46. Reduction of N-methyl-N'-nitro-N-nitrosoguanidine-Induced Mutations by Cobalt Chloride in *Escherichia Coli*

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Since the discovery of a potent mutagenic action of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Mandell and Greenberg, 1961), this chemical has been widely used in genetic studies and breeding of microorganisms. It is well known that MNNG is a typical alkylating agent inducing base-change type mutations. We report here our observation that MNNG-induced mutations are remarkably reduced in *Escherichia coli* by the presence of cobalt chloride.

The strain used was *E. coli* B/r WP2 (Witkin, 1956) requiring tryptophane for growth and reversions to prototrophy were studied. B-2 broth containing 10 g Kyokuto beef extract, 10 g Wako polypeptide dry powder and 5 g NaCl in 1000 ml of water and adjusted to pH 7.0 was used for culture of bacteria. Minimum synthetic medium (a modified Vogel Bonner's) was prepared by dissolving 1 g $(\text{NH}_4)_2\text{SO}_4$, 10 g KH_2PO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.5 g trisodium citrate in distilled water and the solution was neutralized by KOH (about 1.94 g required), then filled up to 1,000 ml. Agar medium contains per liter of the above mixture, 4 g of glucose, 15 g of Difco agar and 10 ml of Difco broth (8 g of Difco nutrient broth powder dissolved in 1,000 ml water). Such a semi-enriched medium (MB agar) has been devised and used since long (Witkin, 1956; Kada *et al.*, 1960) for titration of both survivals and induced mutations of WP2 strains under identical conditions.

Cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) was purchased from Wako Co., Ltd. MNNG, a standard sample obtained from Dr. M. Nakadate of National Institute of Hygienic Science, Tokyo, was dissolved in 15/M phosphate buffer (pH 7.0) just before each experiment and added into the bacterial suspension.

Bacteria were grown overnight (about 16 hours) in B-2 broth with aeration at 37°C, washed twice with 15/M phosphate buffer (PB) and resuspended in PB. Each 10 ml of bacterial suspension was

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supplemented with the MNNG stock solution at 0°C to obtain appropriate chemical concentrations and kept at 37°C for 30 minutes or more with slow shaking. The treated cells were washed twice with PB by centrifugation at cold and resuspended in 10 ml of PB. The bacterial suspensions thus obtained were appropriately diluted and plated on MB agar to count survivals and induced mutants.

Effects of cobalt chloride on cellular viability and reversibility to prototrophy of the bacteria, treated or not treated with MNNG, are shown in Table I. In this case, the metal compound was added at different concentrations in MB agar and bacteria were titrated on it. It is shown that the presence of cobalt chloride did not modify significantly the number of viable bacteria not treated with MNNG and the number of Try⁺ colonies per plate decreased considerably, suggesting that at least cobalt chloride itself is not mutagenic. For bacteria treated with MNNG (60 µg/ml), the survival decreased slightly in increasing metal concentrations of the plates, while mutation inductions were reduced drastically in plates at higher metal concentrations.

Table I. Effects of cobalt chloride on lethality and mutation induction in *Escherichia coli* B/r WP2 Try⁻ cells previously treated or not treated with MNNG (60 µg/ml for 30 minutes).

Concentration of CoCl ₂ ·6H ₂ O (µg/ml) in MB agar	Cells not treated with MNNG			Cells treated with MNNG		
	Mean number of Try ⁺ colonies per plate	Number per ml of viable cells	Frequency of Try ⁺ revertants per 10 ⁸ cells*	Mean number of Try ⁺ colonies per plate	Number per ml of viable cells	Frequency of Try ⁺ revertants per 10 ⁸ cells**
0	5.7	4.0×10 ⁹	<1.4	2,335	3.4×10 ⁹	686.8
5	3.0	4.5×10 ⁹	<0.7	2,243	3.5×10 ⁹	640.8
10	0.7	4.8×10 ⁹	<0.2	453	3.0×10 ⁹	151.0
15	1.0	2.9×10 ⁹	<0.4	79.7	2.9×10 ⁹	27.5
20	0.3	3.7×10 ⁹	<0.1	20.0	2.0×10 ⁹	10.0

0.1 ml portions of bacterial suspensions were plated after appropriate dilutions. Means of three plates are shown.

