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Enhancement by transition metals of chromosome aberrations induced by isoniazid

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Isoniazid (isonicotinic acid hydrazide) is an effective antitubercular drug in widespread use [1, 2]. However, isoniazid has been found to induce tumors in mice [1] and chromosome aberrations *in vivo* [3] and *in vitro* [4]. At the same time there is little evidence for a carcinogenic or mutagenic effect in humans [1, 2, 5]. We have recently found that complexes of transition metals enhance unscheduled DNA synthesis induced by isoniazid [6] in cultured human cells. We report here that a manganese–glycine complex strongly enhances chromosome aberrations induced by isoniazid in Chinese hamster ovary (CHO) cells. A copper–glycine complex has a weak stimulatory effect, while iron–EDTA inhibits DNA damage by isoniazid. Chromosome aberrations induced by isoniazid or isoniazid/manganese mixtures are inhibited by simultaneous treatment with reduced glutathione or catalase. These results suggest that manganese may be important in the toxic and carcinogenic effects of isoniazid.

Procedures for the maintenance and preparation of CHO cells for chromosome aberration experiments, as well as harvesting and staining techniques, have been described [7]. Chemicals were obtained from the following sources: isoniazid (m.p. 169–171°), catalase (purified powder from bovine liver) and glutathione (reduced and oxidized forms) from the Sigma Chemical Co., St. Louis, MO; reagent grades of $CuSO_4 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, glycine, and EDTA from the Fisher Scientific Co., Vancouver, Canada. Metal complexes were prepared at 10^{-2} M in water [6] and diluted in medium [Eagle's minimum essential medium (MEM) plus 2.5% fetal calf serum]. Inactivated catalase was prepared by heating an aqueous solution of catalase (1 mg/ml) at 100° for 30 min. Freshly prepared solutions of chemicals in medium were mixed directly in the culture dishes. The order of addition was: glutathione, catalase, metal complex and isoniazid. All concentrations listed in Table 1 refer to final concentrations after mixing. Following a 3-hr treatment period, the cells were rinsed, incubated for 20 hr in growth medium (MEM plus 15% serum) and harvested [7]. In the analysis of chromosome aberrations only distinct breaks and exchanges were scored.

Elevated frequencies of chromosome aberrations were induced by treatment with high concentrations of isoniazid alone (Table 1). Up to 42 per cent of all metaphase figures contained aberrations, while the number of exchanges per metaphase reached a maximum of 2.1 at 2×10^{-2} M isoniazid.

The addition of a manganese–glycine complex at 10^{-5} M final concentration resulted in enhanced toxicity and high frequencies of chromosome aberrations at low concentrations of isoniazid. At 2×10^{-4} M isoniazid, the frequency of aberrations (53 per cent) and the number of exchanges per metaphase (2.5) exceeded the values obtained with 100-fold higher concentrations of isoniazid in the absence of manganese. The isoniazid/manganese mixtures were active at isoniazid concentrations similar to those measured in humans during treatment [2].

Relative to the strong effect of manganese, copper–glycine showed weak enhancement while iron–EDTA had none. There was approximately a 5-fold increase in the cytocidal and cytostatic effects of isoniazid when copper (10^{-4} M) was present. However, a higher frequency of chromosome aberrations was observed only at one concentration of isoniazid (5×10^{-3} M). At all other concentrations a similar level of aberrations was detected with or without added copper. In contrast, iron–EDTA at 10^{-4} M reduced the cytostatic and clastogenic effects of isoniazid.

Chromosome aberrations were not induced by the metal complexes at the concentrations employed in this study (Table 1). Treatment of cells with much higher (mM) concentrations of the metals induced aberrations, primarily chromatid breaks [7]. This is in contrast to the high frequencies of exchange observed in cells treated with isoni-

Table 1. Effects of metal complexes, glutathione and catalase on chromosome aberrations induced by isoniazid

