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METABOLISM OF THE CARCINOGEN CHROMATE BY RAT LIVER MICROSOMES

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SUMMARY

The metabolism of chromate by rat liver microsomes has been studied. Incubation of chromate with microsomes in the presence of the enzyme cofactor NADPH, resulted in reduction of chromate. In the absence of NADPH no reduction occurred. Only a small amount of chromate reduction was seen with NADPH in the absence of microsomes. Time course studies, microsome and NADPH concentration dependence studies resulted in conditions giving complete reduction of chromate. The possible relationship of metabolism of chromate to its carcinogenicity and mutagenicity is discussed.

INTRODUCTION

Epidemiological data have shown that the incidence of respiratory cancer among chromate workers is much higher than that expected in the general population(1). There is presently no conclusive evidence for a particular carcinogenic chromium compound. However, hexavalent chromium compounds such as calcium chromate have been implicated as possible carcinogens by epidemiological and animal studies(2).

Chromium(VI) compounds have also been shown to be mutagens in bacterial systems. Nishioka(3) found potassium chromate and dichromate were mutagenic using the Rec assay in B. Subtilis. The mutagenicity of dichromate was lost in the presence of sodium sulfite, a reducing agent, suggesting that the oxidized state of chromium was required for mutagenicity. Venitt and Levy(4) found that sodium, potassium and calcium chromates were mutagenic in E. coli WP2 (trp^-) strains, whereas potassium chromium(III) sulfate was not

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studies was the replacement of H-Gly-OH by other amino acid and it's ester: H-Sar-OH, H-β-Ala-OH and H-Gly-OMe. The reasons for the study on glycine analogs as follows: sarcosine is only the existence of the N-methyl group of peptide chain and substitution for H-β-Ala-OH is elongated peptide chain of H-Asp(Gly)-OH by one methylene goup. N-terminal β-aspartyl residue was replaced by H-α-aspartyl, H-D-β-aspartyl and Z-β-aspartyl. The reason for the study on aspartic acid analogs as follows: the necessary distance between amino acid carboxyl groups was studied by moving the peptide bond to the α-carboxyl of aspartic acid and the required stereochemistry of the molecule was determined by synthesizing optical isomer of H-Asp(Gly)-OH. On the other hand, it is founded that H-Asp(Gly)-OH is produced from hemoglobin by the action of proteolytic enzyme during the uremia[2,3]. In view of this investigation it was of interest to examine the suppression test of PHA-induced lymphocyte transformation of H-Ser-Asp-Gly-Leu-OH which was modified the C- and N-terminal vicinity being adjacent to H-Asp(Gly)-OH moiety in hemoglobin (β chain: position 72-75)[3].

The conventional method for the peptide synthesis is used in this investigation. The synthetic rout for the tetrapeptide, H-Ser-Asp-Gly-Leu-OH, is illustrated in Fig. 1. H-Leu-OBzl Tos[4] was condensed with Boc-Gly-OH[5] by the HOBt-DCC method[6] to yield Boc-Gly-Leu-OBzl (I). After removal of the Boc group of I with TFA, the resulting dipeptide ester was condensed with Boc-Asp(OBzl)-OH[6] by the similar manner as described I to yield Boc-Asp(OBzl)-Gly-Leu-OBzl (II). After removal of the Boc group of II with TFA, the resulting tripeptide ester was condensed with Boc-Ser(Bzl)-OH by the similar manner as described I to yield Boc-Ser(Bzl)-Asp(OBzl)-Gly-Leu-OBzl (III). The fully protected tetrapeptide (III) was hydrogenated over 5% palladium-carbon in AcOH solution overnight. The hydrogenated product was treated with MSA in the presence

given 0.5 ml i.p. injections of 20 mg sodium dichromate per kg suspended in sesame oil or 0.5 ml sesame oil. Following injection, the rats were given water but no food. The animals were sacrificed after 16-19 hours and the livers were removed. Microsomal pellets were isolated from the livers by the differential centrifugation method described by Pietropaolo and Weinstein(7). Protein concentration of the final microsomal suspension was determined by the Lowry method(8), using BSA as the standard.

