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The mechanism of mutation induction by a hydrogen bond ambivalent, bicyclic N^4-oxy-2′-deoxycytidine in *Escherichia coli*

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ABSTRACT

The triphosphate of the nucleoside deoxyribosyl dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (dP) is known to be incorporated into DNA efficiently by *Taq* polymerase and is a useful tool for polymerase-mediated *in vitro* mutagenesis. It is shown here that dP is a potent mutagen in *Escherichia coli* and *Salmonella typhimurium*. In *E.coli*, this deoxycytidine analog induces both GC→AT and AT→GC transitions. No induced transversions are observed. It is highly mutagenic in wild-type *E.coli*, but this is much reduced in a strain lacking thymidine kinase. Mutagenesis induced by dP is efficiently inhibited by the addition of thymidine. Partially purified thymidine kinase from *E.coli* catalyzes phosphorylation of dP to its 5′-monophosphate. When *E.coli* was grown in the presence of dP, the nucleoside analog was incorporated into its DNA. The content of dP in DNA was dependent on the concentration of dP added to the medium. The incorporation characteristics of the 5′-triphosphate of dP (dPTP) were also studied using *E.coli* DNA polymerase I large fragment. The results confirm that this triphosphate can be incorporated opposite A and G in the template with similar efficiencies. This indicates that dP is metabolized as a thymidine analog and that the resulting triphosphate induces a high rate of mutagenesis through replicational errors.

INTRODUCTION

The deoxyribosyl derivative of dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (**1**, dP) was earlier synthesized as a model compound for one of the conformers of N^4-methoxydeoxycytidine (**2**), which results from the action of methoxyamine on cytosine residues in DNA. The formation of N^4-methoxycytosine is believed to be a major cause of methoxyamine-induced mutagenesis. In dihydropyrimido[4,5-*c*][1,2]oxazin-7-one (P), the exocyclic amino substituent is constrained to an *anti* conformation (with respect to N1), whereas for methoxycytosine, both the *syn* and *anti* orientations can exist. Consequently there is no hindrance in P to hydrogen bonding (1,2). This property, together with the favorable tautomeric state, contributes to the strong pairing of P with both of the natural purines; for this reason it was considered to be a potential mutagen provided it could be metabolized and incorporated into DNA. The triphosphate dPTP causes mutations *in vitro* (3). We have now found that dP has a very potent mutagenic activity on bacteria. The mutagenicity of dP is higher than that of N^4-aminocytidine (**3**), a strongly mutagenic cytidine analog which we have reported previously (4). In the present work we have investigated the mechanism of dP-induced mutagenesis. The structures of these nucleosides are shown in Figure 1. A conference abstract reported briefly on dP mutagenicity (5).

MATERIALS AND METHODS

Escherichia coli strain CSH50 [Δ(*pro-lac*), *ara*$^-$, *thi*$^-$/F′(*traD36*, *proAB*, *lacI*$_Q$ZΔM15)] was a gift of Dr T.A.Kunkel of NIEHS (Research Triangle Park, NC). CC101–106 were kindly provided by Dr J.H.Miller of the University of California (Los Angeles, CA). A thymidine kinase-deficient strain, SH10 (*ilv*, *trpE*, *tonB*, *tdk-1*, *tsx*), was obtained from Dr Akiko Nishimura of the Genetic Stock Center (National Institute of Genetics, Japan).

