homogenate centrifuged at 1000*g* for 15 minutes. The supernatant with an effective concentration of 0.1 corpora cardiaca equivalent per microliter was divided into 200-μl portions and frozen at −20°C until needed. Abdomens were injected near the anterior midline with 0.7 corpora cardiaca equivalent per gram of live weight.

13. At appropriate times, the abdomens were opened along the dorsal midline, the gut removed, and the remainder pinned out under ice-cold Ringer solution [R. G. Weevers, *J. Exp. Biol.* **44**, 163 (1966)]. The nerve cords were rapidly dissected free of contaminating fat body tissue and frozen on Dry Ice. The dissection time ranged from 1.5 to 2 minutes. Extracts were prepared with internal standards of ^{3}H-labeled cyclic nucleotides to monitor recovery. Cyclic AMP and cyclic GMP were separated chromatographically and measured by binding protein assays as previously described [A. M. Fallon and G. R. Wyatt, *Anal. Biochem.* **63**, 614 (1975); B. L. Brown, R. P. Ekins, J. D. M. Albano, *Adv. Cyclic Nucleotide Res.* **2**, 25 (1972)]. The average recovery of cyclic AMP and cyclic GMP was 89 percent and 70 percent, respectively. Tritiated cyclic AMP (22.1 c/mmole) was from New England Nuclear, and the tritiated cyclic GMP (21 c/mmole) was from Amersham/Searle.
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Infidelity of DNA Synthesis in vitro: Screening for Potential Metal Mutagens or Carcinogens

Abstract. *Thirty-one metal salts have been tested for their ability to affect the accuracy of DNA synthesis in vitro. All ten salts of metal carcinogens decreased the fidelity of DNA synthesis. Of the three metals which beforehand were considered to be possible mutagens or carcinogens, only one decreased fidelity. In contrast, 17 noncarcinogenic metal salts did not affect fidelity even when present at concentrations that were clearly inhibitory.*

The role of environmental agents in causing human malignancy (*1*) suggests the possibility that their identification and subsequent elimination from the environment could reduce the incidence of cancer in the general population. In the past, animal studies have generally been used to identify environmental carcinogens. Unfortunately, these studies require several years to complete and may require inordinate capital expenditures to test the plethora of new chemicals introduced annually into the environment. Thus, attention has focused on the development of assays in vitro to screen initially for potential carcinogens. These tests offer the possibility of rapidly and inexpensively examining a multitude of chemicals to select those to be evaluated further in animal systems. As there is a close correlation between carcinogenicity and mutagenicity, most assays in vitro examine the ability of exogenous agents to act as mutagens (*2*). For example, Ames and his colleagues (*2*) have developed tester strains of *S. typhimurium* in which suspected agents are evaluated by their ability to revert previously induced mutations. Over 300 compounds have been studied, and the correlation of mutagenicity in their system with reported carcinogenicity is about 90 percent. However, this test has not been applied for screening metal compounds for mutagenicity, although it and other systems may be potentially adaptable (*3*). Because many metals have been identified as carcinogens (*4*), through occupational exposure as well as in the laboratory, it may be of importance to develop a test system to screen for these carcinogens. We now report an assay in vitro which may be used to screen for potential mutagenic or carcinogenic metal compounds. All of the known metal mutagens or carcinogens (*5*) which have been tested could be identified through this analysis. In addition, three metals were analyzed that beforehand were assigned as possible mutagens (*6*) or carcinogens (*7*); one was found to be positive in this assay. None of the noncarcinogenic metals tested were identified as potential mutagens or carcinogens.

For this analysis we have measured perturbations in the fidelity of DNA synthesis in vitro caused by soluble metal salts. Metals which increased base substitution in this assay in vitro were designated as suspected mutagens or carcinogens in vivo. Each reaction mixture contained a DNA polymerase, a template-primer of restricted base composition, Mg^{2+}, and complementary and noncomplementary deoxynucleoside triphosphates each labeled with different radioactive isotopes. One of the complementary deoxynucleotides was labeled with α-^{32}P of low specific activity (approximately 5 to 20 dpm/pmole), while the noncomplementary deoxynucleotide was labeled with ^{3}H of high specific activity (approximately 10,000 to 50,000 dpm/pmole). From the ratio of radioactive substrates incorporated, we calculated the error frequency, that is, the ratio of noncomplementary to complementary deoxynucleotide incorporation. For these initial studies, we chose to use the DNA polymerase (E.C. 2.7.7.7) from avian myeloblastosis virus (AMV), since this polymerase is well characterized (*8*), incorporates noncomplementary deoxynucleotides as single base substitutions (*9*) and, in common with all purified eukaryotic DNA polymerases, lacks any associated exodeoxynuclease activity which might excise noncomplementary bases after incorporation (*10*). The propensity of this DNA polymerase to make mistakes increased the sensitivity of the assay and emphasized further the inability of noncarcinogenic metal compounds to affect fidelity.

