Mechanistic Studies of the Inhibition of MutT dGTPase by the Carcinogenic Metal Ni(II)

Dale W. Porter,[†] Victor C. Nelson,[‡] Matthew J. Fivash, Jr.,[§] and Kazimierz S. Kasprzak*,[†]

Laboratory of Comparative Carcinogenesis, National Cancer Institute−FCRDC, Building 538, Room 205E, Frederick, Maryland 21702, SAIC Frederick, National Cancer Institute−FCRDC, P.O. Box B, Frederick, Maryland 21702, and Data Management Services, Inc., National Cancer Institute−FCRDC, P.O. Box B, Frederick, Maryland 21702

Received May 13, 1996[⊗]

Promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) levels are increased in DNA of animals exposed to carcinogenic metals, such as Ni(II). Besides being generated directly in genomic DNA, 8-oxo-dG may be incorporated there from 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP), a product of oxidative damage to the nucleotide pool. The *Escherichia coli* dGTPase MutT, and analogous dGTPases in rats and humans, have been suggested as a defense against such incorporation because they hydrolyze 8-oxo-dGTP to 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate (8-oxo-dGMP). MutT and its mammalian counterparts are Mg(II)-dependent enzymes. Ni(II), in turn, is known to interact antagonistically with Mg(II) in biological systems. Thus, we hypothesized that Ni(II) might inhibit the activity of MutT. As an initial examination of this hypothesis, we conducted enzyme kinetic studies of MutT to determine the effect of Ni(II) on MutT activity and the mechanisms involved. As found, Ni(II) inhibited MutT in a concentration-dependent manner when either dGTP or 8-oxo-dGTP was the nucleotide substrate. Ni(II) was determined to be an uncompetitive inhibitor of MutT with respect to Mg(II) when dGTP was the substrate, with apparent K_i of 1.2 mM Ni(II), and a noncompetitive inhibitor with respect to Mg(II) when 8-oxo-dGTP was the substrate, with apparent K_i of 0.9 mM Ni(II). Hence, the two metal cations did not compete with each other for binding at the MutT active site. This makes it difficult to predict Ni(II) effects on 8-oxo-dGTPases of other species. However, based upon the amino acid sequences of human and rat MutT-like dGTPases, their capacity for Ni(II) binding should be greater than that of MutT. Whether this could lead to stronger inhibition of those enzymes by Ni(II), or not, remains to be investigated.

Introduction

Nickel has been established as a carcinogen in humans and experimental animals (*1*), but the molecular mechanisms of tumor induction by this metal remain to be established. Recent evidence suggests that the oxidation of DNA, particularly its guanine residues, is involved in these mechanisms (*2*). *In vitro* investigations in this laboratory have determined that nickel can facilitate oxidation of guanine in 2′-deoxyguanosine and in DNA, forming promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG)[1] (*3*, *4*). DNA isolated from the rat kidney, the target organ for nickel carcinogenesis, following Ni(II) injection, had 8-oxo-dG levels that were approximately 47% higher in comparison to controls (*5*). Besides being generated directly in existing DNA molecules, 8-oxo-dG can be inserted into newly synthesized DNA from oxidatively damaged nucleotide pool, namely, from 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate (8-oxo-dGTP). Ni(II) might be capable of mediating both processes (*2*). However, the contribution of each of them to Ni(II)-induced *in vivo* elevation of 8-oxo-dG in DNA has not been explored.

8-Oxo-dGTP, which may be formed from dGTP in cells under oxidative stress (*6*), was found to be efficiently utilized for 8-oxo-dG incorporation into DNA using *in vitro* replication assays in procaryotic (*7*−*9*) and eukaryotic (*9*) systems. *In vitro* studies have determined that when 8-oxo-dG is present in the DNA template, 8-oxo-dG causes predominantly G→T transversions during replication (*7*), whereas when 8-oxo-dGTP is used as substrate nucleotide during DNA replication, it causes A→C transversions (*9*). Likewise, *in vivo*, when 8-oxo-dG is present in the DNA template strand, it predominantly causes G→T transversions with a mutation frequency of 0.7%, but when 8-oxo-dGTP is used as substrate nucleotide during DNA replication, it causes A→C transversions with a mutation frequency of 16% (*8*). Thus, when 8-oxo-dGTP is present as a nucleotide substrate for DNA synthesis, the mutagenic potential of 8-oxo-dG is much higher. Both of these transversions are due to 8-oxo-dG−dA, mispairing (*7*−*9*) and may lead to malignant transformation of cells.

Studies in *Escherichia coli* have identified two enzymes, MutM and MutY, which can remove mutagenic 8-oxo-dG lesions from DNA (*10*). A third enzyme, MutT, has also been suggested to be involved in the 8-oxo-dG lesion repair system. MutT is an enzyme that was first

* To whom correspondence should be addressed. Telephone: (301) 846-5738; FAX: (301) 846-5946; Internet: kasprkaz@fcrfv2.ncifcrf.gov.
[†] Laboratory of Comparative Carcinogenesis, NCI-FCRDC.
[‡] SAIC Frederick, NCI-FCRDC.
[§] Data Management Services, Inc., NCI-FCRDC.
[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.
[1] Abbreviations: 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxo-dGTP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-triphosphate; 8-oxo-dGMP, 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate; dGTP, 2′-deoxyguanosine 5′-triphosphate; dGMP, 2′-deoxyguanosine 5′-monophosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

S0893-228x(96)00081-1

described in terms of its ability to hydrolyze the 4 common deoxyribo- and ribonucleoside triphosphates to their respective monophosphates with preference for dGTP (*11*). A later study determined that MutT could also hydrolyze 8-oxo-dGTP to 8-oxo-7,8-dihydro-2′-deoxyguanosine 5′-monophosphate (8-oxo-dGMP) (*6*). *E coli* *mutT*$^-$ strains have 100−1000-fold higher mutation rates characterized by A:T→C:G transversions (*12*), the result of a template A pairing with some form of dGTP (*13*), possibly 8-oxo-dGTP. By hydrolyzing 8-oxo-dGTP, the incorporation of 8-oxo-dG during DNA biosynthesis may be reduced. Consequently, it has been suggested that MutT represents the first line of defense against 8-oxo-dG mutagenesis (*10*).

