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Effect of Several Metal Compounds on the Mutagenicity and Deoxyribonucleic Acid Binding of 1-Nitropyrene in *Salmonella typhimurium* TA100

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The pretreatment of the cells with cobaltous acetate, potassium dichromate or zinc chloride was found to increase reverse mutations induced by 1-nitropyrene (1-NP) and binding of 1-NP to deoxyribonucleic acid (DNA) in *Salmonella typhimurium* TA100. The increase of binding of 1-NP to DNA by metal compounds may contribute to the increased mutagenicity. Under the experimental conditions, the pretreatment of the cells with cadmium acetate, nickelous acetate or chromium(III) nitrate did not increase revertant colonies induced by 1-NP.

Keywords——1-nitropyrene; zinc; chromium; cobalt; DNA binding; mutagenicity; *Salmonella typhimurium* TA100; nitroreductase activity

Introduction

There are few reports on modifications of the activities of mutagens by metal ions. In *Salmonella typhimurium* it has been reported that cupric ions enhance reverse mutations induced by ascorbic acid,[1] cupric and ferric ions enhance the inhibition by ascorbate of reverse mutations induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG),[2] cadmium chloride shows a synergistic effect on the mutagenicity of methylnitrosourea or MNNG,[3] and cobaltous chloride works as a potent antimutagen on the reverse mutations induced by 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-1).[4] Recently, mutagenic activity of the mixture of 9-aminoacridine and cobaltous chloride has been found to be much higher than that of 9-aminoacridine alone.[5] In the previous report,[6] we showed the enhancement by zinc acetate of deoxyribonucleic acid (DNA) binding and mutagenicity of 1-nitropyrene (1-NP) which is a nitropolyaromatic hydrocarbon found in diesel exhaust[7] and coal combustion fly ash.[8] The effect of metal ions on the binding to nucleic acids and proteins of nitroaromatic compounds which are mutagenic or carcinogenic has not been studied adequately.

In this report, we deal with the effect of several metal compounds including zinc chloride on the mutagenicity and DNA binding of 1-NP in *S. typhimurium* TA100.

Experimental

Compounds——1-NP (Tokyo Kasei Kogyo, Ltd.) was purified by high performance liquid chromatography (HPLC) using a column of Zorbax-ODS. This compound was tritiated by TR1 (tritium labelling services of Amersham International Ltd., Buckinghamshire). ^{3}H-1-NP was purified before use as described above. The specific activity was 388 mCi/mmol.

Bacterial Strain——*Salmonella typhimurium* TA100 used in the experiments was supplied by Dr. B. N. Ames (University of California, Berkeley).

Mutagenicity Test——The liquid preincubation method,[10] a modification of the test described by Ames *et al.*,[11] was used. As described in the previous report,[6] mixtures of overnight culture (0.1 ml; approximately 5×10^7 cells) and

metal ion in 0.2 M *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES)–NaOH buffer (0.5 ml; pH 7.4) were preincubated for 30 min at 37 °C. After the pretreatment, 1-NP in dimethyl sulfoxide (0.1 ml) was added to the mixtures, and incubation was continued for an additional 30 min at 37 °C. After addition of 2 ml of soft agar (0.8% Difco agar supplemented with 0.1 μmol of L-histidine and 0.1 μmol of D-biotin in 0.6% sodium chloride), the mixtures were poured onto a minimal glucose agar plate with Vogel-Bonner E medium and incubated at 37 °C in the dark for 48 h. The colonies were counted as revertant colonies. Mixtures prepared separately in another tube under the same conditions as those for counting revertant colonies were preincubated, then diluted 10^{-5} times, and 1-NP in dimethyl sulfoxide and metal ion in the buffer were added to the diluted mixture to give the same concentrations in the mixtures as those before dilution. After addition of 2 ml of soft agar, the adjusted mixtures were poured onto a minimal agar plate supplemented with 0.5 mM L-histidine. After incubation at 37 °C in the dark for 48 h, the colonies were counted as survival colonies.

Determination of Incorporation of ^{3}H-1-NP into the Cells——The cells for each sample were collected from 20 ml of overnight culture (containing approximately 5×10^8 cells per ml) by centrifugation at 10000 *g* for 10 min, suspended in 6 ml of 0.2 M BES–NaOH buffer (pH 7.4) containing metal compounds, and then incubated at 37 °C for 30 min. The mixtures after addition of 6.2×10^{-2} μCi of ^{3}H-1-NP (dissolved in 100 μl of methyl alcohol; sp. act., 7.7 mCi/mmol) were incubated for an additional 30 min at 37 °C. After this incubation, the mixtures were centrifuged at 3000 rpm for 15 min and the collected cells were washed with 10 ml of redistilled water, ultrasonically lysed, and centrifuged at 5000 rpm for 5 min to remove intact cells and debris. The supernatant was then centrifuged for 60 min at 105000 *g*. The radioactivity in the pellet and the supernatant obtained by centrifugation at 105000 *g* was measured in a Triton-toluene scintillator by using a liquid scintillation counter (Beckman LSC 5800).

Determination of Incorporation of Metal Ions into the Cells——The cells for each sample were collected from 100 ml of overnight culture by centrifugation at 10000 *g* for 10 min, suspended in 30 ml of 0.2 M BES–NaOH buffer (pH 7.4) containing metal compounds, and then incubated at 37 °C for 30 min. After incubation, the mixtures were centrifuged at 3000 rpm for 15 min and the collected cells were washed with 5 ml of redistilled water three times. The cells were suspended in 5 ml of redistilled water, ultrasonically lysed and centrifuged at 5000 rpm for 5 min to remove intact cells and debris. The supernatant was then centrifuged for 60 min at 105000 *g*. Both the pellet and the supernatant prepared by centrifugation at 105000 *g* for 60 min were used for the determination of metal concentration.

The samples were decomposed by the wet ashing method using sulfuric and nitric acids for samples containing zinc and using nitric acid and hydrogen peroxide for samples containing cobalt or chromium. The concentration of metal was determined by atomic absorption spectrophotometry[12] as follows. After decomposition of organic materials, the acidic sample solution was diluted to an appropriate volume with redistilled water and was analyzed by atomic absorption spectrophotometry (Hitachi 170-50).

Determination of ^{3}H-1-NP Reductase Activity——The cells for each sample were collected from 300 ml of overnight culture by centrifugation at 10000 *g* for 10 min, washed with 0.2 M BES–NaOH buffer (pH 7.4) containing a metal compound and incubated at 37 °C for 30 min. After incubation, the mixtures were centrifuged at 3000 rpm for 15 min and the collected cells were washed with the buffer three times. The cells suspended in 10 ml of the buffer were ultrasonically lysed, the broken cell suspension was centrifuged at 105000 *g* for 60 min, and the supernatant was used for the determination of 1-NP nitroreduction activity. The determination of activity was carried out according to the method of Lu *et al.*[13] as follows. The assay mixtures contained 1-NP (8 μM), reduced nicotinamide adenine dinucleotide (NADH) (36 μM), glucose-6-phosphate (5.16 mM), and G-6-P dehydrogenase (1 unit) in a total volume of 2.5 ml of the buffer plus 200 μl of enzyme preparation. The mixtures were gassed for 5 min with nitrogen and incubated for 60 min at 37 °C. The amount of 1-aminopyrene produced in the mixtures was measured by fluorometry (Hitachi MPF-4 spectrophotometer; excitation 390 nm, emission 445 nm).

Determination of Protein——The amount of protein was estimated by the method of Lowry *et al.*[14] using bovine serum as the reference standard.