* The real frequency of Try⁺ revertants can only be calculated by knowing the number of Try⁺ colonies appeared after residual divisions on the MB plates.

** Since these frequencies are very high, contribution by the Try⁺ revertants appeared after residual divisions on plates may be neglected.

There may exist different explanations for the above observation on the cobalt effects. In the first, we suspect if cobalt chloride might inactivate directly MNNG. This possibility is unlikely because bacteria were plated on cobalt-containing media after MNNG-treatment and washing out the metal. In any case, we treated bacteria with MNNG (50 µg/ml) in the presence of cobalt chloride (20 µg/

Table II. Effect of the presence of cobalt chloride during MNNG-treatment on mutation induction of *Escherichia coli* B/r WP2 Try⁻.

Treatment at 37°C for 50 min		Mean number of Try ⁺ colonies per plate	Number per ml of viable cells	Frequency of Try ⁺ revertants per 10 ⁸ viable cells
MNNG (μg/ml)	CoCl ₂ ·6H ₂ O (μg/ml)			
0	0	7	1.0 × 10 ⁹	3.8
50	0	1,250	0.91 × 10 ⁹	1,374
50	20	1,247	1.0 × 10 ⁹	1,247

0.1 ml portions of washed bacteria were plated after appropriate dilutions.

ml), washed the cells and plated them on cobalt-free medium. High levels of mutation induction were still observed (Table II). For the second possibility, cobalt chloride might have inhibited gene-expression of reverted Try⁺ genes, since in the present experiments the metal compound always existed in the course of colony formation of the Try⁺ cells. To examine this possibility, a number of MNNG-induced Try⁺ clones in absence of cobalt chloride were transferred onto minimal agar medium without tryptophane but containing the metal compound (20 μg/ml). Since all the clones grew well, there is no specificity as to tryptophane expression of the WP2 strain. For the third possibility, growth of the induced mutant cells plated on cobalt-containing plates might have been inhibited due to "the crowding effects", since the cellular intensity is very much higher in plates for mutation counting than those for measuring the survivals. To check this point, the mutagen-treated cells were plated after dilutions on plates with and without cobalt chloride for mutation selection (Table III). The number of induced Try⁺ colonies were drastically reduced in the presence of cobalt chloride at any dilutions, suggesting that the crowding effects are apparently absent. The above observations indicate that cobalt chloride inhibit selectively induction of induced mutant cells; we do not know still the cellular stage where the metal is inhibitory.

Table III. Cellular concentrations for plating and MNNG-induction of reversions in *Escherichia coli* B/r WP2 Try⁻.

Total number of surviving cells plated	Mean number of Try ⁺ colonies appeared on MB agar containing cobalt chloride	
	0 μg/ml	20 μg/ml
1.7 × 10 ⁸	2,302	15.3
1.7 × 10 ⁷	265	7.0
1.7 × 10 ⁶	39	1.7

Cells were treated with MNNG of 50 μg/ml for 60 min and plated at different concentrations on plates.

The final possibility that we are examining is that the cobalt chloride might block induced error-prone function by the chemical mutagen in a similar way that has been supposed in UV-mutagenesis (Radman, 1974, 1975; Witkin, 1977). Our present observation on the higher drug lethal sensitivity on cobalt medium may support the above hypothesis that mutation induction takes place in the course of repair of lethal effects. Studies are in progress to examine such a possibility. We recently found that UV-induced mutagenesis was also inhibited very much by cobalt chloride (Kada and Hara, in preparation).

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