Mg(II) has been shown to prevent causation of pulmonary adenomas by Ni(II), and thus it was suggested that Ni(II) carcinogenesis may be related to the disruption of critical cellular processes which are Mg(II)-dependent (*14*). The enzymatic activity of MutT is Mg(II)-dependent (*6*, *11*). Therefore, it may be susceptible to inhibition by other divalent metals, including carcinogenic Ni(II). A consequence of this inhibition would be an increase in the amount of 8-oxo-dGTP in the cellular nucleotide pool, which may ultimately result in an increase in 8-oxo-dG levels in DNA due to the use of 8-oxo-dGTP as a nucleotide substrate during DNA biosynthesis. As a first investigation into this hypothesis, the effect of Ni(II) on MutT activity had to be established. Therefore, enzyme kinetic studies of MutT were conducted in order to determine the effect of Ni(II) on MutT activity and establish its mechanism.

Experimental Procedures

Chemicals. Ammonium acetate, poly(vinylpyrrolidone) (MW = 40 000), magnesium chloride, dibasic potassium phosphate, and the sodium salts of 2′-deoxyguanosine 5′-monophosphate (dGMP) and 2′-deoxyguanosine 5′-triphosphate (dGTP) were purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA). Acetic acid was obtained from Mallinckrodt (Chesterfield, MO), and methanol was purchased from Baxter (Muskegon, MI). Nickel chloride was obtained from Aldrich Chemical (Milwaukee, WI). All chemicals used in the synthesis of 8-oxo-dGMP and 8-oxo-dGTP were purchased from Aldrich Chemical, except tributylammonium pyrophosphate which was purchased from Sigma Chemical Co.

8-Oxo-dGMP and 8-Oxo-dGTP Synthesis. 8-Bromo-2′-deoxyguanosine was prepared as previously described (*15*). 8-(Benzyloxy)-2′-deoxyguanosine was produced by reacting 8-bromo-2′-deoxyguanosine with sodium benzyl oxide in DMSO (*16*). The product had mp 198−201 °C, which is in agreement with the previously reported value of 201 °C (*16*).

8-(Benzyloxy)-2′-deoxyguanosine 5′-monophosphate was prepared from 8-(benzyloxy)-2′-deoxyguanosine by a modification of a previous method (*17*). 8-(Benzyloxy)-2′-deoxyguanosine was dissolved in pyridine and evaporated several times to remove water. The material was then suspended in trimethyl phosphate (10 μL/mg) and stirred under nitrogen in an ice−methanol bath as anhydrous triethylamine (7 equiv) was added with a syringe through a septum. Then, phosphorus oxychloride (2 equiv) was likewise added, and after 3 min the reaction was quenched by addition of excess ice-cold aqueous triethylamine and the mixture evaporated to a tar under high vacuum. The tar was washed with diethyl ether followed by acetone, then dissolved in water, filtered, and freeze-dried. The material was purified by preparative HPLC using a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm) and eluted with 0.05% ammonium formate (w/v) in 20% aqueous methanol (v/v) at 5 mL/min with peak detection at 280 nm, the first peak being collected. The solvent was evaporated under vacuum and the residue was subjected to the next step without further purification.

To form the sodium salt of 8-oxo-dGMP, the above residue was dissolved in 50% aqueous methanol and hydrogenated for 12 h under 50 psi hydrogen using palladium on carbon as catalyst. The solution was filtered, diluted with water, and freeze-dried. The residue was purified by preparative HPLC using a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm) and eluted with H_2O at 5 mL/min. Peak elution was monitored at 280 nm. The collected peak fraction was evaporated to glass, dissolved in a minimum amount of methanol, filtered, and treated with 400 mg of sodium perchlorate in 20 mL of acetone to precipitate the product. The product was judged pure by HPLC analysis using a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm), which produced one peak with a retention time of 4.2 min, when eluted with 5% ammonium formate (w/v) in 5% aqueous methanol at 5 mL/min with peak detection at 280 nm. The yield, based on bromodeoxyguanosine, was 1%. ^{1}H NMR (DMSO), δ 1.95 (m, 2′-H), 2.90 (m, 2′-H), 3.2−3.8 (m, 4′-H, 5′-H), 4.20 (m, 3′-H), 4.60 (m, 3′-H), 6.0 (m, 1′-H), 6.6 (m, OH), 7.0 (m, OH), 10.5 (m, NH), was consistent with the structure of 8-oxo-dGMP.

8-Oxo-dGTP (sodium salt) was prepared according to the method of Hoard (*18*). A sample of 8-oxo-dGMP as the triethylammonium salt was obtained by subjecting 8-oxo-dGMP, prepared above, to HPLC isolation on a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm) and elution with 1% triethylammonium formate (v/v) in 30% aqueous MeOH at 5 mL/min with peak detection at 280 nm. The collected fraction was evaporated to dryness and then twice dissolved in ethanol and evaporated to remove volatile buffer. The material (175 mg) was then dissolved twice in anhydrous DMF and evaporated under vacuum to remove ethanol. The flask was sealed with a septum and flushed with nitrogen, and then anhydrous DMF (3 mL) was added through a syringe. With stirring, a solution of carbonyl diimidazole (5 equiv) in DMF (3 mL) was added by syringe, and the mixture was stirred overnight. Methanol (8 equiv) was added, the mixture was stirred for 30 min, and then tributylammonium pyrophosphate (5 equiv) in DMF (10 mL) was added with a syringe. After stirring 1 day, the mixture was centrifuged, the supernatant was treated with methanol for 15 min, and the solution was evaporated to dryness under vacuum. The residue was purified by twice subjecting it to preparative HPLC using a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm), and the product was eluted with 0.03% ammonium formate (w/v) in 5% aqueous MeOH (v/v). The collected fraction was evaporated to dryness, dissolved in a minimum amount of methanol, filtered, and then treated with sodium perchlorate (25 mg) in acetone (3 mL) to precipitate the product as the sodium salt (15 mg, 8% yield). The product was judged pure based upon HPLC analysis using a Whatman Partisil M9 10/25 ODS column (10 mm × 25 mm) and elution with 0.03% ammonium formate (w/v) in 5% aqueous MeOH (v/v) at 5 mL/min with peak detection at 280 nm, which showed one peak at 1.5 min.