The effect of each metal compound on the fidelity of DNA synthesis was determined at multiple concentrations between 20 μM and 150 mM as limited by solubility and by the effect of the metal salt on DNA synthesis. Compounds which increased the error frequency by greater than 30 percent at two or more concentrations were scored as positive. Each experiment contained reaction mixtures without additional metal salts as well as reaction mixtures with $CoCl_2$ (4 mM) as a positive control. This cobalt concentration increases the error frequency by at least 50 percent (*11*).

In this study, 31 compounds were tested (Table 1); 22 compounds were tested in triple-blind experiments. The assays, computations, and the designation of the unknown compounds with respect to fidelity were carried out by three separate individuals, each unaware of the compounds to be analyzed. All metal salts assayed in the triple-blind experiments were designated as carcinogens, possible carcinogens, or noncarcinogens prior to the analysis. Of the total metal com-

pounds tested, 12 were scored as positive (decreased fidelity by at least 30 percent). Eight of the metals in these compounds have been shown to be mutagenic or carcinogenic (*5*). This analysis detected Ag, Be, Cd, Co, Cr, Mn, Ni, and Pb (*12*). The evidence for the mutagenicity or carcinogenicity for three metals was considered to be marginal (*6, 7*). Of these metals, Cu^{2+} was positive, while two of the metals, Fe^{2+} and Zn^{2+}, were negative. The concentration of the compounds required to increase the error frequency by greater than 30 percent varied: $AgNO_3$, 30 μM; $Cd(C_2H_3O_2)_2$, 240 μM; $CrCl_2$, 640 μM; and $CoCl_2$, 4 mM. The hypothesis that alterations in fidelity and mutagenicity or carcinogenicity are independent of each other was examined by the Fisher exact test. With the assumption that the marginal results were opposite in sign, the worst possible situation, this hypothesis may be rejected at a $P = 1.6 \times 10^{-4}$ (*13*).

That the metal cation itself was responsible for the increase in base substitution in vitro was demonstrated by comparing different cations with similar anions. With poly(A) · oligo(dT) being used as the template-primer (where A is adenosine and dT is deoxythymidine), $BeCl_2$ (10 mM) increased the error frequency from 1/1128 to 1/75, while $SrCl_2$ (10 mM) did not alter the error frequency. With poly[d(A-T)] being used as the template-primer, cadmium acetate (0.24 mM) increased the error frequency from 1/1828 to 1/825, while potassium acetate (100 mM) did not affect fidelity.

All of the compounds scored as positive diminished and ultimately eliminated synthesis. To demonstrate that the increased error frequencies were not due to diminished synthesis, per se, the metal compounds were grouped into three classes. Representative metal salts from each class are shown in Table 2. Class 1 contained these compounds which increased the error frequency and decreased synthesis (scored as positive); class 2 contained those compounds which did not affect fidelity but nevertheless did decrease synthesis (negative); class 3 contained those compounds which did not affect fidelity and did not appreciably affect synthesis (negative). As is shown in Table 1, compounds which were scored as negative could be assigned to either class 2 or class 3. Of the 19 compounds which did not alter fidelity, 15 were designated as class 2 compounds. Therefore, reduction in synthesis by itself does not appear to be the cause of the observed decrease in fidelity. Furthermore, previous results indicated that denaturation of AMV DNA

