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Effects of Trivalent and Hexavalent Chromium on Lipid Peroxidation in Rat Liver Microsomes

Masanori Yonaha, Yuko Ohbayashi, Nahoko Noto, Emiko Itoh,[1a] and Mitsuru Uchiyama[1b]

Hokkaido Institute of Pharmaceutical Sciences[1a] and National Institute of Hygienic Sciences[1b]

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The effects of heavy metals, especially chromium, on lipid peroxidation in rat liver microsomes were studied. Lipid peroxidation was determined as thiobarbituric acid-reacting materials.

At lower concentrations in the range of 1—100 μM, both hexavalent and trivalent chromium inhibited lipid peroxidation induced by ascorbate or NADPH in microsomes. In the presence of ascorbate, the inhibitions decreased on treatment with sulfhydryl reagents and were partly overcome by the addition of tartaric acid. It is suggested that chromium, presumably in the trivalent form, may bind with sulfhydryl groups of protein, resulting in the inhibition of lipid peroxidation. In the presence of NADPH, hexavalent chromium showed very potent inhibition. Hexavalent and trivalent chromium did not inhibit the electron-transport system in microsomes. The hexavalent form may act as a radical scavenger, since it strongly inhibited carbon tetrachloride-stimulated lipid peroxidation, could reduce 2,2′-diphenyl-β-picrylhydrazyl to some extent in the presence of a reducing agent such as ethanol, and inhibited NADPH-induced peroxidation markedly in the presence of sulfhydryl compounds.

Above 1 mM, hexavalent chromium caused lipid peroxide formation in microsomes, apparently not associated with iron, while trivalent chromium showed an inhibitory effect.

Keywords——chromium; lipid peroxidation; rat liver microsomes; radical scavenger; pro-oxidant; carbon tetrachloride; aminopyrine demethylase; NADPH-cytochrome c reductase; thiobarbituric acid method

Introduction

Lipid peroxidation *in vivo* is thought to result in membrane damange and to play an important role in tissue injuries induced by chemicals; it was reported to be involved in lung damage caused by air pollutants and herbicides, and in acute liver injury caused by CCl_4 and ethanol.[2] Formation of lipid peroxides may also occur in association with heavy metal intoxications, since cadmium chloride [3]and mercuric chloride[4] decrease the activity of glutathione peroxidase, which catalyzes the destruction of organic hydroperoxides.[5]

In *in vitro* experiments, Wills[6] showed that Co^{2+} and Mn^{2+} catalyzed rapid peroxidation of linoleic acid, but Fe^{2+}, Fe^{3+}, Cu^{2+} were much less effective. In some tissues, iron was required as the only metal able to support lipid peroxidation;[7] no other metal ion could replace inorganic iron adequately.[8] In rat liver microsomes, lipid peroxidation induced by the

1) Location: *a) 7-1, Katsuraoka-cho, Otaru, Japan; b) Kamiyoga-1-chome, Setagaya, Tokyo.*
2) L. Plaa and H. Witschi, *Ann. Rev. Pharmacol. Toxicol.*, **16**, 125 (1976).
3) S.T. Omaye and A.L. Tappel, *Res. Commun. Chem. Pathol. Pharmacol.*, **12**, 695 (1975).
4) T. Morimura, *Nihon Univ. J. Med. (Japan. ed.)*, **34**, 825 (1975).
5) C. Little and P.J. O'Brien, *Biochem. Biophys. Res. Commun.*, **31**, 145 (1968).
6) E.D. Wills, *Biochim. Biophys. Acta*, **98**, 238 (1965).
7) A.A. Barber, *Lipids*, **1**, 146 (1966); A.A. Barber, *Radiation Res. Suppl.*, **3**, 33 (1963); J.D. Robinson, *Arch. Biochem. Biophys.*, **112**, 170 (1965).
8) E.D. Wills, *Biochem. J.*, **113**, 325 (1965).

addition of ascorbate or NADPH is inhibited by Co^{2+}, Mn^{2+},[8,9] Cu^{2+}, Zn^{2+} and Hg^{2+},[8] but Utley *et al.*[10] found stimulative effect of Hg^{2+} and *p*-chloromercuribenzoate on ascorbate-induced lipid peroxidation in mouse liver microsomes.

