Toxicity and Mutagenicity of Hexavalent Chromium on *Salmonella typhimurium*

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Four hexavalent and two trivalent chromium compounds were tested for toxicity and mutagenicity by means of the *Salmonella typhimurium*/mammalian-microsome test. All hexavalent compounds yielded a complete inhibition of bacterial growth at doses of 400 to 800 µg/plate, a significant increase of his^+ revertant colonies at doses ranging from 10 to 200 µg, and no effect at doses of less than 10 µg. The distinctive sensitivity of the four *Salmonella* strains tested (TA1535, TA1537, TA98, and TA100) suggested that hexavalent chromium directly interacts with bacterial deoxyribonucleic acid by causing both frameshift mutations and basepair substitutions. The latter mutations, which are prevalent, are amplified by an error-prone recombinational repair of the damaged deoxyribonucleic acid. On the average, 1 µmol of hexavalent chromium yielded approximately 500 revertants of the TA100 strain, irrespective of the compound tested (sodium dichromate, calcium chromate, potassium chromate, or chromic acid). The mutagenic potency of the hexavalent metal was not enhanced by adding the microsomal fraction of rat hepatocytes, induced either with sodium barbital or with Aroclor 1254. The two trivalent compounds (chromium potassium sulfate and chromic chloride), with or without the microsomal fraction, were neither toxic nor mutagenic for the bacterial tester strains.

Chromium, particularly in the trivalent form, is considered to have a low order of toxicity (26). However, clinical studies indicate that individuals exposed for long periods of time to the hexavalent chromium ion can develop tissue necrosis (7, 16). Moreover, both statistical and epidemiological investigations have demonstrated an association between inhalation of chromium compounds and development of lung cancer (6, 7).

Attempts to reproduce and explore the carcinogenic activity of chromium in laboratory animals have demonstrated the oncogenicity of several chromium compounds in rodents (5, 10, 12, 13, 15). However, further studies are required to complete these findings (8).

The introduction of the *Salmonella*/mammalian microsome test has provided a useful tool for detecting carcinogens that cause point mutations (1, 2). The test uses uniquely constructed mutants of *Salmonella typhimurium* as sensitive indicators of deoxyribonucleic acid (DNA) damage and mammalian liver extracts for metabolic conversion to their active mutagenic forms.

In this paper we report the results of investigations on the effects and mechanisms of action of various chromium compounds in the *Salmonella* test system.

MATERIALS AND METHODS

Chromium compounds. Hexavalent chromium was tested as sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$; molecular weight, 298.00) and chromic acid (CrO_3; molecular weight, 99.99), from the Merck Co. (Darmstadt, West Germany), and as calcium chromate ($CaCrO_4$; molecular weight, 156.09) and potassium chromate (K_2CrO_4; molecular weight, 194.20), from the B.D.H. Co. (Poole, England). Trivalent chromium was tested as chromium potassium sulfate [$CrK(SO_4)_2 \cdot 12H_2O$; molecular weight, 499.42) and chromic chloride ($CrCl_2 \cdot 6H_2O$; molecular weight, 266.45), from B.D.H.

Bacterial tester strains. Strains TA1535, TA1537, TA98, and TA100 of *S. typhimurium* were kindly supplied by Bruce N. Ames. These strains contain mutations in the histidine operon, resulting in a requirement for histidine, and are reverted back to prototrophy by mutagens. In addition, they lack the excision repair system ($\Delta uvrB$ mutation) and the lipopolysaccharide barrier that coats the surface of bacteria (*rfa* mutation).

Mutagenesis tests. The *Salmonella*/mammalian-microsome mutagenicity tests have been described in detail by Ames et al. (3). Briefly, the plate incorporation assay was performed by mixing 0.1 ml of an overnight nutrient broth culture of each bacterial tester strain, 0.1 ml of an aqueous solution of each compound, and 2 ml of molten top agar (0.6% Difco agar, 0.5% NaCl) supplemented with 10% of a sterile solution of 0.5 mM L-histidine · HCl-0.5 mM biotin.

The mixture was poured on a minimal glucose agar medium (1.5% Difco agar in Vogel-Bonner medium E [21] with 2% glucose)-solidified layer in petri plates (12 ml of medium in 8- by 2.2-cm glass plates).