Mutation assays

Mutagenic activities of dP on *E.coli* CC101–CC106 were assayed as reported (6). Briefly, an overnight culture of *E.coli* cells was centrifuged and the bacteria obtained were washed twice. The bacteria were plated with dP and top agar containing 0.5% NaCl and 0.2 mg/ml Nutrient Broth (Difco) onto minimal lactose plates containing 5 g lactose, 2 g citric acid monohydrate, 10 g potassium dihyrogen phosphate, 3.5 g sodium ammonium hydrogen phosphate tetrahydrate, 0.2 g magnesium sulfate heptahydrate and 15 g agar per liter. After incubation for 2 days, the numbers of revertant colonies were scored. The numbers of living cells were scored on glucose minimal plates in which the lactose in the lactose minimal plates was replaced with the same concentration of glucose. For the spot test, bacteria were plated onto minimal lactose plates without dP and then paper discs (10 mm in diameter) containing dP were placed on the plates. *Salmonella* reversion tests and mutations to rifampicin resistance were also performed as reported (4,7). For the rifampicin resistance assay, *E.coli* was grown for 4 h

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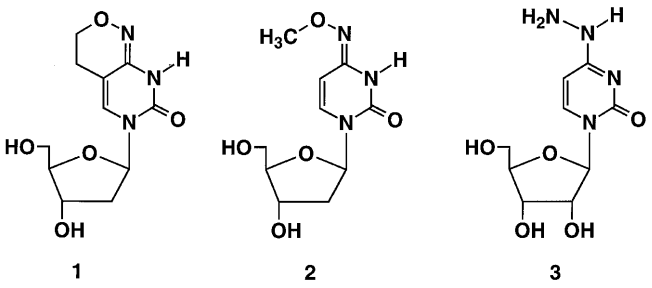

Figure 1. Structure of dP and related compounds: **1**, dP; **2**, N^4-methoxydeoxycytidine; **3**, N^4-aminocytidine.

at 37°C with shaking in liquid LB medium containing 10 g Bacto Tryptone (Difco), 5 g Bacto Yeast Extract (Difco), 10 g NaCl per liter, adjusted to pH 7 with NaOH, supplemented with dP. The bacteria were then plated onto solid LB medium with rifampicin for mutants and onto medium without the antibiotic for survivors after appropriate dilution. The plates were incubated for 2 days at 37°C and the numbers of colonies scored.

Preparation of dP nucleotides

dP (8) and its 5′-triphosphate, 5′-dPTP (3), were synthesized as reported previously. The 5′-monophosphate (5′-dPMP) was synthesized by treating dP (27 mg, 0.1 mmol) with phosphoryl chloride (10 µl, 0.1 mmol) in dry trimethylphosphate (0.25 ml) at 0°C. After 30 min, 5 ml 0.1 M triethylammonium bicarbonate, pH 7.5, were added and the 5′-dPMP was purified by reverse phase HPLC using a Waters C18 (7.8 × 300 mm) column eluted with a linear gradient of 0–10.5% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, over 20 min at a rate of 7.5 ml/min. Samples were monitored at 260 nm. Approximate retention times were 5′-dPMP 9.4 min and dP 16.7 min. Pure 5′-dPMP (27.4 µmol, 27%) was obtained: ^{31}P NMR δ(D_2O) 2.25 p.p.m.

Incorporation of the nucleoside into *E.coli* DNA

One milliliter of *E.coli* CSH50 overnight culture was inoculated into 50 ml LB medium containing 0–10 µg/ml dP. After incubation for 4 h with shaking, *E.coli* cells were collected by centrifugation and washed twice with cold TEG buffer composed of 25 mM Tris–HCl, pH 7.5, 10 mM EDTA and 50 mM glucose. Cells were then suspended in 1.5 ml TEG buffer supplemented with 154 U RNase T1 and 770 µg RNase A and applied to an automated DNA extractor (Genepure 341; ABI Japan, Tokyo) under a procedure recommended by the manufacturer for the preparation of RNA-free DNA. Purified DNA samples (150 µg) in 25 mM Tris–HCl, pH 7.8, containing 25 mM $MgCl_2$ and 12.5 µM coformycin were hydrolyzed at 37°C for 1 h with 100 µg DNase I and further incubated for 3 h after addition of 10 µg snake venom phosphodiesterase, 10 µg alkaline phosphatase and 5 mM Tris–HCl, pH 8.9 (Y.Goto, H.Hayatsu and T.Negishi, unpublished procedure). The resulting nucleosides were fractionated by HPLC using a Waters Novapak C18 column eluted with 10 mM sodium dihydrogen phosphate, 8% methanol. Retention times for dT and dP were 13 and 17 min respectively. The amount of dP was measured by absorption at 320 nm and of the four normal nucleosides at 254 nm. dP has a substantial absorbance at 320 nm (ε = 4200), unlike the normal major nucleosides. For identification of P in DNA, DNA samples were analyzed after digestion to nucleotides. For this purpose, DNA was digested as described above but without the alkaline phosphatase treatment. The nucleotides were fractionated by HPLC using a Waters Novapak C18 column eluted with a linear gradient of 0–28% methanol in 10 mM potassium phosphate buffer, pH 4.0, at a rate of 0.7 ml/min and subsequent isocratic elution with 28% methanol. dTMP and dPMP eluted at 11 and 17 min respectively.

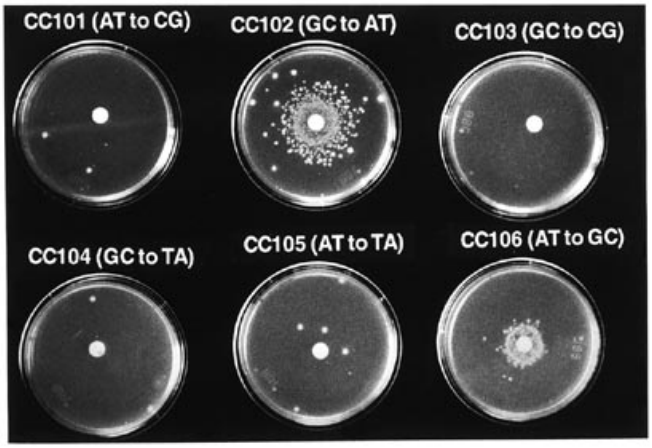

Figure 2. Spot test for dP mutagenicity using *E.coli* CC101–106. Ten micrograms of dP were applied to each disc placed on the center of a plate.

Conversion of dP to 5′-dPMP

Thymidine kinase was partially purified from *E.coli* as reported (9). The reagent mixture for phosphorylation contained 1.5 mM dP (or 1.2 mM thymidine), 10 mM ATP, 10 mM $MgCl_2$, 0.3 mg/ml bovine serum albumin and 70 mM Tris–HCl, pH 7.8. Typically, 75 µl reagent solution were mixed with 25 µl fractionated extract containing 125 µg/ml protein. After an incubation at 37°C, acid-insoluble material was removed from the reaction mixture by the trichloroacetic acid/Freon/trioctylamine procedure (10). dP and dPMP were fractionated with the same gradient as used for nucleotide analysis of the DNA digests. Samples were monitored at 254 nm for thymidine and 320 nm for dP. Retention times for dPMP and dP were 17 and 36 min respectively

Incorporation of dPTP during *in vitro* DNA synthesis

An 18mer oligonucleotide labeled with Texas Red at the 5′-end, 5′-(Texas Red)-TGTAAAACGACGGCCAGT-3′, was purchased from Genset K.K. (Tokyo, Japan) and used as a fluorescent dye primer. The template–primers were obtained by annealing this primer to oligonucleotide templates. Typically, 20 µl of a reaction mixture containing 10 µM dNTP, 1 U Klenow fragment (New England Biolabs), 5 mM $MgCl_2$, 7.5 mM dithiothreitol and 10 mM Tris–HCl, pH 7.5, was incubated at 37°C for 30 min. The enzyme was inactivated by heating at 75°C for 10 min. The elongated products were analyzed in an automated DNA sequencer (Hitachi) after denaturation at 95°C for 3 min.

RESULTS

Mutational specificity of dP

dP shows potent mutagenicity in both *E.coli* and *Salmonella typhimurium*. The mutagenic activity of dP was assayed on a set of *E.coli* tester strains that can distinguish six possible types of

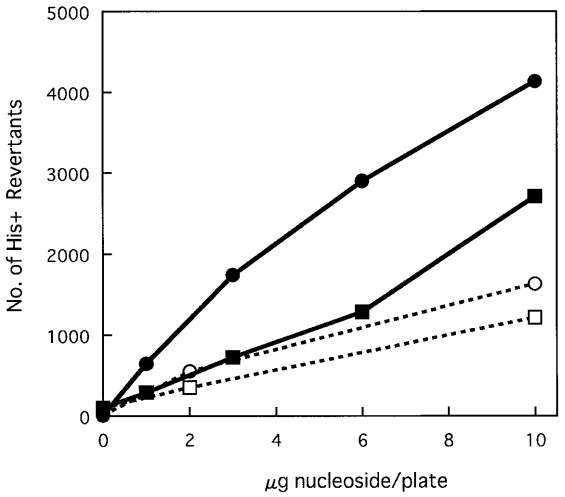

Figure 3. Mutagenic responses of *S.typhimurium* TA100 (■) and TA1535 (●) to dP, compared with those of TA100 (□) and TA1535 (○) to N^4-aminocytidine.

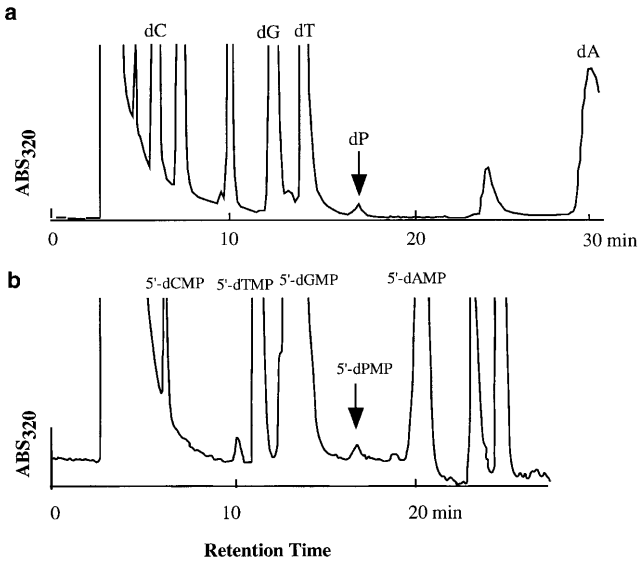

Figure 4. HPLC fractionation of the enzymatic digests of DNA from *E.coli* grown in medium containing 5 μg/ml dP. (**a**) Digested to nucleosides. (**b**) Digested to nucleotides.

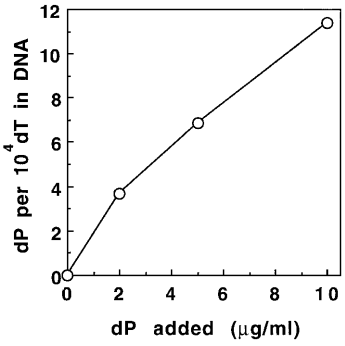

Figure 5. Incorporation of dP into bacterial DNA.