MutT Assay with dGTP as Substrate. Purified MutT, isolated from *E. coli*, was used in the MutT assays described below; it was a gift from Dr. Maurice J. Bessman (The Johns Hopkins University, Department of Biology). All solutions used in the MutT assay were prepared in 100 mM ammonium acetate, the pH of which was adjusted to 7.4 with either NH_4-OH or CH_3COOH. The MutT assay was conducted at 37 °C in 50 μL samples containing the following: 0.15 μg of MutT, 2.5 μg of poly(vinylpyrrolidone), 5−34 mM $MgCl_2$, 0−1.5 mM $NiCl_2$, and 4 mM dGTP. All components, except dGTP, were preincubated for 15 min prior to addition of dGTP to initiate the assay. Aliquots of the sample (10 μL) were removed during a 45 min assay, added to 990 μL of ice-cold ddH_2O to stop the reaction, and kept on ice. The dGMP production was quantified by isocratic HPLC (see below). When $NiCl_2$ was included in the assay, MutT was incubated with all components except $MgCl_2$ and dGTP for 10 min, followed by additional 5 min of incubation

after the addition of $MgCl_2$. The assay was initiated with the addition of dGTP and conducted as in the absence of $NiCl_2$. Under these conditions, the velocity of the MutT-catalyzed reaction was linear with time. The concentration of dGTP was calculated using $\epsilon_{252} = 13\,700\ M^{-1}\ cm^{-1}$ and that of Mg(II)·dGTP complex using log equilibrium constant of 4.13 reported for Mg(II)·GTP (*19*). The concentration of free Mg(II) was calculated by subtracting the concentration of Mg(II) in the form of Mg(II)·dGTP complex from the total concentration of Mg(II).

MutT Assay with 8-Oxo-dGTP as Substrate. All solutions used in the MutT assay were prepared in 100 mM ammonium acetate, the pH of which was adjusted to 7.4 with either NH_4OH or CH_3COOH. The MutT assay was conducted at 37 °C in 50 μL samples containing the following: 0.15 μg of MutT, 2.5 μg of poly(vinylpyrrolidone), 0.25−8.00 mM $MgCl_2$, 0−1.5 mM $NiCl_2$, and 40 μM 8-oxo-dGTP. All components except 8-oxo-dGTP were preincubated for 15 min prior to addition of 8-oxo-dGTP to initiate the assay. Aliquots of the sample (10 μL) were removed during a 10 min assay, added to 990 μL of ice-cold ddH_2O to stop the reaction, and kept on ice. The 8-oxo-dGTP hydrolyzed and 8-oxo-dGMP produced were determined by isocratic HPLC analysis (see below). When $NiCl_2$ was included in the assay, MutT was preincubated for 10 min with all components, except $MgCl_2$ and 8-oxo-dGTP, and then for 5 min with $MgCl_2$. The assay was initiated by the addition of 8-oxo-dGTP and conducted as in the absence of $NiCl_2$. Under these conditions, the velocity of the MutT reaction was linear with time. The concentration of 8-oxo-dGTP was calculated using $\epsilon_{245} = 12\,300\ M^{-1}\ cm^{-1}$ (*20*) and Mg(II)·8-oxo-dGTP complex using log equilibrium constant of 4.13 as reported for Mg(II)·GTP (*19*). The concentration of free Mg(II) was calculated by subtracting the concentration of Mg(II) in the form of Mg(II)·8-oxo-dGTP complex from the total concentration of Mg(II).

High Performance Liquid Chromatography Analysis. The HPLC system consisted of two Shimadzu LC-6A liquid chromatography pumps which were operated with a Shimadzu SCL-6A system controller. Samples (50 μL) of the diluted ice-cold assay solutions were separated using a Beckman Ultrasphere C_{18} column (5 μm particle size, 4.6 mm × 25 cm). The separation of dGTP and dGMP was achieved using isocratic elution with 0.05 M KH_2PO_4 containing 1% aqueous MeOH, at a flow rate of 1.0 mL/min. dGMP was detected by UV absorption at 254 nm with a Shimadzu SPD-6AV UV/vis spectrophotometric detector and quantified from a dGMP standard curve.

The separation of 8-oxo-dGTP and 8-oxo-dGMP was achieved using isocratic HPLC analysis, as well. A 100 μL aliquot of the diluted ice-cold assay solution was eluted with 0.05 M KH_2PO_4 containing 3% aqueous MeOH, at a flow rate of 1.0 mL/min. Both 8-oxo-dGTP and 8-oxo-dGMP peaks were quantified electrochemically at 800 mV with a glassy-carbon electrode using a Princeton Applied Research Model 400 electrochemical detector. Using this system, 8-oxo-dGTP had a retention time of approximately 8.5 min and was well resolved from 8-oxo-dGMP which had a retention time of approximately 13 min (Figure 1). The 8-oxo-dGMP produced was calculated from 8-oxo-dGMP standard curve, constructed each day of analysis, while 8-oxo-dGTP was calculated as a percent loss of the total 8-oxo-dGTP present at the start of the assay.

Biochemical Analysis of Enzyme Kinetic Data. Biochemical analysis of the MutT data utilized three curve fitting procedures. First, the entire data set at each level of Ni(II) was fitted to the Michaelis−Menten equation (*21*) using an implementation of the Levenberg−Marquardt least squares method, and standard errors were calculated using the covariance matrix. Subsequently, parameter estimates from each experiment were determined by fitting the data from each experiment to either the uncompetitive (*22*) or noncompetitive (*22*) inhibitor models using an implementation of the Levenberg−Marquardt least squares method. Parameter means and standard deviations (SD) were calculated, and standard errors (SE) were defined as $[SD/(n)^{1/2}]$, where n is the number of replicate experiments. Finally, linear transformation of the data was performed as described by Cornish-Bowden (*23*), and these transformed data were fitted by linear regression. The software program Origin, Version 4.0 (MICROCAL Software, Northhampton, MA), was used for these curve fitting procedures.

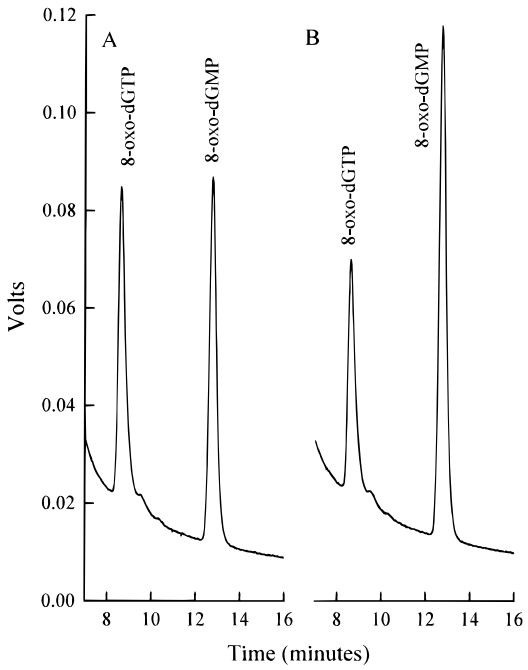

Figure 1. HPLC chromatogram of MutT assay with 8-oxo-dGTP as substrate. HPLC chromatograms of MutT assays of 5 min (A) and 10 min (B) durations are shown.

Statistical Analysis of the Inhibitor Model. The two models assigned to the inhibition data, uncompetitive and noncompetitive, were examined in a detailed statistical analysis procedure in order to provide statistical evidence for the model assignments. The entire set of measurements was fit to either the uncompetitive (*22*) or noncompetitive (*22*) inhibitor models using an implementation of the Levenberg−Marquardt least squares method to estimate each kinetic parameter. The covariance matrix was calculated with the assumption that the model is approximately linear in the region near the minimum sum of squares and was used to determine the standard error of each parameter within the model (*24, 25*). An F statistic was also calculated for each of the model fits. A nonsignificant F statistic ($p > 0.9$) demonstrates that the error between replicate experiments, which is the normally distributed experimental error, was significantly larger than the model-lack-of-fit error, which is the difference between the experimental and predicted values. In addition, examination of the residuals showed no evidence for a patterned error structure in either of the models examined, corroborating the F test result. The software program Mathcad Version 6.0 (Mathsoft Inc., Cambridge, MA) was used for these curve fitting procedures.

Results

HPLC Analysis of the MutT Assays. Using the HPLC conditions described, dGTP had a retention time of 4 min and was well resolved from the dGMP peak, which had a retention time of approximately 10 min. A previous study had determined that dGMP was the only product of the MutT-catalyzed hydrolysis of dGTP (*26*). Thus, this method provided an adequate means to monitor the MutT reaction when dGTP was used as the substrate.

During the MutT assay, the loss of 8-oxo-dGTP was quantitatively coincident with the production of 8-oxo-dGMP (Figure 1). No peak with a retention time between 8-oxo-dGTP and 8-oxo-dGMP was observed, which presumably would correspond to 8-oxo-dGDP. We also

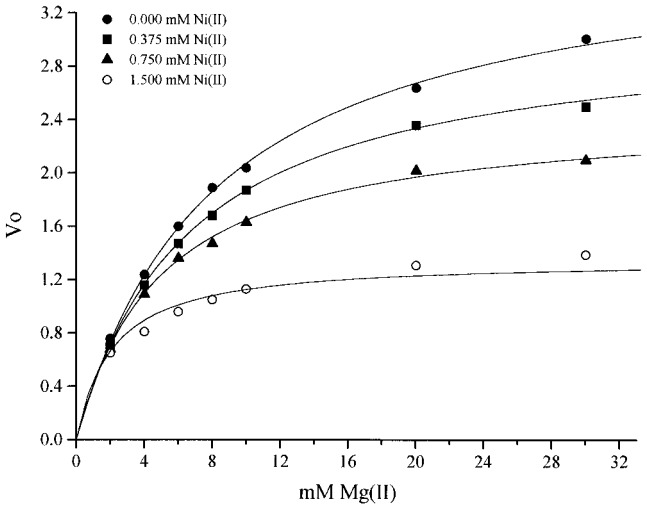

Figure 2. Mg(II) activation of MutT with dGTP as substrate. MutT initial velocity, V_0 [nmol of dGMP produced/(μg of protein·min)], was plotted versus the free Mg(II) concentration, and the data were fitted to the Michaelis−Menten equation using nonlinear regression analysis as described. Values are means of three experiments.

Table 1. Estimates of $K_{m\ app}$ and $V_{max\ app}$ for MutT with dGTP as Substrate[a]

mM Ni(II)	$K_{m\ app}$ [mM Mg(II)]	$V_{max\ app}$ [nmol of dGMP produced/(μg of protein·min)]
0.000	8.22 ± 0.71	3.78 ± 0.13
0.375	6.67 ± 0.46	3.12 ± 0.09
0.750	4.93 ± 0.39	2.46 ± 0.07
1.500	2.07 ± 0.32	1.36 ± 0.06

[a] Kinetic parameter values were determined by fitting the MutT kinetic data to the Michaelis−Menten equation by nonlinear regression analysis as described. Values represent means ±SE, $n = 3$.

considered the possibility that 8-oxo-dG might be formed, from either the direct hydrolysis of 8-oxo-dGTP or a further degradation of 8-oxo-dGMP. Consequently, the HPLC analysis of some of the MutT assays in the absence and presence of Ni(II) was modified to detect 8-oxo-dG, which under the HPLC conditions described has a retention time of approximately 27 min. However, no 8-oxo-dG production was detected. Thus, the only product of the MutT-catalyzed hydrolysis of 8-oxo-dGTP appears to be 8-oxo-dGMP, both with and without Ni(II) in the assay.

Mechanisms of Ni(II) Inhibition with dGTP as the Nucleotide Substrate. In order to conduct a biochemical analysis of the MutT kinetic data with dGTP as the nucleotide substrate, plots of initial velocity of MutT as a function of the free Mg(II) concentration with increasing concentrations of Ni(II) were fitted to the Michaelis−Menten equation (Figure 2). Examination of these plots shows that MutT was activated by Mg(II), which is consistent with a previous report (*27*). Furthermore, in the presence of Ni(II), increasing concentrations of Mg(II) did not affect the degree of inhibition (Figure 2). The kinetic parameters obtained from the Michaelis−Menten plots (Table 1) indicate that Ni(II) caused both the apparent K_m ($K_{m\ app}$) and apparent V_{max} ($V_{max\ app}$) of MutT for Mg(II) to decrease in a concentration-dependent manner, and the decrease was approximately the same for both parameters. The characteristics of the Michaelis−Menten plots (Figure 2) and the kinetic parameters determined from these plots (Table 1) are indicative of

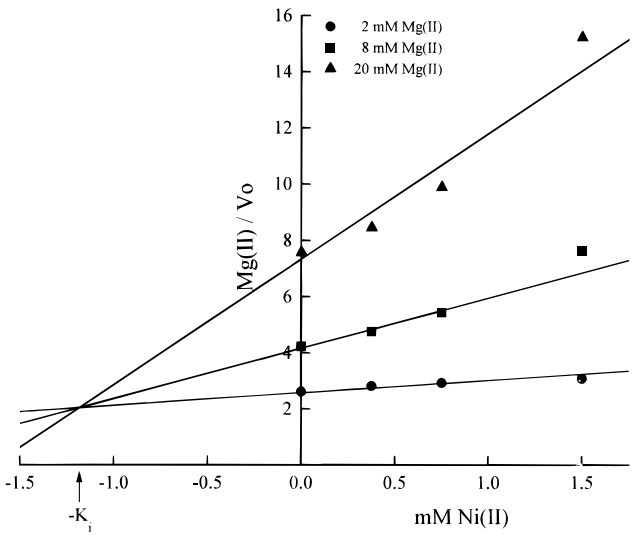

Figure 3. Cornish-Bowden replot of the MutT data with dGTP as substrate. The free Mg(II)/V_0 ratio was plotted versus the Ni(II) concentration, and the data were fitted using linear regression analysis. Mg(II) concentrations shown in the legend are free Mg(II) concentrations, calculated as described. Values are means of three determinations. The correlation coefficients (R^2) were 0.93 for 2 mM Mg(II), 0.97 for 8 mM Mg(II), and 0.95 for 20 mM Mg(II).

Table 2. Estimates of $K_{m\ app}$, $V_{max\ app}$, and $K_{i\ app}$ for MutT Data Fit to the Uncompetitive Inhibitor Model with dGTP as Substrate

error type	$K_{m\ app}$ [mM Mg(II)]	$V_{max\ app}$ [nmol of dGMP produced/(μg of protein·min)]	$K_{i\ app}$ [mM Ni(II)]
replication[a]	7.75 ± 0.45	3.78 ± 0.06	1.19 ± 0.03
model[b]	7.72 ± 4.87	3.76 ± 1.05	1.16 ± 0.78

[a] Values were determined by fitting each experiment replicate to the uncompetitive inhibitor model equation by nonlinear regression analysis as described. Values represent means ± SE, $n = 3$. [b] Values were determined by fitting the entire results of the MutT experiments to the uncompetitive inhibitor model equation by nonlinear regression analysis as described. Values represent means ± SE, $n = 3$.

uncompetitive inhibition, and thus suggest that Ni(II) is an uncompetitive inhibitor with respect to Mg(II). To demonstrate further the uncompetitive mechanism, a replot of the MutT data as described by Cornish-Bowden (*23*) was made (Figure 3). The characteristics of the Cornish-Bowden plot, with the lines intersecting above the *x*-axis in quadrant II of the graph, are consistent with those of an uncompetitive inhibitor and estimated apparent K_i ($K_{i\ app}$) of 1.2 mM Ni(II).

As further evidence of the uncompetitive inhibition mechanism, the MutT data were examined using statistical methods. Specifically, the initial velocity of MutT as a function of the free Mg(II) concentration was fitted to the equation that describes this type of inhibition by nonlinear least squares regression, and estimates of the kinetic parameters were obtained (Table 2). Examination of the estimates of $K_{m\ app}$, $V_{max\ app}$, and $K_{i\ app}$, calculated from replicate experiments (replication error type), demonstrate that the experiments were reproducible. Subsequently, the entire set of measurements was fitted to the uncompetitive model equation, and estimates of $K_{m\ app}$, $V_{max\ app}$, and $K_{i\ app}$ within the model fit (model error type) were calculated. An *F* statistic was calculated and was nonsignificant, indicating there was insufficient evidence to reject this model as a description of the data.

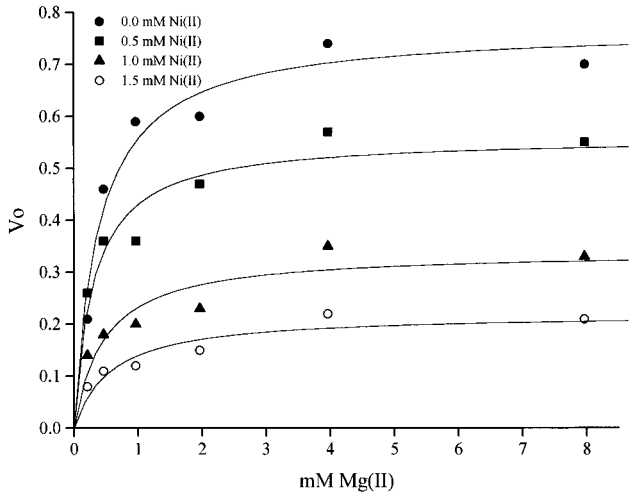

Figure 4. Mg(II) activation of MutT with 8-oxo-dGTP as substrate. MutT initial velocity, V_0 [nmol of 8-oxo-dGMP produced/(μg of protein·min)], was plotted versus the free Mg(II) concentration, and the data were fitted to the Michaelis–Menten equation using nonlinear regression analysis as described. Values are means of three experiments.

Table 3. Estimates of $K_{m\ app}$ and $V_{max\ app}$ for MutT with 8-Oxo-dGTP as Substrate[a]

mM Ni(II)	$K_{m\ app}$ [mM Mg(II)]	$V_{max\ app}$ [nmol of 8-oxo-dGMP produced/(μg of protein·min)]
0.0	0.38 ± 0.07	0.77 ± 0.03
0.5	0.30 ± 0.08	0.56 ± 0.03
1.0	0.46 ± 0.13	0.34 ± 0.02
1.5	0.57 ± 0.25	0.22 ± 0.03

[a] Kinetic parameter values were determined by fitting the MutT kinetic data to the Michaelis–Menten equation by nonlinear regression analysis as described. Values represent means ± SE, $n = 3$.

The results from both the biochemical and statistical analyses of the data indicated that Ni(II) is an uncompetitive inhibitor with respect to Mg(II) when dGTP is the nucleotide substrate for MutT. The estimates of $K_{i\ app}$ obtained from the nonlinear regression analyses of the uncompetitive model (Table 2) are in good agreement with that estimated from the biochemical analysis using the Cornish-Bowden plot (Figure 2).

Mechanisms of Ni(II) Inhibition with 8-Oxo-dGTP as the Nucleotide Substrate. Kinetic studies of MutT were also conducted using 8-oxo-dGTP as the nucleotide substrate. The biochemical analysis of these data was done by plotting the initial velocity of MutT as a function of the free Mg(II) concentration. The data were fitted to the Michaelis–Menten equation, and similarly to the results obtained for dGTP, MutT was found to be activated by Mg(II) (Figure 4). Increasing concentrations of Mg(II) did not affect the degree of inhibition by Ni(II) (Figure 4). When included in the assay, Ni(II) decreased $V_{max\ app}$ in a concentration-dependent manner, but $K_{m\ app}$ was not significantly affected (Table 3). This suggested that Ni(II) might be a **noncompetitive** inhibitor with respect to Mg(II). To obtain further evidence of the noncompetitive mechanism, the MutT data were replotted (Figure 5) as described by Cornish-Bowden (*23*). The characteristics of the Cornish-Bowden plot, with the lines intersecting essentially at the *x*-axis in quadrant II of the graph, are indicative of a noncompetitive inhibitor with an estimated $K_{i\ app}$ of approximately 0.9 mM Ni(II).

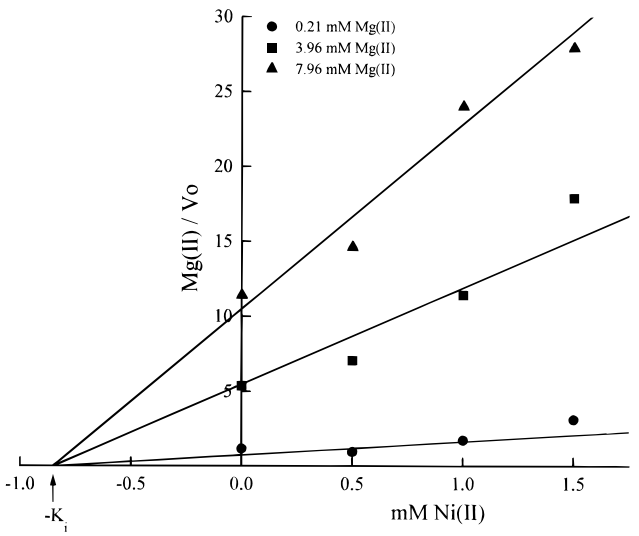

Figure 5. Cornish-Bowden replot of the MutT data with 8-oxo-dGTP as substrate. The free Mg(II)/V_0 ratio was plotted versus the Ni(II) concentration, and the data were fitted using linear regression analysis. Mg(II) concentrations shown in the legend are free Mg(II) concentrations, calculated as described. Values are means of three determinations. The correlation coefficients (R^2) were 0.77 for 0.21 mM Mg(II), 0.94 for 3.96 mM Mg(II), and 0.96 for 7.96 mM Mg(II).

Table 4. Estimates of $K_{m\ app}$, $V_{max\ app}$, and $K_{i\ app}$ for MutT Data Fit to the Noncompetitive Inhibitor Model with 8-Oxo-dGTP as Substrate

error type	$K_{m\ app}$ [mM Mg(II)]	$V_{max\ app}$ [nmol of 8-oxo-dGMP produced/(μg of protein·min)]	$K_{i\ app}$ [mM Ni(II)]
replication[a]	0.39 ± 0.03	0.79 ± 0.02	0.85 ± 0.05
model[b]	0.43 ± 0.82	0.79 ± 0.48	0.92 ± 1.26

[a] Values were determined by individually fitting each experiment replicate to the uncompetitive inhibitor model equation by nonlinear regression analysis as described. Values represent means ± SE, $n = 3$. [b] Values were determined by fitting the entire results of the MutT experiments to the uncompetitive inhibitor model equation by nonlinear regression analysis as described. Values represent means ± SE, $n = 3$.

To obtain additional support for the assignment of the noncompetitive inhibition mechanism, the MutT initial velocity estimates were examined as a function of the free Mg(II) concentration, fitted to the equation that describes noncompetitive inhibition by nonlinear least squares regression, and estimates of the kinetic parameters were obtained (Table 4). Estimates of $K_{m\ app}$, $V_{m\ app}$, and $K_{i\ app}$ determined from replicate experiments (replication error type) demonstrated that the experiments were reproducible. Next, the entire set of measurements was fitted to the noncompetitive model equation, and estimates of $K_{m\ app}$, $V_{max\ app}$, and $K_{i\ app}$ (model error type) were calculated. An F statistic was calculated and determined to be nonsignificant. Hence, there was insufficient evidence to reject this model. Consequently, the statistical analysis of the model fit indicated that the noncompetitive model was a good description of the data.

Both the biochemical and statistical analyses of the kinetic data with 8-oxo-dGTP as the nucleotide substrate determined that Ni(II) was a noncompetitive inhibitor of MutT with respect to Mg(II). In addition, the estimates of $K_{i\ app}$ obtained from the nonlinear regression analyses were in good agreement with those estimated from the Cornish-Bowden plot.

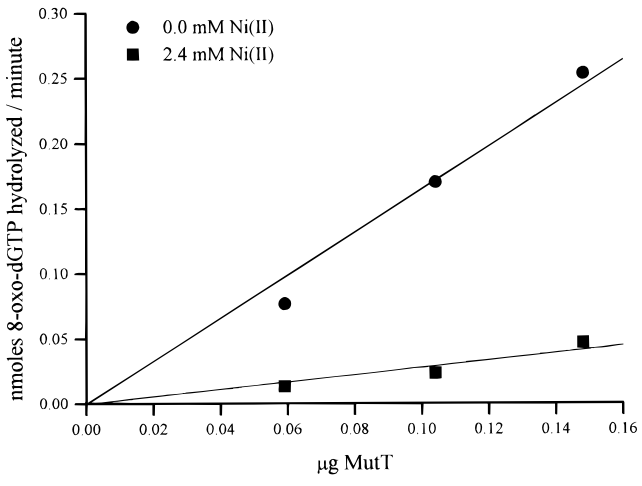

Figure 6. Demonstration of Ni(II) reversibility. MutT was assayed as described in the text, except the concentration of free Mg(II) was constant at 8 mM. The data were fitted by linear regression. Values are means of three determinations. The correlation coefficients (R^2) were 0.99 for 0 mM Ni(II) and for 0.95 for 2.4 mM Ni(II).

Inhibitors which irreversibly inactivate enzymes are sometimes incorrectly described to be noncompetitive inhibitors. A plot of μg of MutT versus nmol of 8-oxo-dGTP hydrolyzed/min was made from the results of assays with and without Ni(II) (Figure 6) in order to demonstrate that Ni(II) was a reversible noncompetitive inhibitor of MutT, and not an irreversible one. The line generated from the assays of MutT with Ni(II) had a smaller slope in comparison to the control, but both lines passed through the origin. These characteristics are consistent with those for a reversible noncompetitive inhibitor (*22*).

Discussion

For the purpose of the present study, two novel procedures were successfully developed: the synthesis of 8-oxo-dGTP, and a quick HPLC assay for separation and quantitation of dGTP, 8-oxo-dGTP, and their hydrolysis products. The first was undertaken because of very low yield of the free radical-driven synthetic procedure of 8-oxo-dGTP used by others (*6*) and the relatively large quantities of this substrate needed to complete our present and future mechanistic studies with 8-oxo-dGTPases. The HPLC assay with UV or electrochemical detection (*28*), unlike previously published colorimetric or radioisotopic assays for MutT (*6*, *26*), allowed for fast, simultaneous quantitative analysis of the substrate and more than one product (if any) of the MutT-catalyzed hydrolysis.