Determination of ^{3}H-1-NP Binding to DNA in the Cells——The cells for each sample were collected from 500 ml of overnight culture by centrifugation at 10000 *g* for 10 min, suspended in 1500 ml of 0.2 M BES–NaOH buffer (pH 7.4) containing a metal compound (0—0.2 mM) and then incubated for 30 min at 37 °C. The mixtures after addition of 4 μmol of ^{3}H-1-NP (dissolved in 1.6 ml of methyl alcohol; 388 or 342 mCi/mmol) were incubated again for an additional 30 min at 37 °C. After incubation, the mixtures were centrifuged at 3000 rpm for 15 min and the collected cells were washed with the BES buffer three times. The cells were suspended in 25 ml of 0.15 M NaCl–0.01 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and the isolation of DNA was carried out according to the method of Marmur[15] as follows. The suspension was lysed with 2% sodium dodecye sulfate (SDS) at 60 °C for 10 min. After cooling of the mixture, 5 M $NaClO_4$ solution was added to give a 1 M final concentration. The same volume of $CHCl_3$–isoamyl alcohol (24 : 1) was added, and the mixture was shaken for 45 min, and centrifuged at 10000 *g* for 5 min. The supernatant containing nucleic acids was separated and mixed with 2 volumes of ethyl alcohol to precipitate nucleic acids. The nucleic acids were redissolved in 9 ml of a solution containing 0.015 M sodium chloride and 0.0015 M sodium citrate, mixed with the same volume of $CHCl_3$–isoamyl alcohol (24 : 1) for deproteinization, shaken for 20 min and then centrifuged at 10000 *g* for 5 min. This deproteinization was carried out three times. The DNA was further purified by ribonuclease (RNase) treatment (50 μg/ml for 30 min), deproteinized with $CHCl_3$–isoamyl alcohol

(24:1) and then precipitated with ethyl alcohol. The precipitate was redissolved in 9 ml of a solution containing 0.015 M sodium chloride and 0.0015 M sodium citrate, mixed with 1 ml of 3 M sodium acetate–0.001 M EDTA (pH 7.0) to remove polysaccharides, and then mixed with 5.4 ml of isopropyl alcohol to precipitate DNA. The purified DNA was redissolved in an appropriate volume of the solution containing 0.015 M sodium chloride and 0.0015 M sodium citrate and used for the determination of radioactivity. The amount of DNA was determined by the method of Burton.[16]

Gel Filtration of DNA Adducts on Sephadex G-25——DNA adducts formed by ^{3}H-1-NP in the cells were separated by the method of Marmur,[15] redissolved in 1 M NaCl solution and applied to a Sephadex G-25 column (0.8 × 5 cm). The samples were eluted with water.

Results

Effect of Metal Compounds on the Mutagenicity of 1-NP

The number of revertant colonies induced by 1-NP (2 μM) was increased approximately 2-fold by the pretreatment of the cells with 0.1 mM potassium dichromate, cobaltous acetate or zinc chloride in the preincubation mixtures, under the experimental conditions employed, as shown in the left panel of Fig. 1. The extent of mutagenic activity increase caused by zinc chloride was similar to that by zinc acetate. The surviving colonies under the same conditions decreased to approximately 85% on addition of zinc chloride, slightly on addition of potassium dichromate and to approximately 45% on addition of 0.2 mM cobaltous acetate. As shown in the left panel of Fig. 2, the pretreatment of the cells with some other metal compounds such as chromium (III) nitrate, nickelous acetate and cadmium acetate produced no significant increase of the revertant colonies induced by 1-NP, while the surviving colonies under the same conditions decreased slightly on addition of chromium (III) nitrate or nickelous acetate, and markedly (to approximately 54%) on addition of 0.2 mM cadmium acetate.

As shown in the right panel of Figs. 1 and 2, the numbers of revertant colonies were