Chromate Incubation with Microsomes. All solutions were buffered at pH=7.4 with 0.05M TRIS·HCl. The initial concentration of chromate (from potassium dichromate, which is in the chromate form at pH 7.4) in the assay system was 4.0×10^{-4}M. The desired concentration of NADPH was achieved by adding the correct amount of a freshly prepared 0.025M NADPH solution to the reaction mixture. The reaction was started by adding aliquots of the standard NADPH solution to the assay system to give the desired final concentration of NADPH. Final volume of the reaction mixture was 2.0 ml. The mixtures were incubated at 37°C for the indicated time period with shaking.

Following incubation, the reaction was stopped by extracting the microsomal protein from the aqueous solution by vigorously vortexing the mixture with an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuging for 15 min. at 1500g(9). The upper aqueous phase was carefully removed and extracted four times more, after which no protein precipitate was visible in the organic layer.

Chromium and NADPH Determination. Following protein extraction, the concentration of chromate and the concentration of NADPH in the aqueous solution were determined spectrophotometically by measuring the absorbance of each solution at 340nm and 400nm on a Perkin-Elmer Coleman 561 spectrophotometer. The absorbance readings were corrected by subtracting readings determined for solutions which originally contained microsomes, but no chromate or NADPH, and which had been incubated and extracted as above. The extinction coefficients of Cr(VI) and NADPH at 340nm and 400nm were determined by measurements on standard solutions. Using these extinction coefficients (Chromate, $\varepsilon_{340}=1.64\times10^{3}M^{-1}\ cm^{-1}$, $\varepsilon_{400}=1.76\times10^{3}M^{-1}\ cm^{-1}$, NADPH, $\varepsilon_{340}=6.2\times10^{3}M^{-1}\ cm^{-1}$, $\varepsilon_{400}=1.1\times10^{2}M^{-1}\ cm^{-1}$) and the corrected absorbances of each solution at the two wavelengths, the concentration of chromate and NADPH were calculated.

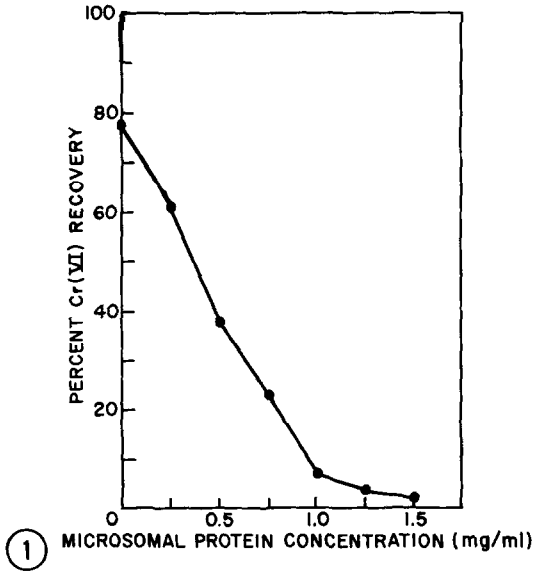
The total chromium concentration of the extracted solution was determined by atomic absorption measurements using a Perkin-Elmer 503 atomic absorption spectrophotometer equipped with an HGA-2100 graphite furnace. Average recovery of total chromium in the aqueous solution was >90% and was independent of the percent Cr(VI) recovery.

In all experiments the values given represent the mean of duplicate determinations.

CAUTION: Chromate is a carcinogen and should be handled with care.

RESULTS AND DISCUSSION

Incubation of chromate with rat liver microsomes and NADPH resulted in reduction of hexavalent chromium to trivalent chromium.



