# EPR Study on the Interaction of Hexavalent Chromium with Glutathione or Cysteine: Production of Pentavalent Chromium and its Stability

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## Abstract

The reduction of hexavalent chromium (Cr(VI)) by glutathione was studied by EPR spectrometry and comparing it with that by cysteine. The characteristics of production of the pentavalent chromium (Cr(V)) species were studied. Two Cr(V) species, which were characterized by $g$ values of 1.995–1.996 and 1.985–1.986, were detected at pH values above 5.0, whereas at pH 3.0 and 4.0 a single species of Cr(V) with a $g$ value of 1.989–1.990 was found. The Cr(V) species were relatively long-lived and most stable at pH 7.0, where signals of two Cr(V) species were observed for more than 30 min. The intensities of the Cr(V) signals were pH-dependent, increasing with an increase in pH from 3.0 to 8.0. At neutral pH, the signal corresponding to the species of the larger $g$ value increased markedly with an increase in glutathione concentration. Stable production of Cr(V) by glutathione was also confirmed by EPR measurements at 77 K. On the other hand, the characteristics of Cr(V) generation by cysteine were quite different. Production of the Cr(V) species was confirmed by a sharp single signal with a $g$ value of 1.984–1.987. The signal intensity corresponding to Cr(V) generation did not change much with a change in pH from 3.0 to 6.0; at pH 7.0 only a small signal was observed, and at pH 8.0 no signal was observed. Moreover, the life-time of the Cr(V) signal was shorter than that observed for the reduction with glutathione. These results suggest that Cr(V) may be stabilized in glutathione solution by its suitable redox potential and ligand structure.

## Introduction

Hexavalent chromium (Cr(VI))** is known to have toxicity and carcinogenicity to organisms [1, 2]. It has been revealed that Cr(VI) is taken up through anion channels and then reduced to the final product of trivalent chromium (Cr(III)) by cellular reductants [3, 4]. Recently, many studies have been made to reveal the process of reduction, and pentavalent chromium (Cr(V)) has been found as a stable intermediate by EPR spectrometry in the interaction of Cr(VI) with various biological substances, such as rat liver microsome in the presence of NaDPH [5], ribonucleotides and related molecules [6], humic acid [7], soil fulvic acid [8] and milk and some of its constituents [9]. Since glutathione is present at high concentrations (0.8–8 mM) in the cytoplasm of various cells [10], it must also be involved in the reduction of Cr(VI), possibly with the production of Cr(V) as an intermediate. Actually, the generation of Cr(V) by reduction with glutathione has recently been reported [11–13]. Goodgame and Joy observed the formation of two major species of Cr(V), which were relatively long-lived, with a few other trace signals detected at room temperature at physiological pH [13]. However, it is still not clear whether the stable generation of two major Cr(V) species can be found over a wider pH range. Moreover, it is also unknown whether the generation of stable Cr(V) can be found in the reaction of Cr(VI) with other biologically important compounds like cysteine. Therefore, to elucidate the characteristics of Cr(V) production in the reaction of thiol compounds with Cr(VI), we investigated by EPR spectrometry the pH-dependency of the production of Cr(V) over a wide pH range (pH 3.0–8.0) for the reaction of Cr(VI) with glutathione or cysteine and observed the generation of stable Cr(V) species.

## Experimental

Potassium dichromate, potassium chromate and L-cysteine were purchased from Nakarai Chemicals Co. (Kyoto, Japan). The reduced form of L-glutathione was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

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**Abbreviations: Cr(VI), hexavalent chromium; Cr(V), pentavalent chromium; Cr(III), trivalent chromium; EPR, electron paramagnetic (spin) resonance; MOPS, 3-(*N*-morpholino)propane sulfonic acid.

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In the measurement of EPR spectra during the reaction of Cr(VI) with glutathione or cysteine, equal volumes of solutions of Cr(VI) and glutathione were mixed and their EPR spectra were measured at room temperature and at 77 K (liquid nitrogen temperature) after incubation of the samples. The final concentration of Cr(VI) was 10 mM in each sample. The pH-dependence of the reaction of Cr(VI) with glutathione or cysteine was examined in 1 M acetic acid–sodium acetate buffer (pH 3.0–5.0) or 1 M MOPS–NaOH buffer (pH 6.0–8.0). EPR spectra were recorded with a JES FE1XG (X-band) spectrometer with 100 kHz field modulation at room temperature and 77 K. A Takeda Riken TR522 frequency counter was used for calibration and Li–TCNQ ($g$ = 2.00252) and Mn(II) in MgO ($\Delta H_{3-4}$ = 86.9 G) were used as standards. Measurements were carried out at an output-power of 5 mW.

## Results

The process of reduction of Cr(VI) by glutathione was investigated in a molar ratio of 1 to 3, because theoretically 3 mol of glutathione are required to reduce 1 mol of Cr(VI) in aqueous solution. As shown in Fig. 1, on addition of glutathione to Cr(VI) at pH 7.0, two sharp consecutive signals with $g$ values of 1.986 and 1.996 appeared in the EPR spectrum, suggesting the generation of Cr(V) species. The $g$ values correspond well with those due to Cr(V) species reported previously [11–13]. The latter (lower-field) signal had a line-width of 9.3 G, but the former (higher-field) signal became sharper and larger when a modulation of 2 G was used in the measurement of the spectra (Fig. 1), where the line-width was about 1.9 G. This result is consistent with that of Goodgame and Joy [13], although we did not find other trace signals. In the absence of glutathione and/or Cr(VI), no EPR signal was observed. Nor, on addition of the oxidized form of glutathione, were any EPR signals observed (data not shown).

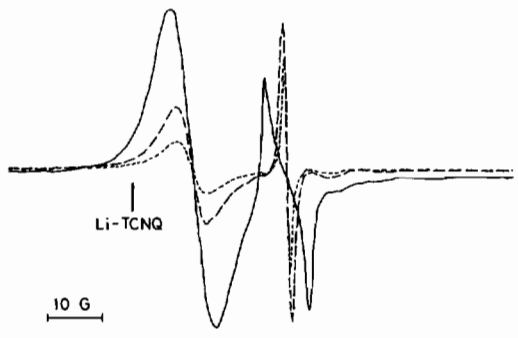


Fig. 1. EPR signals of Cr(V) at room temperature (22 °C) in a mixture of 10 mM Cr(VI) and 30 mM glutathione at pH 7.0 with a modulation of 8 G, ——; 2 G, – – –; 1 G, ----. Spectra were measured at an amplitude of 320.

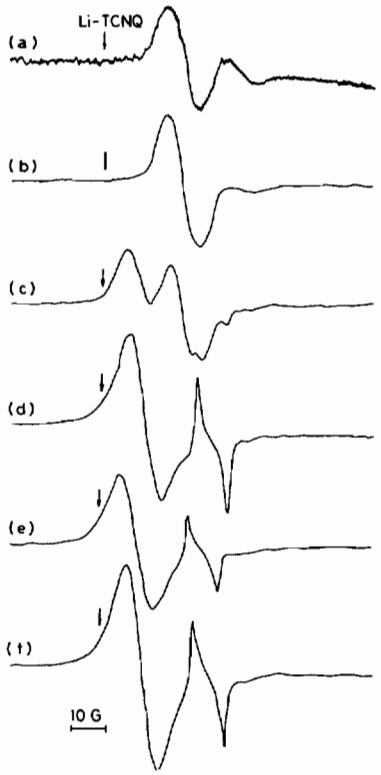


Fig. 2. pH-dependence of EPR signals of Cr(V) in a mixture of 10 mM Cr(VI) and 30 mM glutathione: (a) pH 3.0; (b) pH 4.0; (c) pH 5.0; (d) pH 6.0; (e) pH 7.0; (f) pH 8.0. Spectra were measured at room temperature at an amplitude of 3200 (a), 500 (b and c), 630 (d), or 320 (e and f) with a modulation of 8 G in about 2.5 min after mixing Cr(VI) and glutathione.

We then examined the pH-dependency of the production of Cr(V) species at pH values from 3.0 to 8.0. We analyzed EPR spectra at a modulation of 8 G, which is preferable to detect the signal with the $g$ value at lower-field. As shown in Fig. 2 (a and b), at pH values of 3.0 and 4.0, a single sharp signal with a $g$ value of 1.989–1.990 and line-width of 8.5–9.0 G was observed. This spectrum is quite different from that observed at pH 7.0. The stable broad signal was also observed at pH 3.0, concomitantly with the sharp signal (data not shown), which seemed to be due to the generation of trivalent chromium (Cr(III)). This broad signal can be observed only at pH values lower than 4.0 [14]. The intensity of the Cr(V) signal at pH 4.0 was about eight times greater than that at pH 3.0.

The EPR spectra changed markedly at pH values above 5.0, and especially above 6.0, showing two consecutive signals with $g$ values of 1.985–1.986 and 1.995–1.996, as found at pH 7.0, suggesting the generation of two Cr(V) species. The maximum

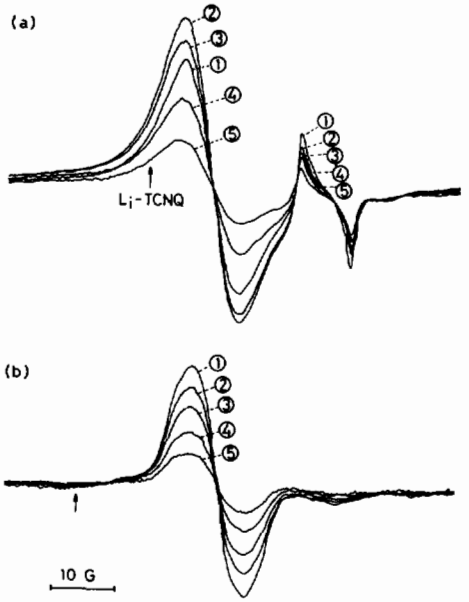


Fig. 3. Time course of change in EPR signal of Cr(V) in a mixture of 10 mM Cr(VI) and 30 mM glutathione at pH 7.0 (a) and at pH 4.0 (b). (a) 1, 4.5 min; 2, 9.2 min; 3, 11.8 min; 4, 22.5 min; 5, 30.6 min; (b) 1, 2.5 min; 2, 4.7 min; 3, 7.2 min; 4, 12.3 min; 5, 20.8 min after mixing Cr(VI) and glutathione. Spectra were measured at an amplitude of 320 (a) or 500 (b) with a modulation of 8 G at room temperature.

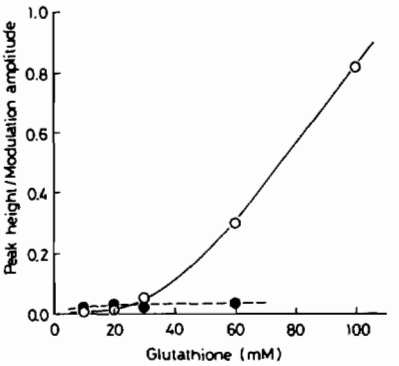


Fig. 4. Effects of glutathione concentration on the intensities of EPR signals of Cr(V) at pH 7.0: ○, signal of $g = 1.996$; ●, signal of $g = 1.986$. The maximum intensities of the signals are plotted.

intensities of both signals, especially the latter, increased with an increase in pH. The intensity of the latter signal at pH 8.0 became 3.6 times larger than that at pH 6.0, whereas that of the former increased only 1.8 times.

These Cr(V) species were relatively long-lived. The stabilities of the Cr(V) species seemed to be highest at pH 7.0, where the half-lives of both the signals were 23–30 min, as calculated by the signals shown in Fig. 3a. The intensity of the signal with the larger $g$ value reached its maximum 9 min after mixing, but decreased rapidly after that. On the other hand, the signal with the smaller $g$ value decreased gradually although its generation seemed to be rapid. At other pH values signal intensities decreased more promptly after rapid generation of the Cr(V) species, as shown in Fig. 3b for the result at pH 4.0.

Furthermore, since two signals were observed at pH 7.0, we examined the effect of glutathione concentration on the signals at this pH. As reported by Goodgame and Joy [13], we ascertained that the major Cr(V) species at high glutathione:Cr(VI) ratio is characterized by the EPR signal at $g = 1.996$, whereas the dominant species at equimolar ratios is that with a signal at $g = 1.985$. Furthermore, we found that, as shown in Fig. 4, the intensity of the signal at $g = 1.996$ increases greatly with an increase in glutathione concentration, being about 16 times more with 100 mM glutathione than 30 mM glutathione, whereas the signal intensity at $g = 1.986$ was scarcely affected by the glutathione concentration. Therefore, the production of the species with the smaller $g$ value was saturated at even low glutathione concentrations. On the other hand, the species with the larger $g$ values seemed to be produced in an increasing amount corresponding to the increase in glutathione concentration.