Since some metal ions have been reported to show either stimulative or inhibitory action on lipid peroxidation in tissues, we tested various metals to determine whether they are stimulative or inhibitory for lipid peroxidation in liver whole homogenate. Cd^{2+}, Ni^{2+}, Hg^{2+} had stimulative effects, while chromium was found to be inhibitory.

Bunyan *et al.*[11,12] reported that hexavalent chromium ($Cr_2O_7^{2-}$, CrO_4^{2-}) as well as Co^{2+} and Mn^{2+} prevented dialuric acid-induced hemolysis and formation of peroxides in vitamine E-deficient erythrocytes. However, no effect of chronmium on lipid peroxidation has ever been reported in microsomes. The present paper reports the inhibitory action of trivalent and hexavalent chromium at low concentrations on lipid peroxidation in rat liver microsomes and the pro-oxidant activity of hexavalent chromium at higher concentrations.

Materials and Method

Materials——The following metal compounds were used; $HgCl_2$, $CdCl_2$, $Pd(CH_3COO)_2$, Na_2CrO_4, $MnSO_4$, $Cu(CH_3COO)_2$, $FeSO_4$ (Wako Pure Chemical Industries, Ltd.), and $CrCl_3$, $CoCl_2$, $ZnCl_2$, $NiCl_2$, $NaAsO_2$, $NaHAsO_4$ (Kanto Chemical Co., Inc). NADPH, $NADP^+$, glucose-6-phosphate-Na, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Ltd. Cytochrom c (II-A) was purchased from Sigma Chemical Co., and thiobarbituric acid was obtained from E, Merck. Carbon tetrachloride (UV-Sol), from Kanto Chemical Co., was distilled with all-glass apparatus, and the distillate obtained was used in the experiments. All chemicals and reagents employed were of commercial reagent-grade quality.

Preparation of Microsomal Suspensions——Male rats of the Wistar strain were maintained on rat chow MF (Oriental Yeast Co., Ltd.). Rats (6—8 weeks old) were lightly anesthetized with ether, then the liver was perfused with ice-cold saline, removed, and homogenized in 3 volumes of 0.05 M Tris-KCl (0.14 M) buffer (pH 7.4). The microsomal fraction was prepared by the method of Chvapil *et al.*,[13] and the microsomes obtained were suspended in 0.05 M Tris-KCl (1.0 ml/g liver). Protein was determined by the method of Lowry *et al.*[14]

Lipid Peroxidation——The incubation mixture[13,15] contained 0.2 ml of microsomal suspension, 0.2 ml of ascorbate (5 mM) or cofactors (NADPH, 2.0 mM; glucose-6-phosphate, 20 mM; $MgSO_4$, 50 mM; nicotinamide, 200 mM), 0.2 ml of metal compound, and an aliquot of 0.05 M Tris-KCl (pH 7.4) in a final volume of 2.0 ml. Incubations were carried out aerobically at 37° for 60 min. When necessary, anaerobic conditions were produced by passing a stream of nitrogen which had been bubbled through alkaline pyrogallol.

Assays——Lipid peroxides were measured by the thiobarbituric acid (TBA) method of Uchiyama *et al.*[16] A portion (0.5 ml) of the incubation mixture was treated with 3 ml of phosphoric acid (1%) and 1 ml of TBA (0.6%), and the whole was heated for 45 min in a boiling-water bath. The mixture was then cooled, and the colored pigment was extracted with 4 ml of *n*-butanol. The optical density of the *n*-butanol layer was determined at 535 and 520 nm using a Shimadzu UV-300 spectrophotometer. Aminopyrine demethylase and NADPH-cytochrome c reductase activities were assayed according to the method described by Mazel.[17]

Unless otherwise specified, all values are means of three determinations.

Results and Discussion

Effects of Various Metals on Lipid Peroxidation

The effects of 10 heavy metals, arsenite, and arsenate on the rate of lipid peroxide formation in rat liver whole homogenate were compared (Fig. 1). The effects of metal ions were

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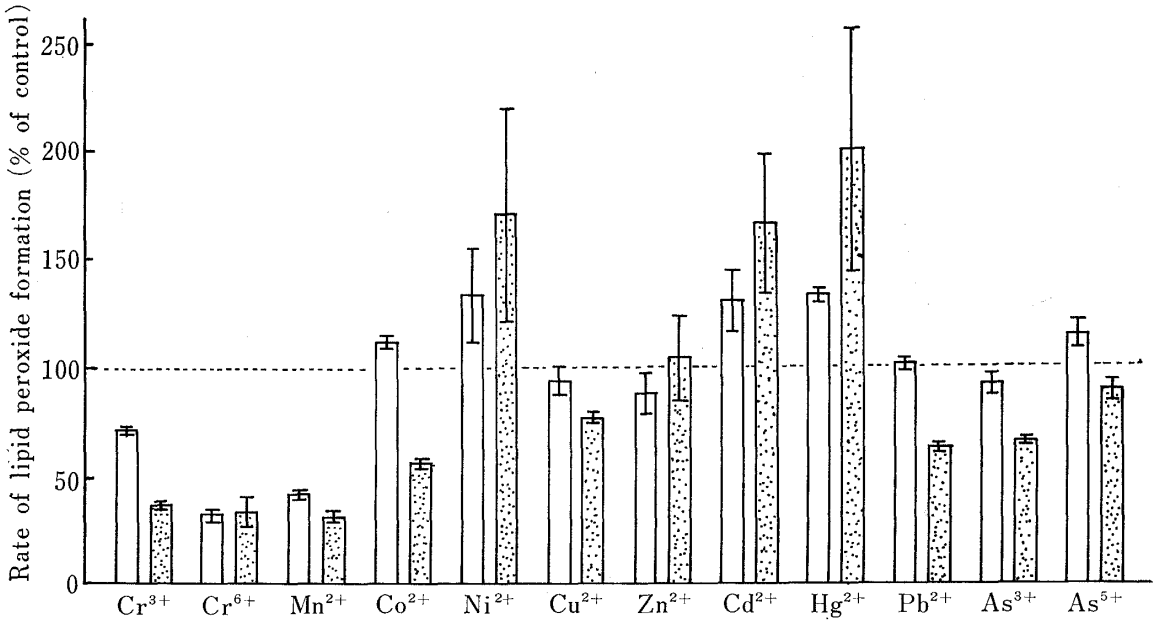

Fig. 1. Effects of Different Metals on Lipid Peroxide Formation by Rat Liver Whole Homogenate

The rate of peroxide formation is expressed as percentages of control values without the addition of metals. Data represent the means ±SD of results from three to nine determinations.
□, 6 μM; ▨, 60 μM.

classified into three groups as follows: (1) stimulative, Ni^{2+}, Cd^{2+}, Hg^{2+}; (2) inhibitory, Cr^{3+}, Cr^{6+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+}, As^{3+}; (3) ambiguous, Zn^{2+}, As^{5+}.

Effects of Different Concentrations of Chromium on Lipid Peroxidation

The effects of chromium over the range of 1—100 μM were studied on enzymatic and non-enzymatic peroxidation in rat liver microsomal suspensions. At these concentrations, lipid peroxidation induced by both ascorbate and NADPH was inhibited by trivalent chromium

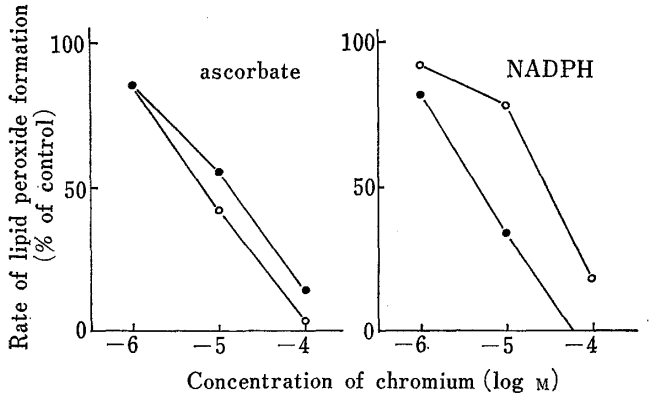

Fig. 2. Effects of Various Concentrations of Chromium on Lipid Peroxide Formation Induced by Ascorbate and NADPH in Microsomal Suspensions

The rates of peroxide formation are expressed as percentages of control values without the addition of chromium. Optical densities ($OD_{535-520}$/mg protein) obtained in the presence of ascorbate or NADPH without the addition of chromium were 0.695 and 0.151, respectively. The optical density measurements in the presence of NADPH and 100 μM hexavalent chromium gave negative values.
—●—, Cr^{6+}; —○—, Cr^{3+}.

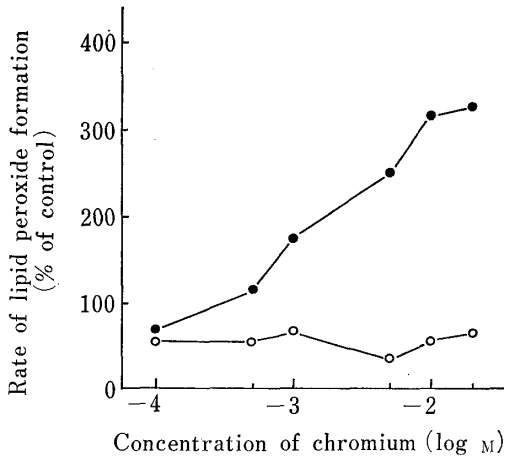

Fig. 3. Lipid Peroxide Formation by Hexavalent Chromium at Higher Concentrations in Microsomal Suspensions

Lipid peroxide formation by the addition of chromium alone in the absence of stimulators of lipid peroxidation (ascorbate or NADPH) was measured. The rates of peroxide formation are expressed as percentages of control values without the addition of chromium.
—●—, Cr^{6+}; —○—, Cr^{3+}.

as well as by the hexavalent form (Fig. 2). The inhibition of lipid peroxidation by trivalent chromium in the presence of ascorbate was more potent than that by hexavalent chromium, but in the presence of NADPH, the inhibitory effects were reversed.

Above 1 mM chromium, lipid peroxidation in the absence of potent stimulators such as ascorbate or NADPH was still inhibited by trivalent chromium, whereas lipid peroxide formation increased with increase in the concentration of hexavalent chromium added (Fig. 3). Lipid peroxidation induced by hexavalent chromium, at high concentrations remained the same in the presence or absence of ascorbate or NADPH.

We next examined whether lipid peroxide formation at high concentrations of hexavalent chromium was caused by oxygen bound to chromium or molecular oxygen in air (Fig. 4). Lipid peroxidation induced by hexavalent chromium was appreciably diminished under a stream of nitrogen.

In vitro lipid peroxidation in tissues is inhibited by EDTA,[18] but lipid peroxidation induced by hexavalent chromium (10 mM) could not be inhibited by EDTA. These results indicate that lipid peroxidation induced by hexavalent chromium is not related to iron in microsomes, but is presumably due to the action of hexavalent chromium itself as a strong oxidizing agent. Therefore, it is considered that free radicals of fatty acid should be produced by hexavalent chromium during peroxidation, since lipid peroxidation by hexavalent chromium was stimulated in air.

Hexavalent chromium compounds such as chromic acid or chromates are usually irritating and toxic to all tissues. Workers in the chrome industry exhibit perforation and ulceration of the nasal septum, and ulcers on the hand or in areas where dust can accumulate.[19]