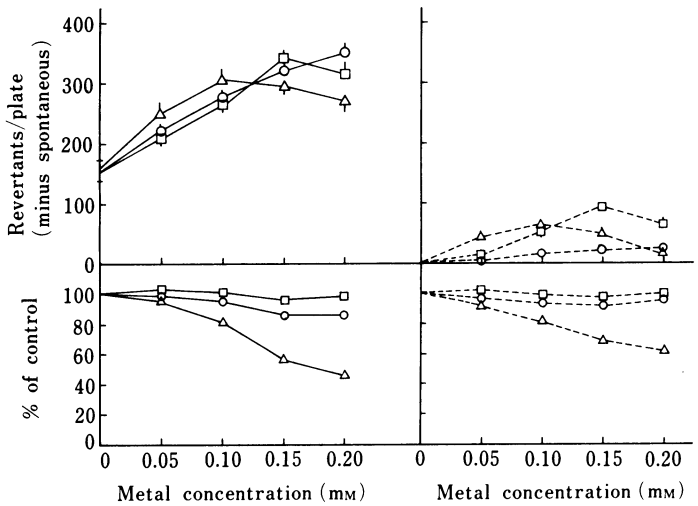

Fig. 1. Effect of Potassium Dichromate, Cobaltous Acetate and Zinc Chloride on the Mutagenicity of 1-NP in *S. typhimurium* TA100

1-NP (2 μM) was added to the preincubation mixtures. The number of spontaneous his^{+} revertant colonies was 140 ± 4.3 and the number of his^{-} surviving colonies corresponding to 100% survival was 597 ± 18.5. The experiments were reported two additional times with the same results. The left panel (——) shows the mutagenicities on treatment with each metal and 1-NP, and each point is the mean ± S.D. of 4—6 plates in one experiment. The right panel (- - - -) shows the mutagenicities on treatment with each metal alone, and each point is the mean ± S.D. of 3—5 plates in one experiment. The symbols are as follows: potassium dichromate (□); cobaltous acetate (△); zinc chloride (○).

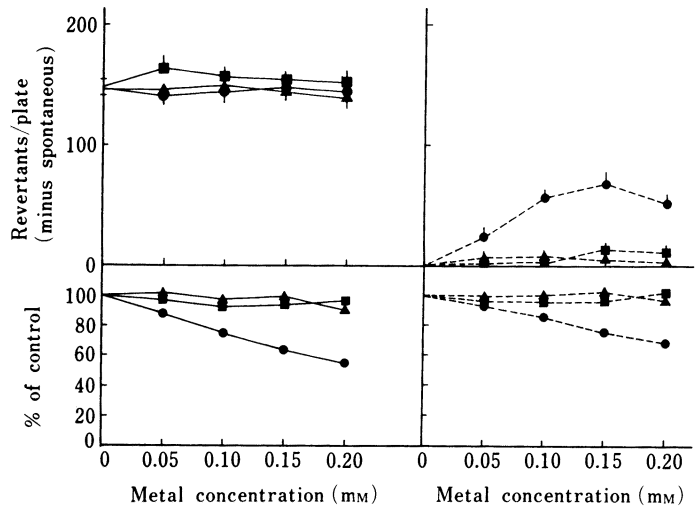

Fig. 2. Effect of Chromium(III) Nitrate, Nickelous Acetate and Cadmium Acetate on the Mutagenicity of 1-NP in *S. typhimurium* TA100

1-NP (2 μM) was added to the preincubation mixtures. The number of spontaneous his^+ revertant colonies was 152 ± 6.2 and the number of his^- surviving colonies corresponding to 100% survival was 691 ± 26.4. The experiments were repeated two additional times with the same results. The left panel (——) shows the mutagenicities on treatment with each metal and 1-NP, and each point is the mean ± S.D. of 4—6 plates in one experiment. The right panel (– – – –) shows the mutagenicities on treatment with each metal alone, and each point is the mean ± S.D. of 3—5 plates in one experiment. The symbols are as follows: chromium(III) nitrate (■); nickelous acetate (▲); cadmium acetate (●).

increased by potassium dichromate, cobaltous acetate, cadmium acetate or zinc chloride alone among the metal compounds used in the experiments, although none of them caused a more than 2-fold increase of spontaneous revertant colonies under the experimental conditions tested.

The revertant colonies induced by 1-NP were not changed by addition of magnesium acetate. Furthermore, the combined effect of magnesium acetate and zinc chloride on the mutagenicity of 1-NP was examined. The addition of magnesium had no effect on the increase of mutagenic activity of 1-NP by zinc.

