

# Metal Salts as Promoters of *in Vitro* Morphological Transformation of Hamster Embryo Cells Initiated by Benzo(a)pyrene

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## ABSTRACT

The hamster embryo cell bioassay has been used to study the effect of metal salts on morphological transformation. A synergistic enhancement of the transformation frequency was found for the combined treatment with organic carcinogens [benzo(a)pyrene, *N*-hydroxy-2-acetylaminofluorene, and 4-nitroquinoline 1-oxide] and nickel sulfate, cadmium acetate, or potassium chromate. Chromic chloride and zinc chloride did not induce transformation themselves, and they had no effect on the transformation frequency when tested in combination with benzo(a)pyrene.

The synergistic effect between benzo(a)pyrene and nickel sulfate or cadmium acetate was also apparent when the cells were treated sequentially with the chemicals. When the cells were first exposed to benzo(a)pyrene, both nickel sulfate and cadmium acetate showed a promotion-like effect similar to that obtained with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. Moreover, when 12-*O*-tetradecanoylphorbol-13-acetate or benzo(a)pyrene were used as promoting agents, both nickel sulfate and cadmium acetate were able to initiate morphological transformation. The data suggest that the metal salts are more potent as promoters than they are as initiators. The present findings may be of importance in relation to carcinogenicity of metal compounds to humans.

## INTRODUCTION

Experimental cancer research as well as epidemiological studies have provided evidence that several inorganic metal compounds are involved in carcinogenesis (4, 14-16, 25, 31, 33, 37). Humans are exposed to inorganic carcinogenic substances in considerable amounts through occupation and air pollution as well as from cigarette smoking (1, 2, 11-13, 24). The role of these compounds in the development of human cancer may be significant.

Most experimental data on animal metal carcinogenesis involve induction of tumor at the site of the injection (22, 32, 34). The finding that it is difficult experimentally to induce in animals the tumor seen in humans after exposure to metals suggests that inorganic metal compounds are not primary carcinogens themselves but rather may act as cocarcinogens. Metal ions are known to be of great importance for the activity of many enzymes, and the possibility exists that they may act by influencing the metabolism of carcinogenic chemicals (9, 22, 35, 36) or DNA synthesis or repair (21). Another possibility is that they act as promoters in carcinogenesis.

*In vitro* bioassays have proved to be a useful tool for studying

mechanisms in carcinogenesis. Several groups have shown that metal compounds associated with human carcinogenicity will transform mammalian cells (4-6, 8, 14, 27, 29). Few experimental studies have been carried out thus far concerning the cocarcinogenic effect of metals. Recently, we have found that nickel sulfate enhances the transformation frequency of hamster embryo cells in the presence of BP<sup>2</sup> and that cigarette smoke extract can act as a promoter of the transformation of cells initiated by BP (28-30). In the present work, the role of metal ions on morphological transformation has been studied in more detail.

## MATERIALS AND METHODS

**Cell Cultures.** Primary cultures of Syrian hamster embryos (Wright, Chelmsford, Essex, United Kingdom) at 14 days of gestation were prepared and cryopreserved in liquid nitrogen as described by Pienta *et al.* (27). Mass cultures were grown in Dulbecco's modification of Eagle's minimum essential medium, supplemented with 10% fetal bovine serum (Gibco Biocult, Paisley, United Kingdom) at 37° in a 10% CO<sub>2</sub> atmosphere. Ampuls with cryopreserved cells were used as stock cultures in the transformation assay.

**Test Chemicals.** BP, TPA, and insulin (crystalline, bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, Mo.). NQO and N-OH-AAF were obtained from the National Cancer Institute Chemical Repository, Bethesda, Md. The metal salts used were of the highest analytical purity from E. Merck (Darmstadt, Germany). The chemicals were dissolved in DMSO and diluted with warm complete medium to the desired concentration shortly before use. The final concentration of DMSO was consistently less than 0.2%. In separate experiments, DMSO was not found to affect the experimental results.

**Transformation Assay.** The bioassay procedure previously described was used with small modifications (6, 26). Essentially, a feeder layer of  $6 \times 10^4$  X-irradiated cells (5000 R) was seeded in 3 ml complete medium [Dulbecco's modification of Eagle's minimum essential medium supplemented with 20% fetal bovine serum and insulin (2 µg/ml); no antibiotic was used] on a 60-mm Petri dish. The next day, 200 or 250 target cells in 1 ml medium were seeded, and test chemicals were added 24 hr later, double strength in 4 ml medium. Eight to 9 days after seeding of the target cells, the dishes were washed with Dulbecco's phosphate-buffered saline (Flow Laboratories, Ayrshire, United Kingdom), and the colonies were fixed with methanol and stained with Giemsa before counting and examination. In experiments in which promotion was studied, the incubation period with test chemicals was divided into 2 parts.