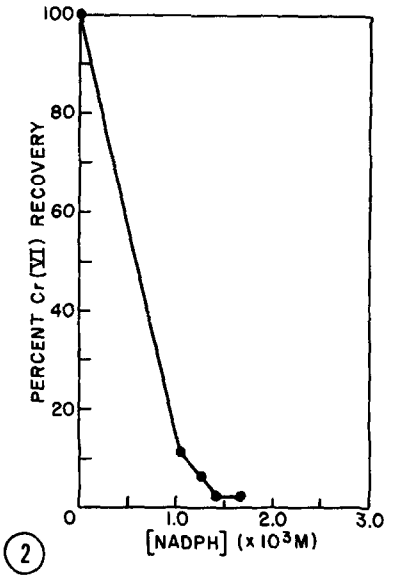


Figure 1. Effect of microsomal protein concentration on the recovery of chromate from solutions incubated in the presence of NADPH. Conditions used were: 0.05M TRIS·HCl, pH 7.4, [NADPH] = 1.66×10^{-3}M, $[CrO_4^{2-}]_0 = 4.0 \times 10^{-4}$M, 37°C with shaking for 6 hours. Microsomes were isolated from rats that had been previously injected with sodium dichromate (20 mg/kg).

Figure 2. Effect of NADPH concentration on the recovery of chromate from solutions incubated in the presence of microsomes. Conditions used were: 0.05M TRIS·HCl, pH 7.4, $[CrO_4^{2-}]_0 = 4.0 \times 10^{-4}$M, 1.5 mg/ml microsomal protein (from sodium dichromate injected rats), 37°C with shaking for 6 hours.

During this reaction the intense absorption band of chromate at 373nm ($\varepsilon_{373}=4.7\times10^{3}M^{-1}\ cm^{-1}$) disappeared and a new band characteristic of chromium(III) appeared at 580nm ($\varepsilon_{580}=24M^{-1}\ cm^{-1}$). Figure 1 shows that the amount of chromate reduced depended on the concentration of microsomes. With a microsomal protein concentration of 1.5 mg/ml essentially complete loss of chromate was observed after a six hour incubation period. The amount of chromate reduction was also dependent on the concentration of NADPH (Figure 2). Maximum conversion to chromium(III) occurred at a NADPH concentration of 1.44×10^{-3}M upon incubation with 1.5 mg/ml microsomes for six hours.

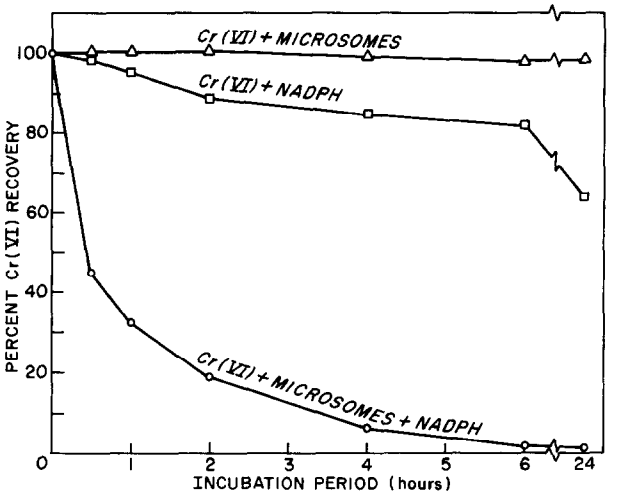


Figure 3. Time course studies on the recovery of chromate from solutions incubated with rat liver microsomes and/or NADPH. All solutions contained 0.05M TRIS·HCl, pH 7.4, $[CrO_4^{2-}]_0$ = 4.0×10^{-4}M and (O) 1.5 mg/ml microsomal protein, [NADPH]= 1.44×10^{-3}M; (□) [NADPH] = 1.44×10^{-3}M; and (Δ) 1.5 mg/ml microsomal protein. Microsomes were isolated from rats previously injected with sesame oil. All incubations were done at 37°C with shaking.

Time course studies (Figure 3) in the presence of 1.5 mg/ml microsomes and 1.44×10^{-3}M NADPH showed that chromate was completely reduced to chromium(III) after six hours incubation. Incubation of chromate with microsomes in the absence of NADPH led to complete recovery of chromate even after 24 hours incubation. This indicated that chromate did not oxidize any components of the microsomal system under these conditions. In the absence of microsomes chromate was reduced at a very slow rate by NADPH. Less than 20% of the chromate was reduced after incubation for six hours with NADPH, compared with greater than 98% reduction with NADPH and microsomes present. These results suggest that a NADPH requiring enzyme or enzyme system is involved in the in vitro metabolism of chromate. Many such enzymes are present in microsomes, e.g., the mixed function oxidases.