Table 1. The effect of metal salts on the fidelity of DNA synthesis in vitro. Each of the metal salts was tested at a minimum of seven different concentrations. The change in error frequency at the highest metal concentration used is shown. Compounds were scored as positive if, during this titration, the error frequency was increased by greater than 30 percent at two or more concentrations as compared to simultaneous reactions containing only Mg^{2+} as the metal cation. Compounds were scored as negative if they did not fulfill these criteria. The fidelity of DNA synthesis when poly[d(A-T)] was used as the template was measured in a reaction mixture (total volume 0.05 ml) which contained 100 mM tris-maleate (pH 7.6); 60 mM KCl; 5 mM $MgCl_2$; 50 μM dATP; 50 μM [α-^{32}P]dTTP (5 to 20 dpm/pmole); 50 μM [^{3}H]dGTP or [^{3}H]dCTP (10,000 to 50,000 dpm/pmole); 1 μg of poly[d(A-T)]; and 0.5 μg of AMV DNA polymerase (*17*). For reactions which used poly(C) · oligo(dG) as the template, the incubation mixtures were altered to include 3 mM $MgCl_2$; 10 mM KCl; 20 μM [α-^{32}P]dGTP (5 to 20 dpm/pmole); and 20 μM [^{3}H]dATP (10,000 to 50,000 dpm/pmole). For reactions which used poly (A) · oligo(dT) as the template, the incubation mixtures were altered to include 50 mM tris-HCl (pH 8.0); 5 mM $MgCl_2$; 20 mM KCl; 5 mM dithiothreitol; 2 μg of bovine serum albumin; 26 μM [α-^{32}P]dTTP (12 dpm/pmole); and 23 μM [^{3}H]dCTP (50,000 dpm/pmole). The purity of the labeled deoxynucleoside triphosphates was determined by chromatography and by measuring their relative effectiveness as substrates for sea urchin nuclear DNA polymerase. All reaction mixtures were incubated for 60 minutes at 37°C. Incorporation of the radioactive deoxynucleotides into an acid-insoluble precipitate was determined after precipitating the polynucleotide product with 1.0N perchloric acid and solubilizing with 0.2M NaOH three times and the collecting the acid-insoluble material on filter paper (*9*). All assays were performed in duplicate and the averages determined. Error frequencies were calculated only when the incorporation of the radioactive deoxynucleotides was at least two times the zero time controls. The maximum change in error frequency was calculated by dividing the highest error frequency during the titration by the error frequency determined without the added metal within the same experiment. The metal concentration in parenthesis is that which yielded the largest observed change in error rate. All metal salts assayed in the triple-blind experiments were designated as carcinogens, marginal carcinogens, or noncarcinogenic prior to their identification. Control experiments were performed to eliminate the possibility that increased misincorporation was due simply to a preferential precipitation of noncomplementary deoxynucleotides. The incorporation of the noncomplementary nucleotide without incubation was determined for each metal designated as positive at the highest concentration used: no metal added, 137 count/min; Ag (0.03 mM), 131; Be (10 mM), 143; Cd (0.04 mM), 127; Co (4 mM), 145; Cr (0.64 mM), 116; Cu (0.12 mM), 140; Mn (10 mM), 127; Ni (8 mM), 113; Pb (4 mM), 145. The noncomplementary deoxynucleotide used was dGTP with poly[d(A-T)] as the template. Furthermore, nearest neighbor anaylsis of metal-induced infidelity with Be (*14*), Co and Mn (*18*) has demonstrated that noncomplementary deoxynucleotides were incorporated in phosphodiester linkage as single base substitutions.

Compound	Class	Template	Δ Error frequency (metal concentration, mM)	Decreased fidelity	Carcinogenic or mutagenic
*$Al_2(SO_4)_3$	2	[d(A-T)]	0.99 (10)	−	−
*$AgNO_3$	1	[d(A-T)]	1.85 (0.03)	+	+
*$Ba(C_2H_3O_2)_2$	2	[d(A-T)]	0.94 (10)	−	−
$BaCl_2$	2	poly(A)	1.17 (10)	−	−
$BeCl_2$	1	poly(A)	15 (10)	+	+
$CaCl_2$	2	poly(A)	1.28 (5)	−	−
*$Cd(C_2H_3O_2)_2$	1	poly(C)	2.22 (0.24)	+	+
*$CdCl_2$	1	poly(C)	1.35 (0.04)	+	+
$CoCl_2$	1	poly(C)	8.37 (4)	+	+
*$CrCl_2$	1	[d(A-T)]	3.70 (0.64)	+	+
*CrO_3	1	[d(A-T)]	3.83 (16)	+	+
*$Cu(C_2H_3O_2)_2$	1	poly(C)	1.83 (0.12)	+	±
*$CuCl_2$	1	poly(C)	2.90 (0.08)	+	±
*$FeCl_2$	2	poly(C)	1.14 (4)	−	±
*$KC_2H_2O_2$	3	poly(C)	0.98 (100)	−	−
*KCl	2	[d(A-T)]	1.11 (150)	−	−
*K_2HPO_4	2	[d(A-T)]	0.74 (40)	−	−
*KH_2PO_4	2	[d(A-T)]	1.05 (80)	−	−
*$MgSO_4$	3	poly(C)	1.11 (10)	−	−
$MnCl_2$	1	poly(C)	3.75 (10)	+	+
*$NaC_2H_3O_2$	2	poly(C)	1.00 (100)	−	−
*NaCl	2	[d(A-T)]	0.81 (120)	−	−
*$NaHCO_3$	2	[d(A-T)]	0.96 (40)	−	−
*NaOH	3	[d(A-T)]	1.17 (10)	−	−
*NH_4COOH	2	[d(A-T)]	0.83 (100)	−	−
$(NH_4)_2SO_4$	2	poly(C)	0.94 (120)	−	−
$NiCl_2$	1	poly(C)	1.92 (8)	+	+
*$PbCl_2$	1	poly(C)	1.48 (4)	+	+
*$RbCl_2$	2	[d(A-T)]	0.90 (20)	−	−
$SrCl_2$	3	poly (A)	0.86 (10)	−	−
*$ZnCl_2$	2	[d(A-T)]	1.06 (0.4)	−	±

*Compounds tested in triple-blind experiments.