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<sup>2</sup> The abbreviations used are: BP, benzo(a)pyrene; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NQO, 4-nitroquinoline 1-oxide; N-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; DMSO, dimethyl sulfoxide; CdAc<sub>2</sub>, cadmium acetate.

At the end of the first period, the dishes were washed twice with Dulbecco's phosphate-buffered saline before adding new medium for the second period.

Morphological transformation is defined as altered colony morphology consisting of criss-crossing and piling up of cells not observed in the control (7, 27). All experiments have been repeated 3 to 5 times with consistent results. One set of experimental data has been presented.

## RESULTS

**Synergism between BP and NiSO<sub>4</sub>.** The data in Chart 1 show the effect of nickel sulfate and BP on the morphological transformation of Syrian hamster embryo cells. The transformation frequency increased with increasing BP concentration (Chart 1A). The concentration range covers a factor of 100. It can be shown by plotting the data in a linear scale that the transformation frequency increases linearly with the BP concentration. NiSO<sub>4</sub>·6H<sub>2</sub>O at a concentration of 5 μg/ml gave a transformation frequency of 0.5%. However, when nickel sulfate was tested in combination with BP, a large enhancement of the transformation frequency was observed. Thus, for BP (0.08 μg/ml) in the presence of nickel sulfate, the transformation frequency was 3.2% compared to an expected frequency of 1%. At a 0.8-μg/ml concentration of BP, the transformation frequency for the combined exposure had increased to about 10% which is 7 times higher than that expected from experiments with the individual compounds. At higher BP concentrations, the frequency decreased probably due to toxic effects. For BP concentrations up to 0.8 μg/ml, the cloning efficiency was more than 80% of the control, while at higher BP concentrations, the cell growth was heavily reduced and a number of toxic processes appeared.

The data in Chart 1B show a similar experiment where the concentration of nickel sulfate was varied in the absence and presence of BP (0.8 μg/ml). A linear increase in the transformation frequency for nickel sulfate can be demonstrated by plotting the data in a linear scale. These data also show the pronounced synergistic effect on transformation frequency for the combined treatment of nickel sulfate and BP.

**Effect of Different Metal Salts on Transformation by BP.** Table 1 shows the effect on the transformation frequency of the combined treatment with different metal salts and BP. The BP concentration was in all experiments 0.8 μg/ml. BP alone

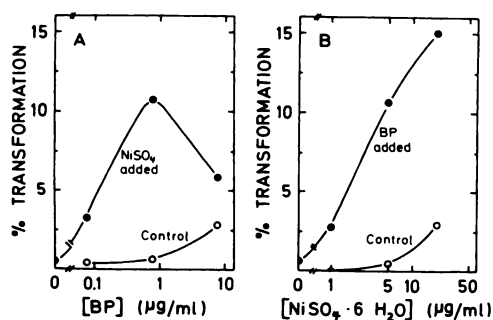


Chart 1. Effect of BP and NiSO<sub>4</sub> on transformation frequency. A, effect of the concentration of BP in the absence and presence of NiSO<sub>4</sub>·6H<sub>2</sub>O (5 μg/ml). B, effect of the concentration of nickel sulfate in the absence and presence of BP (0.8 μg/ml). At least 6 dishes were used for each experimental point. The transformation frequency was determined on the basis of the total number of surviving colonies.

Table 1

Morphological transformation of hamster embryo cells by BP and metal salts given separately and in combinations

Six dishes were used for each concentration. The transformation frequency is calculated as the number of transformed colonies divided by the total number of surviving colonies multiplied with 100. The following concentrations were used: BP, 0.8 μg/ml (3.2 μM); NiSO<sub>4</sub>·6H<sub>2</sub>O, 5.0 μg/ml (19 μM); CdAc<sub>2</sub>·2H<sub>2</sub>O, 0.5 μg/ml (1.9 μM); K<sub>2</sub>CrO<sub>4</sub>, 0.5 μg/ml (2.6 μM); CrCl<sub>3</sub>·6H<sub>2</sub>O, 3.0 μg/ml (11.3 μM); ZnCl<sub>2</sub>, 3.0 μg/ml (22 μM).