base changes. These strains are reverted by a specific type of base change, as indicated in Figure 2: CC102 and CC106 can be reverted from *lac*⁻ to *lac*⁺ by GC→AT and AT→GC transitions respectively, while CC101, CC103, CC104 and CC105 are reverted only by transversions (11). First, the mutagenic specificity was screened by the use of spot tests described previously, the results of which are shown in Figure 2. The nucleoside solution was applied to discs on minimal glucose agar plates spread with *E.coli*. After incubation for 2 days, two strains (CC102 and CC106) out of six gave positive responses, as indicated by growth of colonies on the respective plates. These results show that dP can induce GC→AT and AT→GC transitions specifically with similar efficiency. This mutagenic specificity is consistent with the view that the compound acts as a mutagen of the nucleoside analog type, causing mutations through replication errors. A quantitative study showed that it induced three revertants/nmol in the assay using CC102 and six revertants/nmol with CC106. Figure 3 shows its mutagenicity in *S.typhimurium* TA100 and TA1535, tester strains detecting base change mutations with and without respectively the R factor plasmid, which can enhance mutagenic responses toward some chemicals (12). No such enhancing effect for dP mutagenesis was observed, as in the case of N^4-aminocytidine. The mutagenicity of dP in the *E.coli* and *S.typhimurium* strains was greater than that of N^4-aminocytidine and much greater than N^4-aminodeoxycytidine, which we have reported earlier (4). It should also be noted that dP has almost no toxicity at the doses at which the experiments were performed. Thus, no killing or reduction in surviving fractions was observed in all the mutation assays performed. These findings prompted us to study the mechanism of how dP causes mutations.

Incorporation into bacterial chromosomal DNA

If dP causes mutations by ambiguous base pairing during replication, dP must be metabolized into dPTP and then incorporated into DNA. In order to confirm incorporation of dP into bacterial DNA, *E.coli* cells were grown in the presence of the nucleoside. The DNA extracted from the cells was analyzed by HPLC after digestion to nucleosides (Fig. 4a) and to nucleotides (Fig. 4b). In both systems, the DNA was shown to contain peaks co-migrating with authentic dP or 5′-dPMP. The peak corresponding to dP did not result from decomposition of free dPMP or dP contaminating the DNA fraction, because the peak was not observed in DNA samples treated only with phosphatase, without DNase and PDase. Figure 5 shows that the content of dP in DNA from bacteria grown in medium containing dP had an approximately linear relationship to the concentration of dP added to the growth medium.

Metabolism of dP

The next question was which enzyme converts dP to 5′-dPMP. This should be a key enzyme for dP metabolism in *E.coli*. Thymidine kinase is most likely to phosphorylate dP, because *E.coli* lacks deoxycytidine kinase (13). To confirm the involvement of thymidine kinase in dP mutagenesis, we grew cells of *E.coli* strains proficient (*tdk*⁺, CSH50) and non-proficient in thymidine kinase (*tdk*⁻, SH10) in medium containing dP and then applied them to plates containing rifampicin to score mutants resistant to this antibiotic. As shown in Figure 6, dP had potent mutagenicity towards the *tdk*⁺ strain, but, in contrast, had very little mutagenic activity in the *tdk*⁻ strain. The mutagenicity of N^4-aminocytidine, which may be phosphorylated by uridine-cytidine kinase (7), was

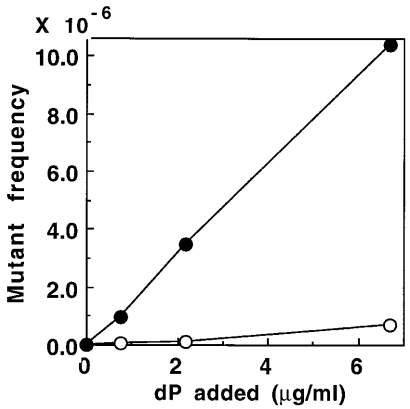

Figure 6. Mutagenicity of dP on *E.coli* strains with (●) and without (○) thymidine kinase.

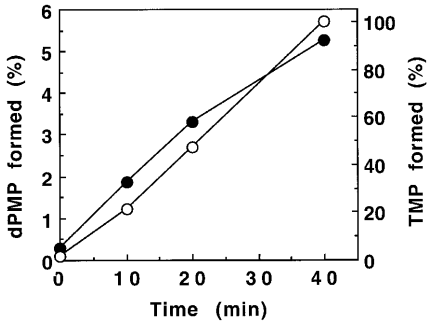

Figure 7. Conversion of dP to dPMP (●) and thymidine to TMP (○) by *E.coli* extract.

no different in the *tdk*⁻ strain from that in the *tdk*⁺ strain (data not shown). The effect of thymidine, a possible competitor for thymidine kinase, was studied next. When 100 μg/ml thymidine were present in a culture of wild-type *E.coli* with 6 μg/ml dP, the mutant frequency was reduced to only 4% of that with dP alone. These results indicate that phosphorylation of dP by thymidine kinase is the major first step of dP metabolism.