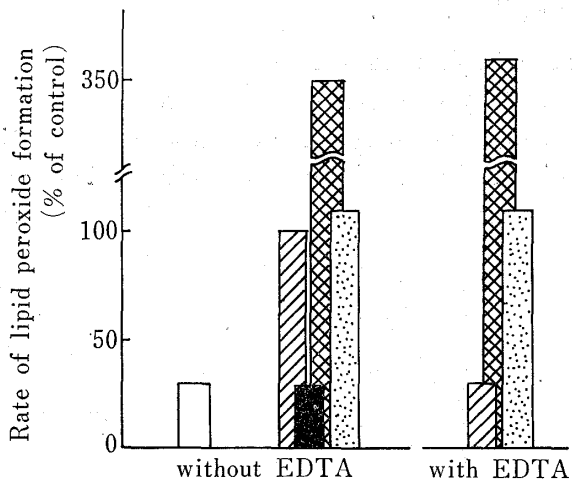

Fig. 4. Lipid Peroxidation Induced by Hexavalent Chromium (10 mM) with or without EDTA in Microsomal Suspensions under a Stream of Nitrogen

EDTA was added at a concentration of 0.1 mM. The rates of peroxide formation are expressed as percentages of control values without chromium and EDTA under air. □, no incubation; ▨, without Cr^{6+} under air; ■, without Cr^{6+} under nitrogen; ▩, with Cr^{6+} under air; ▩, with Cr^{6+} under nitrogen.

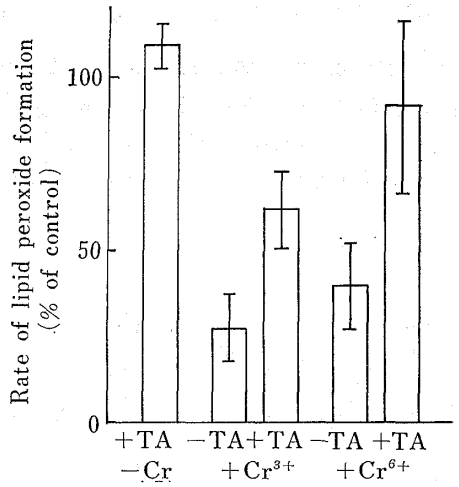

Fig. 5. Effects of Tartaric Acid (TA) on Chromium Inhibition of Lipid Peroxide Formation Induced by Ascorbate in Microsomal Suspensions

The concentrations of chromium and tartaric acid were 10 μM and 0.3 mM, respectively. The rates of peroxide formation are expressed as percentages of control values without the addition of chromium and tartaric acid. Data represent the means ±SD of results from three separate experiments.

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Whether these tissue impairments are associated with direct lipid peroxidation by hexavalent chromium is of considerable interest.

Inhibitory Action of Chromium on Ascorbate-induced Lipid Peroxidation

When hexavalent chromium is reduced to the trivalent state by reducing agents, the resulting trivalent chromium can be chelated by various complexing agents, ascorbic acid[20] and tartaric acid.[21] The inhibition of lipid peroxidation by both trivalent and hexavalent chromium was partly eliminated by the addition of tartaric acid (Fig. 5). It is suggested that the valence state of chromium inhibiting lipid peroxidation may be trivalent, because ascorbate-induced lipid peroxidation was strongly inhibited by Cr^{3+}. The apparent inhibitory action of hexavalent chromium might then be due to trivalent chromium formed as a result of partial reduction by ascorbate.

Chromium in the trivalent form easily binds with proteins, such as egg albumin and human plasma protein[22] and transferrin,[23] while the hexavalent form is first reduced to the trivalent form, then naturally binds in the same way.[22,23] Sodium chromate, on the other hand, forms stable chromium (III) complexes by reductive chelate formation with chelating agents containing sulfuhydryl groups, such as penicillamine and cysteine.[24] Chromate also binds to proteins such as hemoglobin and serum albumin, probably *via* sulfhydryl groups.[24] The effects of sulfhydryl reagents added prior to the addition of chromium on the inhibition of lipid peroxidation by chromium in the presence of ascorbate was investigated (Fig. 6). The inhibition of lipid peroxidation by chromium was almost removed by the addition of NEM. Mercuric chloride had almost the same effect as NEM. Although lipid peroxide formation was stimulated in boiled microsomes, the diffrrence of inhibitory effect of chromium in the absence or presence of sulfhydryl reagents was indistinct. These results indicate that the inhibitory action of chromium on lipid peroxidation may involve binding with protein sulfhydryl groups. Utley *et al.*[10] suggested that sulfhydryl agents may produce a change in the tertiary structure of microsomal Fex, thereby making the protein-bound iron available for