Addition	Total no. of colonies	Cloning efficiency (%)	Total no. of transformed colonies	Transformation frequency (%)
Control	450	30	0	0
BP	270	18	2	0.7
NiSO <sub>4</sub>	390	32	1	0.3
BP + NiSO <sub>4</sub>	198	17	19	9.6
CdAc <sub>2</sub>	360	24	1	0.3
BP + CdAc <sub>2</sub>	156	10	10	6.4
K <sub>2</sub> CrO <sub>4</sub>	210	14	0	0
BP + K <sub>2</sub> CrO <sub>4</sub>	126	8	5	4.0
CrCl <sub>3</sub>	336	22	0	0
BP + CrCl <sub>3</sub>	276	18	0	0
ZnCl <sub>2</sub>	420	28	0	0
BP + ZnCl <sub>2</sub>	270	18	1	0.4

gave a transformation frequency of 0.7%. It is apparent from the table that a synergistic enhancement of the transformation frequency was obtained for NiSO<sub>4</sub>, cadmium acetate, and K<sub>2</sub>CrO<sub>4</sub>. On the other hand, the presence of CrCl<sub>3</sub> as well as ZnCl<sub>2</sub> did not affect the transformation frequency induced by BP. Moreover, none of these metal salts induced transformation. The experiments with chromium support the previous findings that it is hexavalent chromium which is associated with carcinogenesis (14, 20, 31).

**Combined Exposure to NiSO<sub>4</sub>, Cadmium Acetate, N-OH-AAF, and NQO.** In Chart 2, cadmium and nickel salts have been tested in the presence of the carcinogens NQO and N-OH-AAF. The carcinogens alone gave a transformation frequency of less than 0.5%. Probably due to the low transformation frequency, it was difficult to obtain a reproducible increase in the transformation frequency with increasing concentrations of NQO and N-OH-AAF. When the cells were exposed to the metal salts and the carcinogens in combination, a large enhancement of the transformation frequency was observed. With NQO (0.01 μg/ml) (Chart 2A) and N-OH-AAF (1 μg/ml) (Chart 2B), the transformation frequencies in the presence of the metal salts were approximately 5 times higher than expected on the basis of experiments with the individual compounds. Since the 2 carcinogens are activated by different mechanisms, the data suggest that the metal salts affect not the metabolism of the carcinogens but rather the carcinogenic process.

**NiSO<sub>4</sub> and Cadmium Acetate as Promoters of Morphological Transformation.** In order to study the mechanism of the synergistic effect in more detail, experiments were carried out in which the cells were sequentially exposed to BP and NiSO<sub>4</sub> or cadmium acetate. The results are presented in Table 2. When BP alone was present only during the first period, the transformation frequency was 0.3%. Nickel sulfate and cadmium acetate gave a transformation frequency of 0.3 and 1.4%, respectively, when present only during the last period.

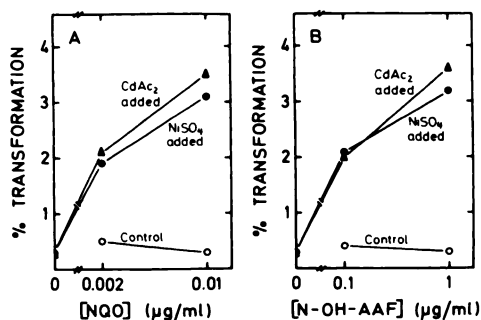


Chart 2. Effect of  $\text{NiSO}_4$  and cadmium acetate ( $\text{CdAc}_2$ ) on transformation, tested separately and in combination with NQO (A) and N-OH-AAF (B). The transformation frequency was measured in the absence and presence of  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  ( $5 \mu\text{g/ml}$ ) and  $\text{CdAc}_2 \cdot 2\text{H}_2\text{O}$  ( $0.5 \mu\text{g/ml}$ ).

Table 2

Promotion and initiation effects of metal salts on morphological transformation of hamster embryo cells

The transformation frequency is calculated as the number of transformed colonies divided by the total number of surviving colonies multiplied with 100. The following concentrations were used:  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ ,  $5.0 \mu\text{g/ml}$  ( $19 \mu\text{M}$ );  $\text{CdAc}_2 \cdot 2\text{H}_2\text{O}$ ,  $0.5 \mu\text{g/ml}$  ( $1.9 \mu\text{M}$ ); BP present during Period 1,  $0.05 \mu\text{g/ml}$  ( $0.20 \mu\text{M}$ ); BP present during Period 2,  $0.01 \mu\text{g/ml}$  ( $0.04 \mu\text{M}$ ); TPA,  $0.1 \mu\text{g/ml}$  ( $0.16 \mu\text{M}$ ).