The above results showed that a relatively long-lived Cr(V) species was produced during the reaction of Cr(VI) with glutathione, although its chemical form at neutral pH appeared complex. To see whether this is also produced on reaction of Cr(VI) with another thiol compound, we studied the reaction of cysteine with Cr(VI). As shown in Fig. 5, a sharp signal with a $g$-value of 1.984–1.987 and linewidth of 8.1 to 18.2 G was observed at pH 3.0–7.0, suggesting the generation of a single species of Cr(V). At pH 3.0, the signal was similar to that in the Cr(VI)–glutathione system. There, the stable broad signal due to Cr(III) was also observed concomitantly with the Cr(V) signal as observed in glutathione solution. The signal intensity due to Cr(V) increased with an increase in pH, but the increase was much less than in the system containing glutathione. Moreover, the spectral pattern did not change with change in pH. The signal was small at pH 7.0 and was not observed at pH 8.0 (data not shown). Thus its pH-dependency was quite different from that in the Cr(VI)–glutathione system. Moreover, even at pH values where the generation of Cr(V) species was observed, its half-life was considerably less than that in the system containing glutathione, as shown in Table I.

We also examined the EPR spectrum of the penicillamine–Cr(VI) system and found that it was similar to that of the cysteine–Cr(VI) system. Therefore, a big difference was found for the generation and stability of Cr(V) species between glutathione

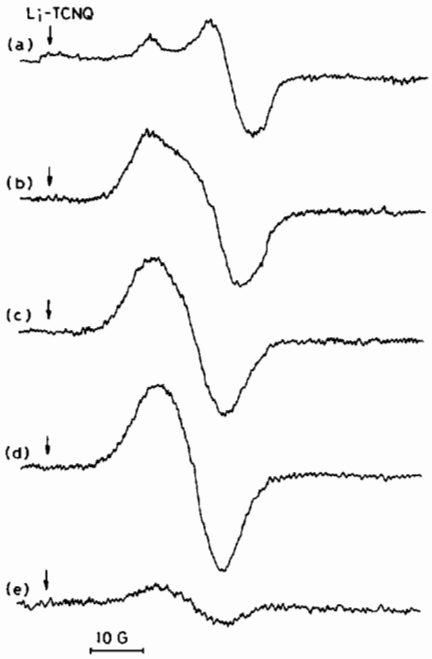


Fig. 5. pH-dependence of EPR signals of Cr(V) in a mixture of 10 mM Cr(VI) and 30 mM cysteine: (a) pH 3.0; (b) pH 4.0; (c) pH 5.0; (d) pH 6.0; (e) pH 7.0. Spectra were measured at room temperature at an amplitude of 2000 (a–d) or 3200 (e) with a modulation of 8 G after about 2.0 min after mixing Cr(VI) and cysteine.

TABLE I. Half-lives of EPR Signals of Cr(V) in a Mixture of 10 mM Cr(VI) and 30 mM Cysteine at Room Temperature

| pH | $t_{1/2}$ (min)[a] |
|---|---|
| 3.0 | 8 |
| 4.0 | 5 |
| 5.0 | 4 |
| 6.0 | 6 |
| 7.0 | 3 |

[a]Half-life ($t_{1/2}$) is defined here as the time for the intensity of the signal to become half the maximal from the beginning of the reaction.

and cysteine (or penicillamine) as a ligand. To investigate the characteristics of the Cr(V) species in more detail, we then observed the spectra of frozen samples at 77 K. As shown in Fig. 6, a single sharp signal was observed with an anisotropy at $g_{\parallel} = 2.015$ and $g_{\perp} = 1.990$ 5 min after mixing 10 mM Cr(VI) and 100 mM glutathione at pH 7.0. These values are similar to those of Kawanishi *et al.* [12] and Galleev and Usmanov [15]. The shape of the spectrum did not change with an increase in glutathione concentration, but the signal intensity increased markedly with an increase in glutathione concentration, just as the signal with the larger $g$ value at room temperature. When this mixture containing excess glutathione was