A previous study, which used radioisotope methods, reported that dGMP was the only product of the MutT-catalyzed hydrolysis of dGTP (*26*). In our experiments, we considered the possibility that Ni(II) might alter the hydrolysis toward dGDP and/or dG, or their respective 8-oxo analogues. However, no such products were found in the presence or absence of Ni(II). Thus, the results reported here are consistent with those previously reported for MutT.

MutT assays published previously used Tris as the buffering species (*6*, *26*) and bovine serum albumin (*6*) as a MutT carrier protein. Both Tris (*29*) and albumin (*30*) have high affinities for Ni(II) at pH 7.4, and because of their concentrations in the assay mixture would have a substantial capacity to bind Ni(II). This binding could potentially obscure interactions between Ni(II) and MutT, and thus should be minimized. Therefore, the assay of MutT in the present study employed an ammonium acetate buffer system in place of Tris and poly(vinylpyrrolidone) as a substitute for bovine serum albumin. Ammonium acetate was chosen because the ammonium ion (*31*) and acetate ion (*32*) both have weak Ni(II) binding in comparison to Tris. An examination of poly(vinylpyrrolidone) structure indicates that it does not have any apparent Ni(II) binding sites. Indeed, spectrophotometry did not reveal formation of a detectable complex species between poly(vinylpyrrolidone) and Ni(II) at pH 7.4. MutT activity was stable when diluted with poly(vinylpyrrolidone) during day-long experiments, whereas carrier-free MutT rapidly lost activity.

To assign inhibitor mechanisms for Ni(II) in these studies, two lines of independent analyses were used. First, the data were examined using biochemical analysis methods. Subsequently, using nonlinear least squares regression analysis, the data were fitted to equations which described the inhibition mechanism indicated by the biochemical analysis. The use of two independent lines of analysis, instead of relying on either one alone, provides more compelling evidence for the assignments of inhibitor mechanisms for Ni(II) in these studies.

MutT, assayed with either dGTP or 8-oxo-dGTP, was found to be activated by Mg(II), exhibiting Michaelis−Menten kinetic behavior when MutT activity was plotted versus free Mg(II) concentrations. In concordance with previous results (*6*), MutT in our assays appeared to have a much higher affinity for 8-oxo-dGTP than dGTP. Ni(II) decreased the activity of MutT with either nucleotide substrate. To determine the inhibition mechanisms, biochemical and statistical analysis methods were independently applied to the kinetic data. Both methods led to a conclusion that when MutT was assayed with dGTP, Ni(II) acted as an **uncompetitive** inhibitor with respect to Mg(II). In contrast, when MutT was assayed with 8-oxo-dGTP, both the biochemical and statistical analyses suggested that Ni(II) was a **noncompetitive** inhibitor with respect to Mg(II).

The uncompetitive and noncompetitive models predict that Ni(II) binds to MutT at a site distinct from the Mg(II) binding site and that Ni(II) binding induces conformational changes in MutT that affect substrate hydrolysis. The noncompetitive model also predicts the possibility that Ni(II) may bind very close to the active site, so that it sterically blocks the nucleotide substrate access to this site. The exact position(s) of Ni(II) binding on MutT cannot be deduced from these experiments. However, the amino acid composition of *E. coli* MutT used in these studies includes 3 histidine and no cysteine residues (*11*, *33*). Because histidine residues are known to be strong ligands for Ni(II) binding in proteins, it seems likely that they would provide binding site(s) for Ni(II) on MutT.

It is difficult to generalize the conclusions of this experiment and discuss possible relevance of the results to Ni(II) mutagenicity and carcinogenesis in mammalian cells. To our best knowledge, there is no data published on the development or lack of A→C transversion mutations in Ni(II)-treated cells. Such transversions, characteristic for 8-oxo-dGTP mutagenicity (*7*, *9*), could be associated with 8-oxo-dGTPase inhibition in Ni(II)-treated cells. The relatively high K_i values for Ni(II), found in this study, seem to preclude a significant effect

of nontoxic Ni(II) concentrations on the enzyme. However, phagocytosis and intracellular dissolution of particulate nickel compounds, essential for strong nickel carcinogenesis (*34*), may provide local Ni(II) concentrations high enough to affect enzyme activity. Also, there are 7 histidine and 1 cysteine residues in the rat 8-oxo-dGTPase (*35*) and 3 histidine and 2 cysteine residues in the human 8-oxo-dGTPase (*36*). Cysteine residues are stronger ligands for Ni(II) in comparison to histidines. Thus, both the rat and human 8-oxo-dGTPases have more and stronger putative Ni(II) (and some other transition metals) binding sites in comparison to MutT, that might suggest stronger interaction with Ni(II). Nonetheless, the questions whether or not interaction of Ni(II) with mammalian 8-oxo-dGTPases does in fact lead to their inhibition, and whether the magnitude of such inhibition allows for 8-oxo-dG incorporation into DNA and results in development of the predicted A→C transversion mutations in Ni(II)-treated animals, remain to be answered.

Acknowledgment. The authors would like to thank Dr. Maurice J. Bessman (The Johns Hopkins University, Department of Biology) for the generous gift of the MutT used in this study. We would also like to thank Dr. Wojciech Bal for his assistance in searching the IUPAC Stability Constants Database, Release 2 (IUPAC and Academic Software, 1995).

References

(1) *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*. (1990) Chromium, Nickel and Welding, IARC, Lyon.

(2) Kasprzak, K. S. (1995) Possible role of oxidative damage in metal-induced carcinogenesis. *Cancer Invest*. **13**, 411−430.

(3) Kasprzak, K. S., and Hernandez, L. (1989) Enhancement of hydroxylation and deglycosylation of 2′-deoxyguanosine by carcinogenic nickel compounds. *Cancer Res*. **49**, 5964−5968.

(4) Kasprzak, K. S., North, S. L., and Hernandez, L. (1992) Reversal by nickel(II) of inhibitory effects of some scavengers of active oxygen species upon hydroxylation of 2′-deoxyguanosine *in vitro*. *Chem.-Biol. Interact*. **84**, 11−19.

(5) Kasprzak, K. S., Diwan, B. A., Konishi, N., Misra, M., and Rice, J. M. (1990) Initiation by nickel acetate and promotion by sodium barbital of renal cortical epithelial tumors in male F344 rats. *Carcinogenesis* **11**, 647−652.

(6) Maki, H., and Sekiguchi, M. (1992) MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis. *Nature* **355**, 273−284.

(7) Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxo-dG. *Nature* **349**, 431−434.