Table 2. Characterization of compounds added to fidelity reaction. All reactions were performed as described in Table 1. Error frequencies were calculated as single base substitutions. The noncomplementary deoxynucleotide was dCTP with poly[d(A-T)] as the template; dATP was the noncomplementary deoxynucleotide with poly(C).

Metal	Concentration (m*M*)	Template	Complementary deoxynucleotide incorporation (pmole)	Noncomplementary deoxynucleotide incorporation (pmole)	Error frequency
		Class 1 metal compounds			
	0	Poly[d(A-T)]	192	0.27	1/711
$CrCl_2$	0.08	Poly[d(A-T)]	133	0.23	1/578
$CrCl_2$	0.16	Poly[d(A-T)]	169	0.32	1/528
$CrCl_2$	0.24	Poly[d(A-T)]	127	0.35	1/363
$CrCl_2$	0.64	Poly[d(A-T)]	71	0.37	1/192
	0	Poly(C)	565	0.68	1/831
$CuCl_2$	0.02	Poly(C)	385	0.44	1/875
$CuCl_2$	0.04	Poly(C)	130	0.18	1/722
$CuCl_2$	0.08	Poly(C)	54	0.10	1/540
$CuCl_2$	0.12	Poly(C)	36	0.08	1/450
		Class 2 metal compounds			
	0	Poly[d(A-T)]	225	0.23	1/978
$Ca(C_2H_3O_2)_2$	0.8	Poly[d(A-T)]	217	0.22	1/986
$Ca(C_2H_3O_2)_2$	2.4	Poly[d(A-T)]	78	0.08	1/975
$Ca(C_2H_3O_2)_2$	4.0	Poly[d(A-T)]	39	0.04	1/975
$Ca(C_2H_3O_2)_2$	4.8	Poly[d(A-T)]	17	0.02	1/850
	0	Poly[d(A-T)]	192	0.27	1/711
K_2HPO_4	8	Poly[d(A-T)]	179	0.22	1/814
K_2HPO_4	16	Poly[d(A-T)]	180	0.23	1/783
K_2HPO_4	32	Poly[d(A-T)]	107	0.13	1/823
K_2HPO_4	40	Poly[d(A-T)]	48	0.05	1/960
		Class 3 metal compounds			
	0	Poly(C)	480	0.65	1/738
$KC_2H_3O_2$	4	Poly(C)	484	0.66	1/733
$KC_2H_3O_2$	20	Poly(C)	423	0.56	1/755
$KC_2H_3O_2$	40	Poly(C)	378	0.51	1/741
$KC_2H_3O_2$	80	Poly(C)	379	0.51	1/743
	0	Poly(C)	303	0.44	1/689
$MgSO_4$	4	Poly(C)	299	0.50	1/598
$MgSO_4$	6	Poly(C)	398	0.63	1/632
$MgSO_4$	8	Poly(C)	349	0.55	1/635
$MgSO_4$	10	Poly(C)	360	0.58	1/621

polymerase by urea (4*M*), or by ethanol (20 percent) drastically reduced synthesis, but did not appreciably affect fidelity (*14*). These cumulative results suggest that few factors can alter the fidelity of DNA replication, and they underscore the importance of those chemicals which do alter fidelity. The correlation between alterations in fidelity in vitro and mutagenicity and carcinogenicity in vivo are in accord with suggestions that infidelity during polymerization may cause mutations (*15*). Furthermore, this correlation suggests that eukaryotes may be unable to excise noncomplementary deoxynucleotides after incorporation into DNA.

We have presented evidence that alterations in the fidelity of DNA synthesis can be used to screen metal compounds for their suspected mutagenicity or carcinogenicity. This system can serve as a complement to preexisting assays in vitro, so that additional classes of carcinogens can now be detected. Many chemical carcinogens require metabolism by host enzymes for conversion to their active forms (*16*). The use of a microsomal enzyme preparation (*2*) coupled with this system may provide a supplementary test to screen many types of carcinogens.

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