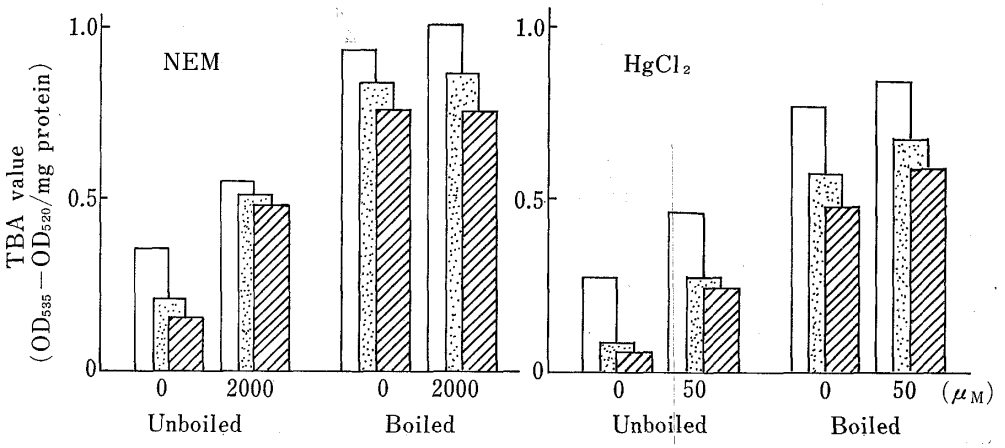

Fig. 6. Effect of Sulfhydryl Reagents on Chromium Inhibition of Lipid Peroxide Formation Induced by Ascorbate in Unboiled and Boiled Microsomal Suspensions

Boiled microsomes were prepared by heating microsomes for 10 min in a boiling-water bath, followed by homogenization. Mercuric chloride and N-ethylmaleiimide (NEM) were added at concentrations of 0.05 mM and 2 mM, respectively, before the addition of chromium.
□, without Cr; ▨, with 10 μM Cr^{6+}; ▨, with 10 μM Cr^{3+}.

20) M.H. Samitz, D.M. Schneider, and S.A. Katz, Arch. *Environ. Health*, **17**, 44 (1968).
21) M.H. Samitz, S. Gross, and S. Katz, *J. Invest. Derm.*, **38**, 5 (1962).
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24) Y. Sugiura, Y. Hojo, and H. Tanaka, *Chem. Pharm. Bull.*, **20**, 1362 (1972).

catalysis of peroxidation. On the other hand, the inhibition of ascorbate-induced lipid peroxidation by chromium may be due to the envelopment of iron in the protein involved in lipid peroxidation as a result of binding with the sulfhydryl groups.

Effect of Chromium on Drug Metabolizing Enzymes

The oxidation of aminopyrine and the reduction of cytochrome c in liver microsomes were examined to determine whether chromium inhibited the microsomal electron-transport chain in connection with the inhibitfon of NADPH-induced lipid peroxidation by chromium (Fig. 7). Trivalent and hexavalent chromium, at concentrations below 100 μM, did not affect the activity of NADPH-cytochrome c reductase, while the aminopyrine demethylase activity was stimulated about 20% by chromium, compared with the control. Therefore, the possibility can be excluded that the inhibition of NADPH-induced lipid peroxidation by chromium may be caused by the prevention of electron flow in the electron-transport chain in liver microsomes. Some enzymes containing cytochrome P-450 in liver microsomes were inhibited by peroxidation,[25] probably by toxic lipids formed.[26] In contrast, as reported by Kamataki[27] and Wills,[28] drug-metabolizing activities were stimulated by EDTA, which inhibited lipid peroxidation in microsomes, and the increase of the aminopyrine demethylation activity by chromium may occur in a similar manner.

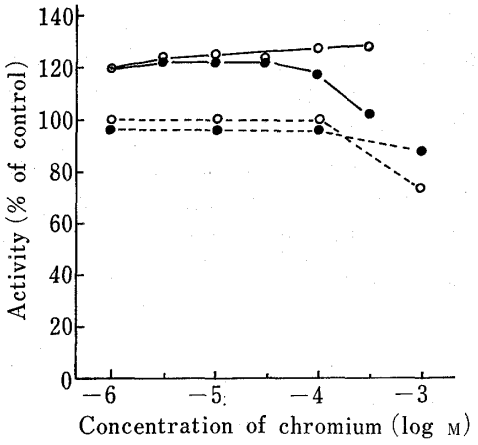

Fig. 7. Effects of Various Concentrations of Chromium on Aminopyrine Demethylase and NADPH-Cytochrome c Reductase Activities in the Microsomal Suspensions

Incubations were carried out aerobically at 37° for 25 min. The enzyme activities in the presence of chromium were taken as control values.
Aminopyrine demethylase: —●—, Cr^{6+}; —○—, Cr^{3+}.
NADPH-cytochrome c reductase: ···●···, Cr^{6+}; ···○···, Cr^{3+}.

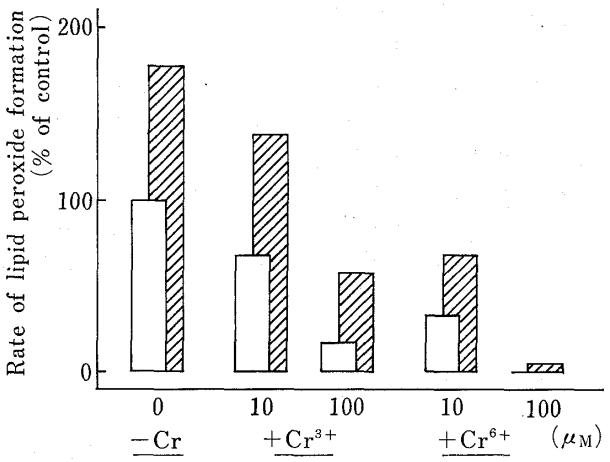

Fig. 8. Effect of Chromium on Lipid Peroxide Formation Stimulated by Carbon Tetrachloride in Microsomal Suspensions

CCl_4 (5 μl) was added to the incubation medium containing NADPH in the case of carbon tetrachloride-stimulated peroxidation. The rates of peroxide formation are expressed as percentages of control values in the presence of NADPH without the addition of chromium.
□, without CCl_4; ▨, with CCl_4.

Effect of Chromium on Free Radicals

As reported by Recknagel *et al.*[29] and Slater *et al.*,[30] lipid peroxide formation in liver microsomes was stimulated by the addition of carbon tetrachloride in the presence of NADPH. We examined whether carbon tetrachloride-stimulated lipid peroxidation is inhibited by

25) E.D. Wills, *Biochem. J.*, **123**, 983 (1971); J. Högberg, A. Bergstrand, and U. Jakobsson, *Eur. J. Biochem.*, **37**, 51 (1973).
26) A.M. Hruszkewycz, E.A. Glende, and R.O. Recknagel, *Toxicol. Appl. Pharmacol.*, **46**, 695 (1978).
27) T. Kamataki, Y. Hojo, and H. Kitagawa, *Biochem. Pharmacol.*, **22**, 3199 (1973).
28) E.D. Wills, *Biochem. J.*, **113**, 333 (1969).
29) R.O. Recknagel and A.K. Ghoshal, *Lab. Invest.*, **15**, 132 (1966).
30) T.F. Slater and B.C. Sawyer, *Biochem. J.*, **123**, 805 (1971).

chromium (Fig. 8). Carbon tetrachloride is transformed to $\cdot CHCl_3$ in the presence of NADPH in microsomes, and reaction of the radical results in peroxidation of the membrane lipids.[34] Trivalent and hexavalent chromium inhibited lipid peroxidation in the presence of carbon tetrachloride, and in the case of hexavalent chromium, very potent inhibition of peroxidation was observed. The results suggest that hexavalent chromium may act as a free radical scavenger in part.