(8) Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. (1992) 8-Hydroxyguanosine, an abundant form of oxidative DNA damage, causes G-T and A-C substitutions. *J. Biol. Chem*. **267**, 166−172.

(9) Pavlov, Y. I., Minnick, D. T., Izuta, S., and Kunkel, T. A. (1994) DNA replication fidelity with 8-oxodeoxyguanosine triphosphate. *Biochemistry* **33**, 4695−4701.

(10) Grollman, A. P., and Moriya, M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet*. **9**, 246−249.

(11) Bhatnagar, S. K., and Bessman, M. J. (1988) Studies on the mutator gene, mutT of *Escherichia coli*. *J. Biol. Chem*. **263**, 8953−8957.

(12) Yanovsky, C., Cox, E. C., and Horn, V. (1966) The unusual mutagenic specificity of an *E. coli* mutator gene. *Proc. Natl. Acad. Sci. U.S.A*. **55**, 274−281.

(13) Schaaper, R. M., and Dunn, R. L. (1987) *Escherichia coli* MutT mutator effect during *in vitro* DNA synthesis. *J. Biol. Chem*. **262**, 16267−16270.

(14) Kasprzak, K. S., and Poirier, L. A. (1985) Effects of calcium(II) and magnesium(II) on nickel(II) uptake and stimulation of thymidine incorporation into DNA in the lungs of strain A mice. *Carcinogenesis* **6**, 1819−1821.

(15) Gannett, P. M., and Sura, T. P. (1993) An improved synthesis of 8-bromo-2-deoxyguanosine. *Synth. Commun*. **23**, 1611−1615.

(16) Bodepudi, V., Shibutani, S., and Johnson, F. (1992) Synthesis of 2′-deoxy-7,8-dihydro-8-oxoguanosine and 2′-deoxy-7,8-dihydro-8-oxoadenosine and their incorporation into oligomeric DNA. *Chem. Res. Toxicol*. **5**, 608−617.

(17) Toorchen, D., and Topal, M. (1983) Mechanisms of chemical mutagenesis and carcinogenesis: effects on DNA replication of methylation at the O^6-guanine position of dGTP. *Carcinogenesis* **4**, 1591−1597.

(18) Hoard, D. E., and Ott, D. G. (1965) Conversion of mono- and oligodeoxyribonucleotides to 5-triphosphates. *J. Am. Chem. Soc*. **87**, 1785−1788.

(19) Sigel, H. (1977) Comparison of the stabilities of binary and ternary complexes of divalent metal ions with the 5′-triphosphates of adenosine, inosine, guanosine, cytidine, uridine and thymidine. *J. Inorg. Nucl. Chem*. **39**, 1903−1911.

(20) Kasai, H., and Nishimura, S. (1984) Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents. *Nucleic Acids Res*. **12**, 2137−2145.

(21) Segel, I. H. (1993) Kinetics of unireactant enzymes. In *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 18−22, John Wiley & Sons, New York.

(22) Segel, I. H. (1993) Simple inhibition systems. In *Enzyme Kinetics. Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 100−160, John Wiley & Sons, New York.

(23) Cornish-Bowden, A. (1974) A simple graphical method for determining inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem. J*. **137**, 143−144.

(24) Draper, N., and Smith, H. (1966) *Applied Regression Analysis*, John Wiley & Sons, New York, NY.

(25) Bates, D. M., and Watts, D. G. (1988) *Nonlinear Regression Analysis & Its Applications*, John Wiley & Sons, New York, NY.

(26) Bhatnagar, S. K., Bullions, L. C., and Bessman, M. J. (1991) Characterization of the mutT nucleoside triphosphatase of *Escherichia coli*. *J. Biol. Chem*. **266**, 9050−9054.

(27) Frick, D. N., Weber, D. J., Gillespie, J. R., Bessman, M. J., and Mildvan, A. S. (1994) Dual divalent cation requirement of the MutT dGTPase. Kinetic and magnetic resonance studies of the metal and substrate complexes. *J. Biol. Chem*. **269**, 1794−1803.

(28) Floyd, R. A., Watson, J. J., Wong, P. K., Altmiller, D. H., and Rickard, R. C. (1986) Hydroxyl free radical adduct of deoxyguanosine: sensitive detection and mechanism of formation. *Free. Radical Res. Commun*. **1**, 163−172.

(29) Fischer, B. E., Haring, U. K., Tribolet, R., and Sigel, H. (1979) Metal ion/buffer interactions. Stability of binary and ternary complexes containing 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and adenosine-5′-triphosphate (ATP). *Eur. J. Biochem*. **94**, 523−530.

(30) Bal, W., Christodoulou, J., Sadler, P. J., and Tucker, A. (1990) Multi-metal binding site on serum albumin. In *Metal Ions in Biology and Medicine* (Collery, P., Poirier, L. A., Littlefield, N. A., and Etienne, J.-C., Eds.) Vol. 3, pp 43−45, John Libbey Eurotext, Montrouge, France.

(31) Nagypal, I., Gergely, A., and Jekel, P. (1969) Extension of the concept of the average number of ligands. A new method for the calculation of stability constants for any maximum number of ligands. *J. Inorg. Nucl. Chem*. **31**, 3447−3457.

(32) Banerjea, D., Kaden, T. A., and Sigel, H. (1981) Enhanced stability of ternary complexes in solution through the participation of heteroaromatic N bases. Comparison of the coordination tendency of pyridine, imidazole, ammonia, acetate, and hydrogen phosphate toward metal ion nitrilotriacetate complexes. *Inorg. Chem*. **20**, 2586.

(33) Akiyama, M., Horiuchi, T., and Sekiguchi, M. (1987) Molecular cloning and nucleotide sequence of the mutT mutator of *Escherichia coli* that causes A:T to C:G transversion. *Mol. Gen. Genet*. **206**, 9−16.

(34) Costa, M. (1995) Model for the epigenetic mechanism of action of nongenotoxic carcinogens. *Am. J. Clin. Nutr*. **61** (Suppl), 666S−669S.

(35) Cai, J.-P., Kakuma, T., Tsuzuki, T., and Sekiguchi, M. (1995) cDNA and genomic sequences for rat 8-oxo-dGTPase that prevents occurrence of spontaneous mutations due to oxidation of guanine nucleotides. *Carcinogenesis* **16**, 2343−2350.

(36) Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) Cloning and expression of cDNA for a human enzyme that hydrolyzes 8-oxo-dGTP, a mutagenic substrate for DNA synthesis. *J. Biol. Chem*. **268**, 23524−23530.

TX9600816