The spot test was performed by adding 10 μl of compounds on sterile 5-mm filter-paper disks at the center of the plate, over the layer of top agar incorporating the bacterial strain.

Revertant colonies were scored after 48 h at 37°C in the dark. Only colonies detected in plates with a normal background of bacterial growth, due to the presence of histidine traces in the top agar, were considered to be revertant.

Both the plate incorporation assay and the spot test were carried out with and without addition of the S-9 mix (0.5 ml) to the top agar. The S-9 mix contains, in a final volume of 1 ml: 5 mM glucose 6-phosphate, 4 mM nicotinamide adenine dinucleotide phosphate, 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate (pH 7.4), and 0.04 to 0.1 ml of S-9 fraction. The latter is the 9,000 × g supernatant of liver homogenates from rats induced orally with sodium barbital or intraperitoneally with a polychlorinated biphenyl mixture (Aroclor 1254). Aroclor 1254 was a gift from W. G. Papageorge (Monsanto Co., St. Louis, Mo.).

RESULTS

The effects of hexavalent chromium compounds on *S. typhimurium* strains showed an apparent shift from toxicity to mutagenicity according to the amounts of compounds tested with bacterial strains.

A representative example of the change from the toxic to the mutagenic response is provided by the results of the spot test. A halo of complete bacterial inhibition is detectable in the area surrounding a paper disk saturated with a chromium solution (Fig. 1). Conversely, a ring of revertant colonies appears around the inhibition area, which provides evidence for the mutagenic activity of the metal.

The plate incorporation assay allowed dose response curves to be constructed. The results of one of these experiments are reported in Fig. 2. The incorporation of 400 to 800 μg of hexavalent chromium compounds into the top agar layer, resulting in a concentration of 200 to 400 μg/ml, produced inhibition of bacterial growth. At lower doses (10 to 200 μg), a mutagenic response was shown by a significantly increased number of revertant colonies, as compared with chromium-free controls. Slight differences were recorded among the compounds tested according to their chromium content. The mutagenic response was found to disappear when the amounts of all compounds were further reduced.

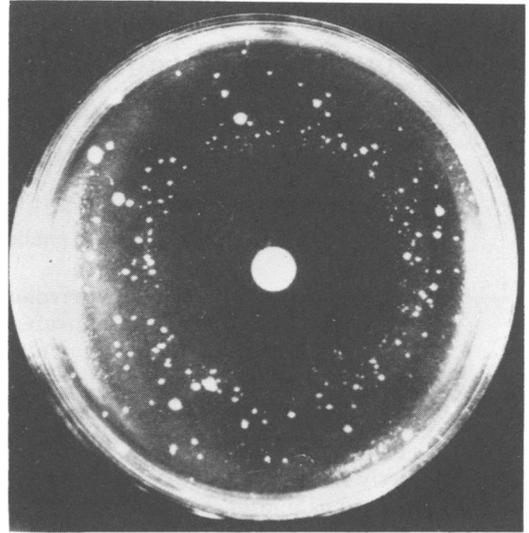The mutagenic effects were detected in plates containing strains TA1537, TA98, and TA100.

FIG. 1. *Example of a spot test performed with hexavalent chromium compounds. Addition of 10 μl of $CaCrO_4$ (40 μg) to the paper disk at the center of the plate results in a large halo of complete bacterial inhibition, which is surrounded by a ring of his$^+$ revertant colonies of strain TA100 of S. typhimurium.*

The latter was the most effective strain in detecting mutagenicity of hexavalent chromium, although it showed the highest spontaneous mutation rate. Conversely, the number of colonies was increased to a lesser extent with strain TA1535 in the presence of hexavalent chromium.

These results were confirmed in a large number of comparative assays. No significant difference could be detected among the four hexavalent compounds under test when the number of chromium-induced revertants was related to the chromium content of each compound (Fig. 3). On the average, 1 μmol of the hexavalent metal was found to account for approximately 500 his$^+$ TA100 revertant colonies, by subtracting spontaneous from chromium-induced revertants.

Addition of various amounts of S-9 mix, prepared either from rats administered sodium barbital or Aroclor, did not result in any further increase of revertants.