We therefore tested whether hexavalent chromium could react with the stable free radical, 2,2′-diphenyl-β-picrylhydrazyl[31] (DPPH·) (Fig. 9). Trivalent chromium at the concentrations tested did not react with DPPH·, but hexavalent chromium reacted at least partially, like tocopherol and N,N-diphenyl-*p*-phenylene diamine, which are known radical scavengers. Hexavalent chromium, however, does not react directly with DPPH·, since it is never oxidized further. It is assumed that the reaction of hexavalent chromium with DPPH· may be coupled with the oxidation of ethanol (used as the solvent). In practice, when acetone was used as the solvent instead of ethanol, hexavalent chromium could not react with DPPH·. Furthermore, it was found that hexavalent chromium inhibited NADPH-induced peroxidation more strongly in the presence of cysteine or glutathione, which showed inhibitory action alone, although no such effect of sulfhydryl compounds was observed on the inhibition by trivalent chromium (Fig. 10). The results presented above suggest that hexavalent chromium may act as a free radical scavenger in the presence of reducing agents. It is possible that the more potent inhibition by hexavalent chromium of NADPH-induced lipid peroxidation may be dependent upon its action as a radical scavenger.

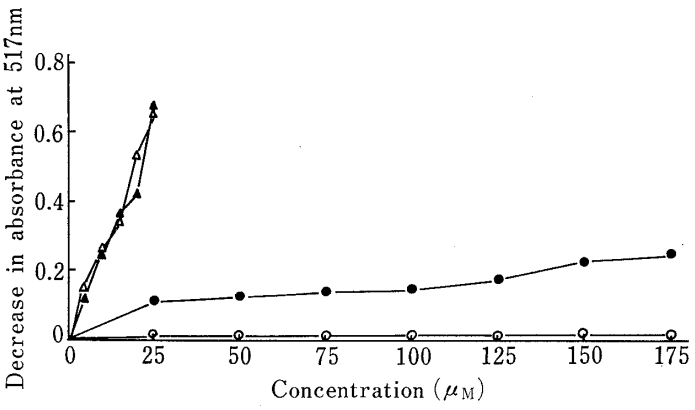

Fig. 9. Reactivities of DPPD, Tocopherol, Cr^{3+}, and Cr^{6+} with a Stable Free Radical, 2,2′-Diphenyl-β-picrylhydrazyl (DPPH·)

The incubation system,[32] which consisted 200 mM acetate buffer (pH 5.5) 1.0 ml, 250 μM DPPH·-ethanol 2.0 ml, distilled water 1.0 ml, and ethanol 1.0 ml, was incubated at 30° for 30 min. DL-α-Tocopherol and N,N′-diphenyl-*p*-phenylene diamine (DPPD) were dissolved in the ethanol, and chromium was dissolved in the distilled water. —△—, DPPD; —▲—, tocopherol; —●—, Cr^{6+}; —○—, Cr^{3+}.

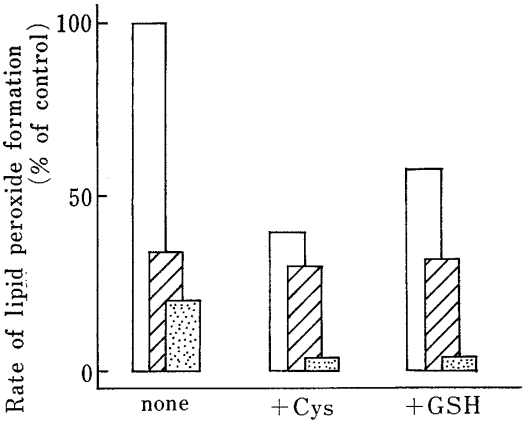

Fig. 10. Effects of Sulfhydryl Compounds on Chromium inhibition of Lipid Peroxide Formation Induced by NADPH in Microsomal Suspensions

Cysteine and glutathione were added each at a concentration of 100 μM. The rates of peroxide formation are expressed as percentages of control values in the presence of NADPH without the addition of chromium. □, without Cr; ▨, with 10 μM Cr^{6+}; ▨, with 10 μM Cr^{3+}.

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