Direct evidence was obtained from the experiments using partially purified thymidine kinase from *E.coli*. dP was treated with the thymidine kinase fraction of an *E.coli* extract in the presence of ATP. HPLC analysis of the reaction mixture demonstrated formation of dPMP. A time course of dPMP formation is presented in Figure 7. Under conditions where 50% of thymidine can be converted to dTMP, 3% of dP was phosphorylated to dPMP.

Base pairing properties of dPTP

Base pairing specificity of dPTP was studied using a DNA chain elongation reaction catalyzed by Klenow fragment. To analyze the incorporation of dPTP opposite adenine, a fluorescently labeled primer was annealed to an oligonucleotide with adenine at position 7 relative to the 5′-end to form primed oligo-'A', as shown in Figure 8a. For incorporation of dPTP opposite guanine, we used primed oligo-'G', which is the same primer annealed to an oligonucleotide with guanine at position 7 relative to the 5′-end. These template–primers were incubated with the Klenow

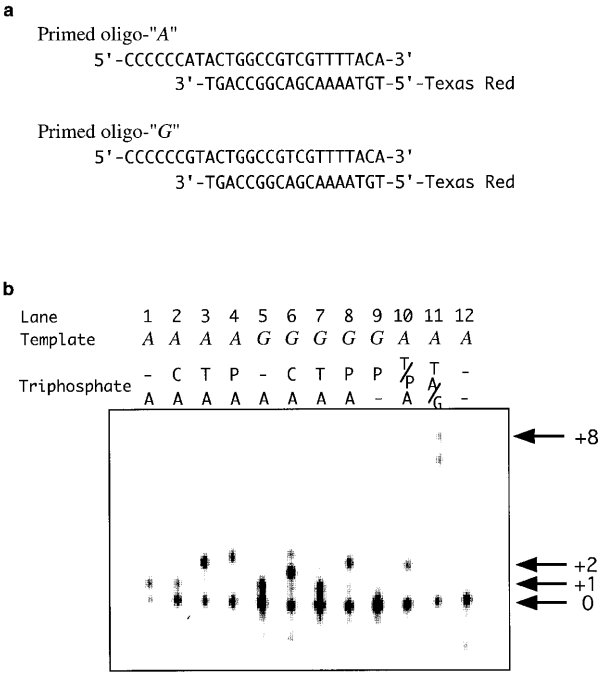

Figure 8. Incorporation of dPTP during DNA synthesis *in vitro*. The primed templates used are shown in (**a**). A, C, T, G and P under Triphosphate show the deoxyribotriphosphates added. The elongated products migrate from top to bottom. The primer itself migrates at position 0 (lane 12).

fragment and requisite triphosphates. Resulting elongation products were analyzed by automated DNA sequencing (Fig. 8b). If only dATP was added as a triphosphate substrate, the primer was elongated by 1 nt (lanes 1 and 5). As shown in lanes 2 and 6, dCTP did not pair with adenine, but with guanine. As shown in lanes 3 and 7, dTTP paired with adenine, but not with guanine. In contrast, dPTP was incorporated opposite both A and G with similar efficiency (lanes 4 and 8). The bands in lanes 3, 4, 6 and 8 correspond to molecules of primer + AT, primer + AP, primer + AC and primer + AP respectively. The mobilities of these 20mers were slightly different from each other because they had different nucleotides at the 3′-end.