The number of revertant colonies growing in plates incorporating trivalent chromium compounds (chromium potassium sulfate and chromic chloride) did not differ significantly from that of spontaneous revertants for any of the bacterial tester strains, over the dose range active for the hexavalent metal. No toxic or mutagenic effect could be detected in either the
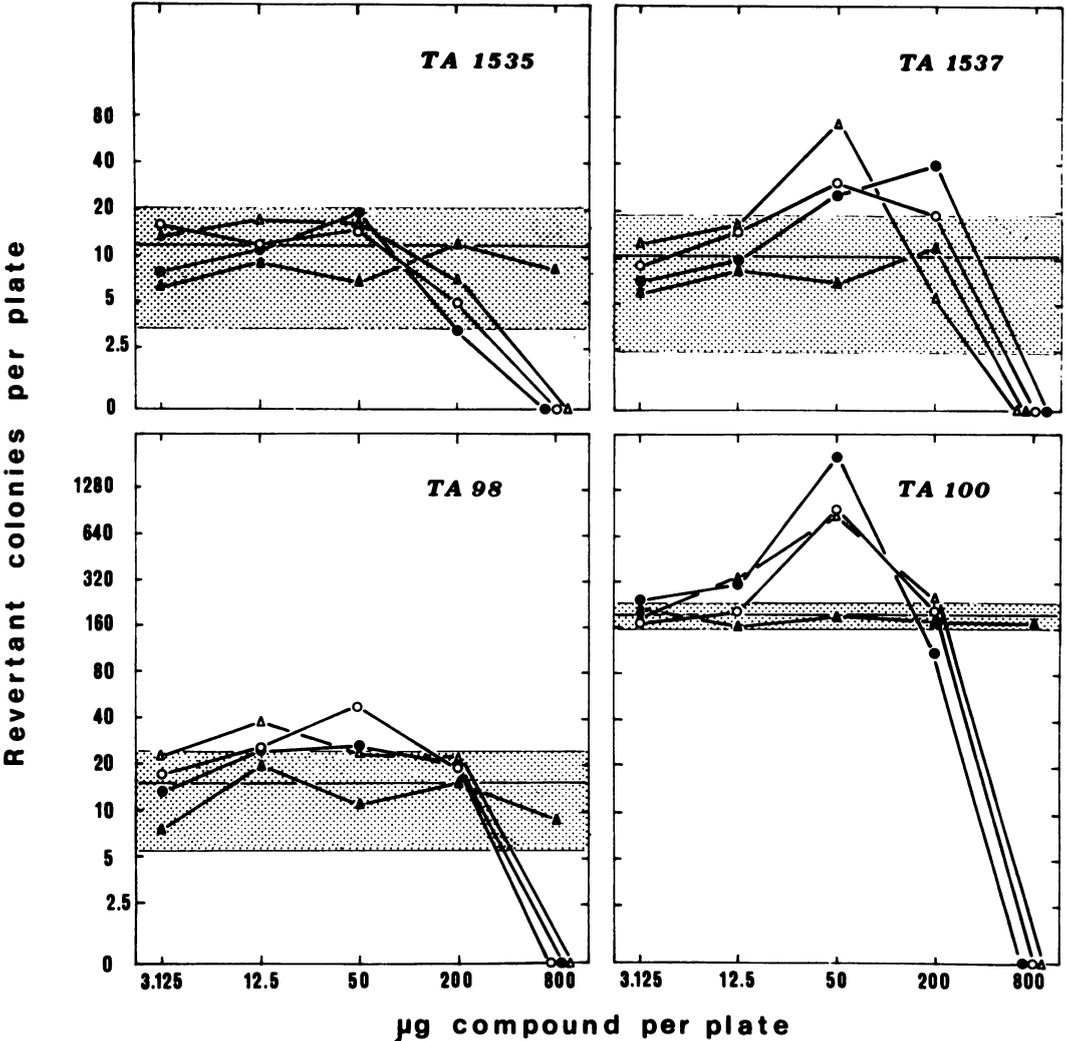
FIG. 2. *Dose response curves showing the activity of various amounts of calcium chromate (○), sodium dichromate (●), chromic acid (△), and chromium potassium sulfate (▲) on strains TA1535, TA1537, TA98, and TA100 of S. typhimurium. The horizontal line indicates the mean of controls (spontaneous revertants), and the shaded area indicates the 95% confidence interval.*

presence or absence of the S-9 mix, or by increasing the dose to 20 mg/plate.