Effect of Potassium Dichromate, Cobaltous Acetate and Zinc Chloride on the Incorporation of ^{3}H-1-NP into the Cells

The increased incorporation of ^{3}H-1-NP into the cells may lead to the increase of the mutagenicity and the DNA binding. Therefore, the effect of metal compounds on the incorporation of ^{3}H-1-NP was examined. The cells were mixed with metal compounds and incubated for 30 min at 37 °C, then ^{3}H-1-NP was added to the mixtures and incubation was continued for an additional 30 min at 37 °C. However, addition of metal compounds to the preincubation mixtures had no significant effect on the incorporation of ^{3}H-1-NP into the cells.

Incorporation of Potassium Dichromate, Cobaltous Acetate and Zinc Chloride into the Cells

The incorporation of metal ions into the cells was measured after the cells had been mixed with metal compounds and incubated for 30 min at 37 °C. The amount of metals in the pellet separated by centrifugation at 105000 *g* for 60 min after removal of intact cells and debris and in its supernatant increased with increase of the metal concentration added, as shown in Fig. 3.

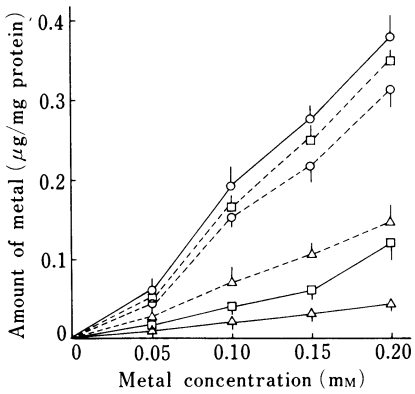

Fig. 3. Incorporation of Metal Ions into the Cells

The amounts of metal ions in the supernatant (----) and the pellet (——) obtained by centrifugation at 105000 *g* for 60 min, after removal of intact cells and debris, are shown. Each point is the mean ± S.D. of 4—6 determinations. The symbols are as follows: potassium dichromate (□); cobaltous acetate (△); zinc chloride (○).

TABLE I. Change of 1-NP Nitroreductase Activity by Addition of Cobaltous Acetate, Potassium Dichromate and Zinc Chloride to the Whole Cells

Metal added (mM)	1-NP nitroreductase activity ($\times 10^{-11}$ mol/mg protein/min)		
	Co(II)	Cr(VI)	Zn(II)
0	0.68 ± 0.03	0.68 ± 0.03	0.68 ± 0.03
0.10	0.64 ± 0.03	0.72 ± 0.03	0.65 ± 0.04
0.20	0.63 ± 0.02	0.67 ± 0.08	0.64 ± 0.05
0.30	0.62 ± 0.04	0.70 ± 0.05	0.64 ± 0.11
0.40	0.65 ± 0.02	0.65 ± 0.01	0.63 ± 0.11

Each value is the mean ± S.D. of 4 or 5 determinations.

Effect of Potassium Dichromate, Cobaltous Actate and Zinc Chloride on the Activity of 1-NP Nitroreductase in the Supernatant

The alteration of 1-NP nitroreductase activity has an effect on the metabolic activation of 1-NP and it may have an increasing effect on the mutagenicity and the 1-NP binding to DNA. Therefore, metal compounds were added to whole cells suspended in the buffer containing metal ions and then the reaction mixtures were incubated at 37 °C for 30 min. After the incubation, the cells were ultrasonically lysed for the preparation of enzymes. The addition of potassium dichromate, cobaltous acetate and zinc chloride to whole cells had no significant effect on the nitroreductase activity, as shown in Table I. Furthermore, the direct addition of these metal compounds to the reaction mixtures also had no significant effect on the nitroreductase activity.