These studies of metabolism of chromate by microsomes coupled

with the epidemiological and animal studies(2), and the results of the mutagenicity assays(3-6) suggest that chromium(III) may be the ultimate carcinogen of chromium. The Loeb assay(6) using a subcellular DNA synthesizing system showed that trivalent chromium caused misincorporation of noncomplementary nucleotides into DNA, whereas hexavalent chromium did not. Cellular assay systems such as E. coli WP2(trp^-) reversion(4), B. Subtilis(his^-) Rec(3) and S. typhimurium(his^-) reversion(5) showed hexavalent chromium was strongly mutagenic whereas trivalent chromium was not mutagenic(3,4) or was much less mutagenic(5). Hexavalent chromium was required for activity in cellular assays, however, our studies suggest that upon entry of chromate into the cells the endoplasmic reticulum bound enzymes will reduce chromate to chromium(III).

Chromium(III) may pass through the cellular membrane only with difficulty. Gray and Sterling(10) found that the red blood cell was impermeable to the chromium(III) cations, whereas the anionic hexavalent chromate readily diffused through the cell membrane. They suggested that chromate was reduced to the cationic trivalent state within the red blood cell. Ehrlich mouse ascites carcinoma cells were also found to be impermeable to chromium(III)(11). Chromate was readily taken up by the tumor cells and intracellularly reduced to the trivalent state. An electron spin resonance signal characteristic of chromium(III) has been found in thyroid tissues treated with dichromate(12).

Microsomal enzymes may be involved in the reduction of chromate to chromium(III) upon entering the cell and, therefore, produce a good electrophile which is capable of binding cellular nucleophiles in proteins and nucleic acids. Chromium(III) bound to nucleic acid has been isolated from tissues previously treated with solutions containing chromium(VI)(13). Chromium bound to protein has been

isolated from cells treated with chromate(10,11,14), even though most of the chromium in the cells (>80%) was coordinated to small organic ligands such as citrate and amino acids(15,16).

In conclusion, metabolism of chromate by microsomal systems occurs in vitro and may be an important factor in determining the mutagenicity and carcinogenicity of chromium(VI) compounds in vivo.

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REFERENCES

1. Enterline, P.E. (1974) J. Occup. Med. 16, 523-526.
2. National Institute for Occupational Safety and Health (1975) Criteria for a Recommended Standard: Occupational Exposure to Chromium(VI), pp. 1-151, U.S. Department of Health, Education and Welfare, Washington, D.C.
3. Nishioka, H. (1975) Mut. Res. 31, 185-189.
4. Venitt, S. and Levy, L.S. (1974) Nature 250, 493-495.
5. Löfroth, G. and Ames, B. (1977) Abstracts Environmental Mutagen Society, p. 30, Environmental Mutagen Society, Colorado Springs, Colorado.
6. Sirover, M.A. and Loeb, L.A. (1976) Science 194, 1434-1436.
7. Pietropaolo, C. and Weinstein, I.B. (1975) Cancer Res. 35, 2191-2198.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Borgen, A., Darvey, H., Castagnoli, N., Crocker, T.T., Rasmussen, R.E., and Wang, I.Y. (1973) J. Med. Chem. 16, 502-506.
10. Gray, S.J. and Sterling, K. (1950) J. Clin. Invest. 29, 1604-1613.
11. Rajam, P.C. and Jackson, A.-L. (1958) Proc. Soc. Exp. Biol. Med. 99, 210-213.
12. Gutierreg, P.L., Sarna, T. and Swartz, H.M. (1976) Phys. Med. Biol. 21, 949-954.
13. Herrmann, H. and Speck, L.B. (1954) Science 119, 221.
14. Ronai, P.M. (1969) Blood, 33, 408-413.
15. Sanderson, C.J. (1976) Proc. Roy. Soc. Lond. B. 192, 221-239.
16. Sanderson, C.J. (1976) Transplantation, 21, 526-529.