Isoniazid concn (M)	Frequency of chromosome aberrations*								
	Isoniazid alone	Isoniazid plus		Isoniazid plus 10^{-5} M Mn(II)†	Isoniazid/Mn(II) plus		Isoniazid plus 10^{-4} M Cu(II)†	Isoniazid/Cu(II) plus catalase	Isoniazid plus 10^{-4} M Fe(III)‡
		Catalase (0.1 mg/ml)	Glutathione (10^{-2} M)		Catalase (0.1 mg/ml)	Glutathione (10^{-2} M)			
1×10^{-1}	M.I.§	5.1‖ (0.22)¶	3.4 (0.14)	Toxic**	6.3 (0.28)	2.7 (0.13)	Toxic	M.I.	2.5 (0.07)
5×10^{-2}	41.0 (0.95)	0.8 (0.00)	0.5 (0.00)		2.9 (0.18)	1.5 (0.01)	M.I.	23.1 (0.25)	1.8 (0.01)
2×10^{-2}	42.1 (2.10)	0.5 (0.00)	0.7 (0.00)		1.2 (0.00)	0.5 (0.00)	M.I.	3.4 (0.00)	1.6 (0.03)
1×10^{-2}	27.0 (0.65)	0.8 (0.00)	0.7 (0.00)				27.5 (0.50)	1.0 (0.00)	
5×10^{-3}	9.6 (0.20)	0.5 (0.00)	0.8 (0.00)				19.3 (0.47)	1.2 (0.00)	
2×10^{-3}	2.8 (0.06)	0.7 (0.01)	0.5 (0.00)		2.0 (0.02)	0.7 (0.01)	2.6 (0.03)	1.0 (0.00)	0.6 (0.00)
1×10^{-3}	0.6 (0.00)			Toxic			1.3 (0.01)	1.5 (0.00)	
5×10^{-4}	1.3 (0.00)	0.7 (0.00)	0.5 (0.00)	M.I.	1.5 (0.00)	0.4 (0.00)	2.4 (0.01)	0.8 (0.00)	1.4 (0.00)
2×10^{-4}	1.5 (0.01)			53.1 (2.53)	0.8 (0.00)	0.7 (0.00)			
1.5×10^{-4}	0.8 (0.00)	0.5 (0.01)	0.5 (0.01)	31.2 (1.75)	0.6 (0.00)	0.5 (0.01)	0.6 (0.00)	0.9 (0.00)	0.5 (0.00)
1×10^{-4}	1.2 (0.00)			18.6 (0.41)					
5×10^{-5}				1.8 (0.06)					
0††	0.6 (0.00)	0.6 (0.00)	1.1 (0.00)	0.8 (0.00)	0.5 (0.00)	1.0 (0.00)	1.0 (0.00)	1.3 (0.00)	1.0 (0.00)

* For each treatment 100–200 metaphase plates were scored for chromosome and chromatid breaks and exchanges.
† Cu(II) and Mn(II) were added as glycine complexes at a molar ratio of metal:glycine = 1:10.
‡ Fe(III) was added as an EDTA complex (Fe: EDTA = 1:2).
§ Mitotic inhibition: less than one metaphase among 5000 cells.
‖ Frequency (per cent) of metaphase figures having at least one aberration.
¶ Average number of exchanges per metaphase plate.
** Toxic: no detectable mitosis and variable cell loss.
†† Controls: cells treated with chemical mixtures lacking isoniazid [e.g. 0.8 (0.00) is the frequency of aberrations induced by the 10^{-5} M manganese complex].

azid and isoniazid/metal mixtures. This indicates that the effect of the metal complexes is catalytic.

Catalase and reduced glutathione (GSH) were highly effective in inhibiting the damaging effects of isoniazid. Catalase (0.1 mg/ml) or reduced glutathione (10^{-2} M) diminished the frequency of chromosome aberrations induced by isoniazid from a peak value of 42 per cent to background levels (about 1 per cent). Moreover, the potent toxic and clastogenic effects of isoniazid/manganese mixtures were nearly abolished by addition of these inhibitors. Only at the highest doses of isoniazid (near 10^{-1} M) were the protective effects of catalase and glutathione not complete. Heat-inactivated catalase or oxidized glutathione (GSSG) at equivalent concentrations did not reduce the damaging effects of isoniazid/manganese mixtures (data not shown).

Partial protection was achieved by the addition of catalase to the isoniazid/copper mixtures. Mitotic inhibition and up to 23 per cent chromosome aberrations were still observed. However the dose–response curve was shifted to about 5-fold higher concentrations of isoniazid. Glutathione could not be used as an inhibitor in solutions containing copper since glutathione/copper mixtures induce aberrations [8].

The three transition metals appear to interact with isoniazid via different reaction mechanisms. Manganese catalyzes the autoxidation of isoniazid to produce approximately equimolar concentrations of hydrogen peroxide [9]. In buffers similar to the culture medium (bicarbonate or phosphate), about 90 per cent of the isoniazid was oxidized in 1 hr [9]. Furthermore, H_2O_2 alone induces chromosome aberrations in CHO cells [7, 8] in the same concentration range (10^{-4}–5×10^{-4} M) as that of isoniazid in the presence of manganese. Catalase protects against the chromosome-damaging action of isoniazid/manganese over a 1000-fold range of isoniazid concentrations (10^{-4}–10^{-1} M). Catalase does not inhibit manganese-catalyzed autoxidation of isoniazid [9]. Heat-inactivated catalase had no inhibitory effect on the clastogenic activity of isoniazid/manganese or H_2O_2 (unpublished results). These observations provide strong evidence that H_2O_2 is the primary damaging agent in isoniazid/manganese mixtures. Free radicals derived from H_2O_2, such as the hydroxyl radical, may be the ultimate reactive species [10].

The protective effect of glutathione is related to its sulfhydryl group [11, 12]. The inhibitory activity of GSH was not observed when the corresponding disulfide (GSSG) was added to the isoniazid/manganese mixtures. In addition, GSH but not GSSG reduced the cytotoxic and clastogenic effects of H_2O_2 (unpublished results). Glutathione reacts directly with H_2O_2, with or without metal catalysis [12]. An alternative explanation, chelation of manganese by glutathione, does not seem to be important since manganese is weakly bound to the sulfhydryl group [12].

In contrast, the copper-catalyzed autoxidation of isoniazid is relatively slow [9]. Hydrogen peroxide was not detected in these mixtures [9, 13] although reactive free radicals were observed [9]. The radicals may cause the toxic [9, 13], cytostatic, and clastogenic effects of isoniazid/copper. However, catalase reduced unscheduled DNA synthesis [6] and provided partial (5-fold) protection against chromosome aberrations (Table 1). This suggests that H_2O_2 is involved in the mechanism of damage. It is likely that H_2O_2 is formed during copper-catalyzed autoxidation of isoniazid but is rapidly consumed in subsequent reactions to form free radicals or to further oxidize isoniazid. Catalase may compete favourably for H_2O_2 at a rate (about 1 M min^{-1}) sufficient to reduce the production of damaging species.

Iron does not catalyze the autoxidation of isoniazid [9]. Morever, iron–EDTA inhibits the chromosome-damaging effect of isoniazid, which may be catalyzed by other trace metals present in the culture medium. Iron may consume H_2O_2 by a catalase-like reaction or by scavenging free radicals [10].

These results with isoniazid differ significantly from those obtained in the DNA repair assay [6]. While the effects of manganese and copper were similar in both assays, unscheduled DNA synthesis was *not* observed in cells treated with freshly prepared isoniazid alone. (As a consequence, the protective effect of iron–EDTA was also not detected in the DNA repair assay.) This difference may result from inhibition of unscheduled DNA synthesis by isoniazid. Isoniazid inhibits post-replication repair of DNA damage [14] and may also block excision repair.

Manganese is a potent agent in enhancing chromosome aberrations, DNA damage [6, 15], and toxicity by isoniazid. The concentrations of manganese in several human tissues exceed 10^{-5} M [16, 17], and there may be accumulation of manganese in disease states, such as alcoholism, which are associated with isoniazid toxicity in man [16]. The availability of tissue manganese to interact with isoniazid and related hydrazides is not known. The quantities of manganese in the diet can vary greatly, and homeostatic control of tissue levels is maintained predominantly by excretion of excess manganese [16]. The amounts of manganese in diets for laboratory rodents are commonly much greater than the amounts in human diets [16]. Thus, variations in manganese levels in different test species or in human diets may account for some of the variable results obtained in studies of mutagenesis and toxicity by isoniazid. Moreover, manganese may also influence tissue damage by the isoniazid metabolite acetylhydrazine [18].

These results strongly suggest that examination of the interactions of metals with ioniazid *in vivo* is warranted. Such investigations will contribute to an increased understanding of the function of transition metals in carcinogenesis and mutagenesis.

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Hepatic mitochondrial cholesterol hydroxylase activity—a cytochrome P-450-catalyzed mono-oxygenation refractory to cobaltous chloride

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It is well known that the administration of cobaltous chloride to rats results in an inhibition of hepatic heme biosynthesis [1–3] and a marked decrease in the concentration of cytochromes P-450 in hepatic microsomes [1–6]. Since hepatic mitochondria have been shown recently to contain a form of cytochrome P-450 which catalyses the 25- and 26-hydroxylations of cholesterol* [7–11], the present study was undertaken to determine if mitochondrial and microsomal cytochromes P-450 are affected similarly by cobaltous chloride.

Glucose-6-phosphate, yeast glucose-6-phosphate dehydrogenase (type VII), NADP, NADPH (type III) and cholesterol (chromatography standard grade) were obtained from the Sigma Chemical Co. (St. Louis, MO). [7(*n*)-^{3}H]Cholesterol (specific activity, 9.5 Ci/mmole) was obtained from Amersham/Searle (Arlington Heights, IL), and the radioactive purity was confirmed to be greater than 98 per cent by thin-layer chromatographic analysis. Ethylmorphine hydrochloride was purchased from Merck & Co., Inc. (Rahway, NJ); thin-layer plastic sheets (5 × 20 cm), precoated with silica gel G, were obtained from Brinkman Instruments, Inc. (Westbury, NY). Metyrapone and aminoglutethimide were gifts from the Ciba–Geigy Corp. (Summit, NJ). Goat anti-bovine adrenal ferredoxin and goat preimmune immunoglobulin (Ig) fractions were obtained as described previously [12]. Sheep anti-rat hepatic microsomal NADPH-cytochrome *c* (P-450) reductase and sheep preimmune IgG fractions were prepared as described previously [13]. All other chemicals employed were of the highest purity available.

Male albino Holtzman rats, 150–250 g, were used throughout these studies. Cobaltous chloride ($CoCl_2 \cdot 6H_2O$) was administered subcutaneously once daily for 4 days at a dose of 60 mg/kg. Control rats received

* Trivial names used are: cholesterol, 5-cholesten-3β-ol; 25-hydroxycholesterol, 5-cholesten-3β,25-diol; 26-hydroxycholesterol, 5-cholesten-3β,26-diol; and Ig, immunoglobulin.

Table 1. Effects of mono-oxygenase inhibitors and adrenal ferredoxin on rat hepatic mitochondrial cholesterol hydroxylase activity

Addition	Concentration	% Conversion in 30 min*
None		1.30 ± 0.06 (100)
Metyrapone	0.1 mM	0.61 ± 0.11† (46)
	1.0 mM	0.18 ± 0.03† (14)
Aminoglutethimide	1.0 mM	0.70 ± 0.12† (53)
	10.0 mM	0.04 ± 0.04† (3)
Adrenal ferredoxin	2.0 mM	5.40 ± 0.37† (392)
Goat anti-adrenal ferredoxin Ig	0.1 mg/mg mitochondrial protein	0.31 ± 0.08† (23)
Goat preimmune Ig	0.1 mg/mg mitochondrial protein	1.03 ± 0.08‡ (80)
Sheep anti-NADPH-cytochrome *c* (P-450) reductase IgG	0.1 mg/mg mitochondrial protein	1.04 ± 0.05‡ (80)
Sheep preimmune IgG	0.1 mg/mg mitochondrial protein	1.08 ± 0.14 (83)

* Each value represents the mean ± S.E. for four determinations with the percentage of control activity given in parentheses.
† $P < 0.001$, compared with the control value.
‡ $P < 0.02$, compared with the control value.