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Studies on the Sulfur-containing Chelating Agents. XXXIII.[1] Interaction of Penicillamine and Its Related Compounds with Chromium Ion and Hemoglobin-bound Chromium

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The chelating agents containing sulfhydryl group, such as penicillamine and cysteine, formed stable chromium (III) complexes by the reductive chelate formation with sodium chromate. The sulfur-containing ligands coordinated with chromium (III) ion through their sulfur atom, and the nephelauxetic effect in chromium (III) complexes occured to a larger extent with sulfur-containing ligands than the other ligands.

The effects of the chelating agents containing sulfhydryl group on the removal of chromium bound to hemoglobin were compared with those of ascorbic acid and EDTA, and the sulfur-containing ligands were superior to ascorbic acid and EDTA, in the rate and the amount of the removal of chromium.

It has been known that chromium (III) is moderately toxic, whereas chromium (VI) is highly toxic to organism.[3] Ascorbic acid and EDTA have been proposed as antidote against the poisoning caused by the inhalation of mist of chromic acid and the allergic response on skin caused by chromate.[4] However, these antidotes are not always effective and their mechanisms of the detoxication have not been clarified. Sodium chromate-^{51}Cr has been widely used as a labelling agent of red blood cells[5] for the diagnostic use, but little has been known on the binding of radioactive chromium with protein. Recently, research for the effective chelating agent as the antidote against various heavy metal poisonings has become urgent requirement in accord with the increase of heavy metal poisonings. In addition, the studies on the binding of metal ion to protein and the removal of metal ions bound to protein by the chelating agent are important for the investigation of the mechanism of detoxication of heavy metal by the chelating agent. Further, the binding of radioactive metal ion to protein has been considered as a basically important problem in the study of the radiopharmaceuticals. On the background mentioned above, we attempted to study the reaction of various chelating agents with both free and protein-bound chromium. We have studied the reactions of penicillamine with various heavy metal ions extensively and reported that penicillamine exhibits the characteristic reductive chelate formation with cupric ion.[6] In the reaction with chromium, the chelate formation accompanied by the reduction of chromium (VI) to chromium (III) is expected by penicillamine through its sulfhydryl group. This paper deals with the investigations on the chelate formation of penicillamine and its related compounds with chromium by electronic spectra and on the binding of chromium to hemoglobin and the effect of various chelating agents for the removal of chromium bound to hemoglobin.

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Experimental

Materials——S-Methylpenicillamine, penicillamine methyl ester and N-acetylpenicillamine were obtained by the method reported in the previous paper.[5] Penicillamine, cysteine, cysteine methyl ester, glutathione, thioglycolic acid, thiomalic acid, valine, methionine, serine, glycine, EDTA and DTPA were commercial reagent grade materials and were purified whenever necessary. Sodium chromate-^{51}Cr (42.6 m Ci/mg), chromic chloride (128.2 m Ci/mg) and L-cysteine-^{14}C (3.5 m Ci/mg) were obtained from Japan Atomic Energy Research Institute, Commissariat A L'Energy Atomique and The Radiochemical Center, respectively. Bovine hemoglobin was purchased from the Man Research Laboratories and its purity was checked by electrophoresis. The bovine serum albumin and myoglobin from horse heart were Sigma's crystallized materials. The pH of the solution was adjusted to about 7.0 with Michaelis buffer (1/30M potassium phosphate-1/30M sodium phosphate).

Spectral Measurements——Solutions of sodium chromate (10^{-2}M) and chelating agents (10^{-1}M) were mixed and the visible-ultraviolet spectra were measured by a Hitachi recording spectrophotometer model EPS-2. The measurements were carried out after heating of the reaction solution for 30 min on water bath in the reaction of chromic chloride.

Gel-filtration Technique——The binding of chromium to protein and its removal from protein were investigated by gel-filtration method, with the application of the previous reported procedure.[7] Dry Sephadex G-25 (8 g) was swelled in distilled water, packed in a column of 30 cm×2.0 cm and equilibrated with Michaelis buffer (pH 7.0). The column was calibrated isotopically with ^{51}Cr-penicillamine chelate and ^{51}Cr-hemoglobin complex. For the investigation of the binding of chromium to protein, the protein solution was reacted with chromium ion in the presence or absence of foreign metal ions at pH 7.0. For the investigation of the removal of chromium by the chelating agents, the hemoglobin solution treated with chromium ion was reacted with chelating agents at pH 7.0. At certain time intervals, the reaction mixture was added to column and eluted with the buffer solution of pH 7.0. For the separation of ternary complex, the Sephadex chromatography was carried out at 2 minutes after mixing the reactants. The radioactivity of the fractions of each 1 ml collected was determined and the absorbance was measured at 575 mμ to determine the concentration of hemoglobin. The radioactivities of ^{51}Cr and ^{14}C were determined with a Fujitsu well type scintillation counter model ATS-621 and a Beckman liquid scintillation counter model LS-233, respectively.

Result and Discussion

The spectral change of the solution of sodium chromate induced by the addition of penicillamine or cysteine which contain free sulfhydryl group was obviously observed as seen in Fig. 1. Whereas, S-methylpenicillamine and EDTA did not induce any spectral change.

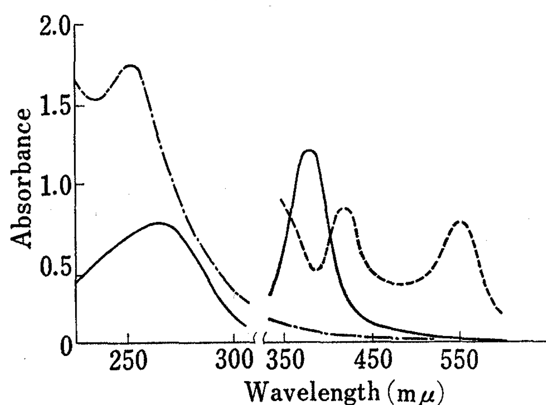

Fig. 1. Absorption Spectra of Penicillamine Chromium Complex at pH 7.0

——: 2.5×10^{-4}M of sodium chromate
– – –: 2.5×10^{-4}M of sodium chromate and 2.5×10^{-3}M of penicillamine
-------: 1.0×10^{-2}M of sodium chromate and 1.0×10^{-1}M of penicillamine

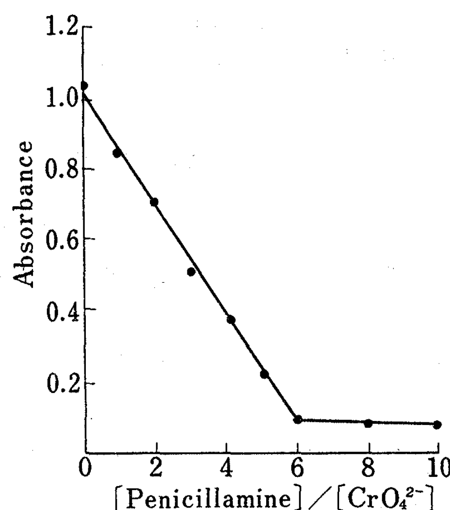

Fig. 2. Change in Absorbance at 371 mμ with Ratio of Penicillamine to Sodium Chromate at pH 7.0

concentration of sodium chromate: 2.5×10^{-4}M

7) Y. Sugiura, Y. Hojo, and H. