäck & Yamashina, 1958; Gladner et al., 1959; Folk et al., 1959). The removal of fibrinopeptide A proceeds at a much faster rate than that of fibrinopeptide B (Bettelheim, 1956); however, the removal of fibrinopeptide A is sufficient for the fibrin polymerization to occur (Laurent & Blombäck, 1958). The investigation of the role of fibrinopeptide release was stimulated by the discovery of fibrinogen clotting enzymes in snake venoms. Reptilase and batroxobin from *Bothrops jararaca* and *Bothrops atrox*, respectively, cleave fibrinopeptide A (Laurent & Blombäck, 1958; Blombäck et al., 1957; Bilezikian et al., 1975) and form fine-structured clots which appear to contain fibrin monomers in an end-to-end arrangement (Laurent & Blombäck, 1958) which can be cross-linked by activated factor XIII (Furlan et al., 1976).

Blombäck et al. (1978) have reported that human fibrin isolated from blood clotted in glass tubes has a Gly/Tyr ratio of 1.3:1, indicating that very little fibrinopeptide B was cleaved by thrombin. The authors suggested that most fibrin formed in vivo would still contain a majority of the fibrinopeptide B. However, at present it is impossible to conclude if fibrinopeptide B is present in intravascular fibrin clots.

Digestion of human cross-linked fibrin by plasmin results in the formation of fragments DD and E and α polymer remnants as the major high molecular weight degradation products (Ferguson et al., 1975; Gaffney & Brasher, 1973; Gaffney et al., 1975; Hudry-Clergeon et al., 1975; Kopeć et al., 1973; Marder et al., 1976; Pizzo et al., 1973a,b). A complex between fragments DD and E, first observed by Gormsen & Feddersen (1973) and later by others (Gaffney & Brasher, 1973; Kopeć et al., 1973), contains one molecule of fragment DD and one molecule of fragment E (Olexa & Budzynski, 1979a). Close analysis of the fragment E from plasmic digests of cross-linked fibrin revealed that there are three species of fragment E, that is, E_1, E_2, and E_3 of molecular weight 60 000, 55 000, and 50 000, respectively (Olexa & Budzynski, 1979b). Fragments E_1 and E_2 can bind with fragment DD, forming the (DD)E complex; however, fragment E_3 cannot. Plasmic digestion of human cross-linked fibrin, which had been formed by extended incubation with thrombin and lacks all of the fibrinopeptides A and B, proceeds according to the following scheme (Olexa & Budzynski, 1979a):

$$(DD)E_1 \rightarrow (DD)E_2 \rightarrow DD + E_3$$

It has been suggested that the (DD)E complex is a nucleus of the fibrin polymerization sites (Hudry-Clergeon et al., 1975; Olexa & Budzynski, 1979b).

Since the differential loss of fibrinopeptide A or B affects the structure of the fibrin clot (Laurent & Blombäck, 1958),

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