Effect of Cobaltous Acetate, Potassium Dichromate and Zinc Chloride on Binding of 1-NP to DNA in the Cells

The effect of addition of metal ions on the binding of 1-NP to DNA in *S. typhimurium* TA100 was examined. After pretreatment of the cells with potassium dichromate, cobaltous acetate and zinc chloride in the preincubation mixtures, the amount of 1-NP binding to DNA increased with increase of the metal concentration as shown in Fig. 4, and increased approximately 2.0-, 1.8- and 2.8-fold, respectively, at the concentration of 0.2 mM. Pretreatment of the cells with nickelous acetate in the preincubation mixtures did not increase the amount of 1-NP binding to DNA at nickel concentrations ranging from 0.05 to 0.20 mM.

Pattern of Gel Filtration of DNA Adducts on Sephadex G-25

DNA-binding species formed by 1-NP in the cells pretreated with metal compounds were

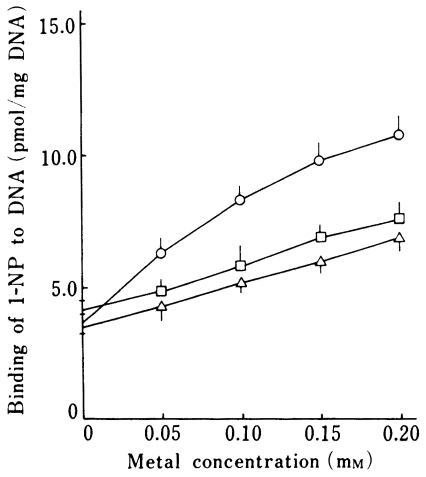

Fig. 4. Effect of Metal Ions on the Binding of ^{3}H-1-NP to DNA in the Cells

Each point is the mean ± S.D. of 4—6 determinations. The symbols are as follows: potassium dichromate (□); cobaltous acetate (△); zinc chloride (○).

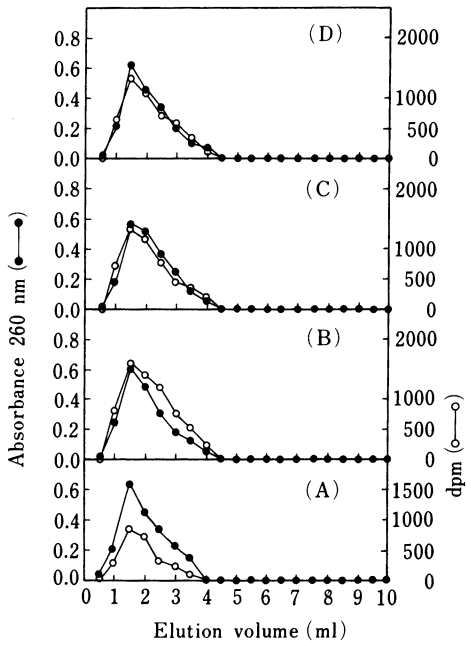

Fig. 5. Gel Filtration of DNA Adducts on Sephadex G-25

The cells were mixed with ^{3}H-1-NP without addition of metal ions (A) and with 0.2 mM zinc chloride (B), cobaltous acetate (C) or potassium dichromate (D) before the incubation with ^{3}H-1-NP. The incubation of mixtures was carried out as described in Experimental. The amount of DNA adducts applied to a Sephadex G-25 column is equivalent to 2 OD_{260}.

compared with those in the cells treated with 1-NP alone. The elution pattern of DNA separated from the cells treated with 1-NP alone is shown in Fig. 5A. The elution pattern of DNA separated from the cells treated with chromium(VI), cobalt(II) or zinc(II) and with 1-NP is similar to that of DNA separated from the cells treated with 1-NP alone. The peak in the patterns of ^{3}H-counts in DNA separated from the cells treated with the metal ions and 1-NP was observed to overlap with that of the absorbance at 260 nm, as shown in Fig. 5B—5D. The ^{3}H-counts was much higher than that in DNA separated from the cells treated with 1-NP alone.