DISCUSSION

The effects of hexavalent chromium on *S. typhimurium his⁻* strains shifted from toxicity to mutagenicity depending on concentration of the metal, without any significant differences among the four compounds under scrutiny.

The mechanisms of chromium-induced mutagenicity can be elucidated by checking the distinctive sensitivity of the *S. typhimurium* strains tested. In fact, strain TA1535, which

was a *hisG46* mutation, detects mutagens causing base pair substitutions, whereas strain TA1537 (*hisC3076* mutation), as well as TA1538 (*hisD3052* mutation), detects various kinds of frameshift mutagens (3). Strains TA98 and TA100, which were obtained from TA1538 and TA1535 mutants, respectively, by transferring a resistance factor (R-factor), are typically reverted by mutagens working through an error-prone recombinational repair (11).

On these bases, the results obtained suggest that the hexavalent chromium ion causes both frameshift errors and basepair substitutions in
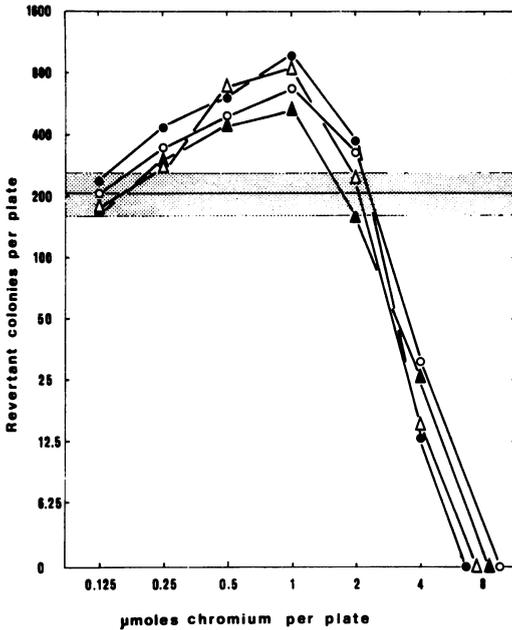
FIG. 3. *Dose response curves showing the activity of hexavalent chromium contained in four compounds (○, calcium chromate; ●, sodium dichromate; △, chromic acid; ▲, potassium chromate) on strain TA100 of S. typhimurium. The horizontal line indicates the mean of controls (spontaneous revertants), and the shaded area indicates the 95% confidence interval.*

bacterial DNA. This is in agreement with the conclusions drawn by Tamaro et al. (18), who investigated the effects of potassium chromate and dichromate on TA1535 and TA1538 strains. The monitoring of TA98 and TA100 strains has additionally shown that the frameshift mutations, which are less pronounced on a quantitative basis, arise from a direct interaction between chromium and bacterial DNA. Conversely, basepair substitutions become much more evident as a consequence of an error-prone recombinational repair of DNA. In fact, the R-factor in strain TA98 did not seem to increase the mutation rate, as was the case with strain TA100.

In previous studies, Venitt and Levy (20) were able to demonstrate basepair substitutions by sodium, potassium, and calcium chromates in *trp⁻* strains of *Escherichia coli*, whereas Nishioka (14) showed that the DNA damage by potassium chromate and dichromate on *Bacillus subtilis* can be repaired through recombinational mechanisms.

Trivalent chromium, tested as chromium potassium sulfate and chromic chloride, did not show any toxic or mutagenic effect on the same

Salmonella strains, even at doses of 20 mg/plate. This is consistent with the current view that trivalent chromium is considerably less toxic than hexavalent chromium (19).

Although the quantitative data obtained with the *Salmonella* model cannot be extrapolated to human cells, the present findings suggest that all the hexavalent chromium compounds so far tested are potentially carcinogenic or genotoxic in vivo, provided that adequate concentrations are reached in tissues.

It is relevant in this connection that in humans all the chromium ingested via food and water (about 60 μg/day) is eliminated with urine and feces. Conversely, practically all the metal inhaled from air is retained in human lungs, which, on the other hand, is the only tissue in which chromium shows a progressive accumulation during life (17). Interestingly, the size of chromate dust (0.35 ± 0.18 μm) is consistent with an easy penetration and retention in the lung (9). Large amounts of the metal (54 to 17,385 μg of chromium per g of tissue ash) were, in fact, detected in the lungs of men exposed for long periods in chromate manufacturing plants, even after some years of exposure withdrawal (4).

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