DISCUSSION

Previous T_m and NMR studies of oligonucleotide duplexes containing dP proved that dP can form stable base pairs with both adenine and guanine (1,2). The ambivalent pairing nature together with efficient conversion to dPTP in *E.coli* cells must be the basis for dP showing potent mutagenicity in *E.coli* and this must be so too for *S.typhimurium*. A probable pathway for the mechanism of this mutagenesis is summarized in Figure 9. The nucleoside is first phosphorylated in bacterial cells by thymidine kinase. All the present data support this view. However, there might be a partial contribution of the free base liberated from dP by the action of cellular thymidine phosphorylase, which catalyzes conversion of thymidine to thymine and 2-deoxyribose-1-phosphate, and a reversal of the reaction. Thus we treated dP with thymidine phosphorylase (Sigma Chemical Co.) and degradation of dP was analyzed by HPLC. Although the velocity of degradation into the putative P base was over three orders of magnitude smaller than the thymidine to thymine reaction

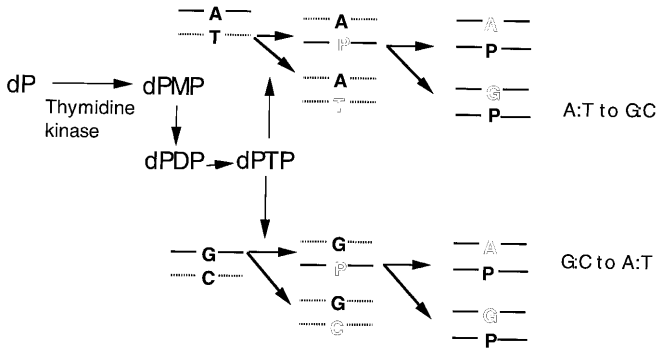

Figure 9. Probable pathway of dP mutagenesis in *E.coli*.

catalyzed by the same enzyme, dP can be metabolized to a state of apparent equilibrium. The putative P base purified by HPLC was found to be non-mutagenic in *E.coli* CC102 and CC106 (E.Mito and K.Negishi, unpublished results). Therefore, a contribution of the product of phosphorylase action to mutagenesis seems to be very unlikely.

5′-dPMP is then converted to 5′-dPTP by cellular nucleotide metabolism. This dPTP is incorporated in place of dTTP opposite an A of template DNA and in place of dCTP opposite a G. Incorporation catalyzed by the Klenow enzyme was efficient in both cases, but slower than that of normal nucleotides into their correct sites, i.e. C opposite G or T opposite A. Zaccolo *et al.* have reported *in vitro* random mutagenesis using dPTP (3). The efficiency of dPTP incorporation opposite A and G by *Taq* polymerase was measured and the results showed that dPTP was incorporated opposite A as efficiently as dTTP; the efficiency of incorporation of dPTP opposite G was 10% of that of dCTP. Here we have shown that the Klenow fragment, an *E.coli* DNA polymerase, can also incorporate dPTP opposite both A and G efficiently.

All these findings indicate that dP is a mutagen of the nucleoside analog type. It causes mutations by ambiguous base pairing during incorporation into DNA and by replication of the template containing it. In DNA synthesis *in vitro*, *Taq* DNA polymerase incorporated only A opposite dP in an oligonucleotide template (14). However, more recently it has been found that when the dP is in other sequence contexts both A and G are incorporated (F.Hill, D.Loakes and D.M.Brown, unpublished results), thus accounting for the observed AT→GC and GC→AT transitions. It is interesting that a nucleoside analog with a bicyclic ring system like dP can be metabolized as a thymidine analog. It will also be interesting to determine whether dP can be phosphorylated by a mammalian thymidine kinase.

In the present paper we have elucidated a major pathway of dP mutagenesis in *E.coli*. dP is the most potent among the known nucleoside analog mutagens in *E.coli*. N^4-Aminocytidine has a comparable mutagenicity, but dP is much more stable than this hydrazino compound. dP can be easily detected by its characteristic absorption at wavelengths >300 nm, where normal major nucleosides have very little absorption. Thus dP may be a useful tool in mutational studies as a standard mutagen specifically inducing transitions.

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