Discussion

We found that pretreatment of the cells with potassium dichromate, cobaltous acetate and zinc chloride increased reversion mutation induced by 1-NP and the binding of 1-NP to DNA. These metal compounds had no significant effect on the incorporation of ^{3}H-1-NP in the cells. Messier *et al.*[17] showed that 1-NP was reduced to reactive metabolites which bound to DNA in intact cells of *S. typhimurium*. Lu *et al.*[13] showed that the 105000 ***g*** supernatant of *S. typhimurium* TA100 contained 1-NP nitroreductase activity. However, the addition of these metal compounds to whole cells or the enzyme sources *in vitro* had no significant effect on the nitroreductase activity under the experimental conditions tested. The metal ions or their complexes such as zinc and cobalt have been reported to transform the B-form of poly d(G-C) to the *Z*-form.[18-20] Therefore, it is considered that they may alter the conformation of DNA

to a form more capable of binding 1-NP.

1-NP-induced mutagenicity was increased by potassium dichromate, cobaltous acetate and zinc chloride, *i.e.*, the metal compounds that increased the binding of 1-NP to DNA. Therefore, the increased binding of 1-NP to DNA induced by the metal compounds may play an important role in the increased mutagenicity. The 1-NP-induced mutagenicity was not increased by nickelous acetate, and the amount of 1-NP binding to DNA was also not increased. Howard *et al.*[21] have reported a good correlation between the extent of 1-NP binding to DNA and the frequency of induced histidine revertants in *S. typhimurium*. They have also reported that DNA-binding species are formed during the metabolic reduction of 1-NP and the major DNA adduct is *N*-(deoxyguanosin-8-yl)aminopyrene.

Zinc and magnesium ions are known to interact with nucleic acids and proteins that are cellular components.[22-25] Therefore, the interaction of magnesium and zinc ions in the cells was examined in the preincubation mixtures by simultaneous addition. However, the addition of magnesium acetate had no effect on the increase of mutagenic activity of 1-NP caused by zinc ions under the experimental conditions tested. Iyehara-Ogawa *et al.*[5] reported the combined mutagenicity of cobaltous chloride and heteroaromatic compounds in *S. typhimurium*. In contrast, Mochizuki and Kada[4] reported the antimutagenic action of cobaltous ions on Trp-P-1-induced mutations in *S. typhimurium*, and in their experiment cobaltous ions were added to the mixtures after preincubation of the test system. In our experiment, the addition of metal ions including cobaltous ions was carried out before the preincubation of the cells with 1-NP in the reaction mixtures. By using rat liver S9 mixtures in the preincubation, both nitroreduction and ring-hydroxylation have been reported to affect the mutagenic activity of 1-NP toward *S. typhimurium* TA100.[26] However, under our experimental conditions without S9 mixtures production of ring-hydroxylated metabolites seems unlikely.

Under the experimental conditions tested, the revertant colonies were slightly increased by potassium dichromate, cobaltous acetate or zinc chloride alone. The number of revertant colonies induced by each metal compound and 1-NP under our experimental conditions appears to be more than the sum of those induced by metal compound alone and 1-NP alone. The pretreatment of the cells with nickelous acetate, cadmium acetate and chromium(III) nitrate did not increase the number of revertant colonies induced by 1-NP. The number of revertant colonies induced by cadmium acetate alone was higher than the spontaneous number (control), but there was no additive effect with 1-NP. The number of revertant colonies induced by cadmium acetate and 1-NP was similar to that with 1-NP alone, while the surviving colonies were found to decrease markedly in the case of cadmium acetate treatment. Under our experimental conditions, cadmium acetate appears to suppress the revertant colonies induced by 1-NP. Mandel and Ryser[3] have reported that the frequencies of mutation induced by MNNG and methylnitrosourea in *S. typhimurium* TA1975 and TA1535 are increased in the presence of cadmium chloride. Dubins and LaVelle[27] have reported the potentiation of mutagenesis of ethyl methanesulfonate and methyl methanesulfonate by nickelous chloride in *S. typhimurium* TA100. They suggest that nickel(II) acts directly as a comutagen. On the other hand, no increase of 1-NP-induced revertant colonies by nickelous acetate was found. Under our experimental conditions nickelous acetate did not appear to act as a comutagen.

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