Additions		Total no. of colonies	Cloning efficiency (%)	Total no. of transformed colonies	Transformation frequency (%)
Period 1 (3 days)	Period 2 (3 days)				
None	None	325	27	0	0
$\text{NiSO}_4$	None	520	26	0	0
$\text{CdAc}_2$	None	528	24	2	0.4
BP	None	301	25	1	0.3
None	$\text{NiSO}_4$	604	22	2	0.3
None	$\text{CdAc}_2$	488	22	7	1.4
None	TPA	518	26	9	1.7
None	BP	265	27	3	1.1
BP	$\text{NiSO}_4$	346	25	17	4.9
BP	$\text{CdAc}_2$	419	21	24	5.7
BP	TPA	398	20	42	10.6
$\text{NiSO}_4$	TPA	552	23	17	3.1
$\text{NiSO}_4$	BP	318	27	6	1.9
$\text{CdAc}_2$	TPA	480	20	25	5.2

However, when the cells were treated with BP in the first period and the metal salts in the second period, the transformation frequency increased to 4.9 and 5.7% for nickel sulfate and cadmium acetate, respectively. These results suggest that  $\text{NiSO}_4$  and cadmium acetate promote transformations initiated by BP. This is further supported by experiments where the tumor promoter TPA was present during the second period instead of the metal salts. It is apparent that the effect of  $\text{NiSO}_4$  and cadmium acetate is similar although not as great as that of TPA (Table 2).

**$\text{NiSO}_4$  and Cadmium Acetate as Initiators of Morphological Transformation.** When  $\text{NiSO}_4$  and cadmium acetate were present during the first period with TPA during the second period, the transformation frequency increased compared to the frequency found when the compounds were tested separately (Table 2).  $\text{NiSO}_4$  and cadmium acetate in the first period with no addition during the second period, gave a transformation frequency of less than 0.5%. For the sequential treatment to nickel sulfate or cadmium acetate and TPA, transformation frequencies of 3.1 and 5.2% were observed. If the cells were exposed to BP only during the second period, a transformation

frequency of 1.1% was obtained. When  $\text{NiSO}_4$  was present during the first period with BP during the second period, the transformation frequency was 1.9%. By comparing these results, it is apparent that the metal salts are more potent as promoters than they are as initiators.

## DISCUSSION

The present results show that nickel sulfate, cadmium acetate, and sodium chromate give rise to a synergistic enhancement of the transformation frequency in combination with different organic carcinogens. Zinc chloride and chromium chloride neither induced transformation themselves nor enhanced the transformation frequency in the presence of BP.

Exposure to nickel compounds in nickel refineries has been shown to cause cancer of the lung and nasal sinuses (11, 26). Data have been presented indicating that hexavalent chromium induces lung cancer and that cadmium compounds may be involved in the induction of cancer of the respiratory tract as well as cancer of the prostate and kidney (17, 18, 20). Zinc salts and salts of trivalent chromium are not associated with human carcinogenesis. Previous studies have indicated a synergistic enhancement of the carcinogenic effect of nickel and cadmium by cigarette smoke (18, 19). The higher incidence of lung cancer in urban compared to rural areas (13) is of interest in this connection. Fly ash from coal-powered plants as well as particles in car emission are covered by a number of different metal compounds and polycyclic aromatic hydrocarbons (2, 3, 10, 23). The potentiating effect of the combined exposure to these compounds may be of importance for the development of cancer.

In the present study on the synergistic effect of combined treatment to  $\text{NiSO}_4$  or cadmium acetate and BP, it was not necessary for the 2 compounds to be present at the same time. Transformation frequency was also enhanced when the metal salts were added after the cells had been treated with BP. These data together with model studies with the tumor promoter TPA indicate that nickel and cadmium salts act as promoters in transformation of cells initiated by BP. The data further show that the metal salts can also act as weak initiators of morphological transformation with TPA as promoter. The possibility therefore exists that the enhancement observed for combined treatment with BP and nickel sulfate or cadmium acetate could result from the promotional effect of BP following metal salt initiation. However, the enhancement observed in the experiment with BP as promoter was very small. Thus, in the cocarcinogenic experiments, it seems likely that the enhancement is mainly due to the promotion-like effect of the metal salts.

Previously, we have demonstrated that cigarette smoke extract enhances the transformation of hamster embryo cells in combination with BP and nickel sulfate and, moreover, that cigarette smoke extract can act as a promoter for transformation initiated by BP (28, 30). The possibility should be considered that promotion effects of metal compounds may be significant in carcinogenesis.

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