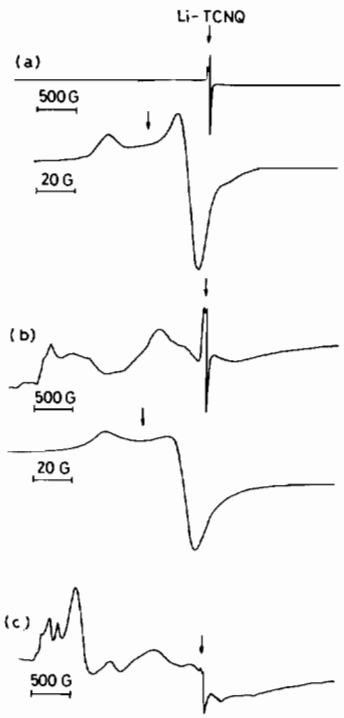


Fig. 6. EPR spectra of Cr(V) at 77 K: (a) a mixture of 10 mM Cr(VI) and 30 mM glutathione at pH 7.0 after 5.0 min preincubation at room temperature; (b) a mixture of 10 mM Cr(VI) and 100 mM glutathione at pH 7.0 after 90 min preincubation; (c) a mixture of 10 mM Cr(VI) and 30 mM cysteine at pH 6.0 after 5.0 min preincubation. The lower charts in (a) and (b) are enlargements of the Cr(V) signals. Spectra were measured at an amplitude of 12.5 (a, upper), 20 (a, lower), 50 (b, upper and lower) or 200 (c) with a modulation of 6.3 G.

allowed to stand for 90 min, the ESR spectrum clearly showed the presence of both Cr(V) and Cr(III) species (wide spectral pattern) (Fig. 6b). The spectrum due to Cr(III) was very similar to that of the Cr(III)–tetraphenylporphyrin complex [16]. After preincubation for 90 min, the intensity of the signal due to Cr(V) decreased markedly, indicating that the Cr(V) had been reduced to Cr(III) by the excess glutathione. When cysteine was used in place of glutathione, even after preincubation for only 5 min, a relatively intense signal due to Cr(III) was observed and the signal due to Cr(V) was very weak. These results indicate that reduction of Cr(VI) to Cr(V) and Cr(III) is more rapid with cysteine than with glutathione.

## Discussion

Judging from our results with two other thiol compounds, cysteine and penicillamine, Cr(V) seems to be stabilized by interaction with glutathione. We found that Cr(V) was produced in a larger

amount and was more stable in the physiological pH region than in the acidic pH region. Moreover, its production seemed to be greater in the presence of excess glutathione than with a stoichiometric amount. Since glutathione is a major thiol compound in mammalian cells [17], Cr(V) may be formed intracellularly through the interaction of Cr(VI) with this ligand, as predicted by its generation in thymocytes [18]. The long-lived Cr(V) generation may be related to the toxicity of chromium, as suggested by Jennette [5] and Goodgame and Joy [13].

The stability of Cr(V) in glutathione solution may be partly related to the redox power of the thiol. The redox potentials of glutathione and cysteine are reported to be −0.23 V and −0.32 V, respectively, at pH 7.0 and 25 °C [19]. Thus, cysteine has a relatively higher reducing potential. However, glutathione seems to have a suitable redox potential for the one-electron reduction of Cr(VI). Moreover, the stability of Cr(V) is probably due to the ability of glutathione as a ligand to form an intermediate Cr(V)–glutathione complex, because glutathione is known to form stable complexes with various metal ions such as Cr(III) [20]. Our EPR studies indicate that at pH values of above 5.0 two forms of Cr(V) species should be present in the solution. Therefore, at least one signal of Cr(V) may be that of a glutathione–Cr(V) complex. There have been many studies of the structures and mechanisms of formation of metal ion–glutathione complexes. However, in general, the formation of complexes of glutathione with metal ions is very complicated, involving dissociation of charged groups [21] and reductive reactions by thiol groups. Moreover, oxidation of glutathione further complicates the reaction. In the complex formation with Cr(V), as well as the reduced form of glutathione, the oxidized form of glutathione generated by the oxidation with Cr(VI) might also be involved, although it does not generate Cr(V), as mentioned it in the Results section. Actually, the complex of divalent copper with oxidized glutathione has been isolated both *in vitro* [22] and *in vivo* [23].

Although two separate EPR signals were observed at room temperature at neutral pH, only a single signal due to Cr(V) was observed at liquid nitrogen temperature. The difference was probably due to the difference in relaxation times of Cr(V) in these two states. Further studies are required on the structure and mechanism of formation of the Cr(V)–glutathione complex during the reaction of Cr(VI) with glutathione.

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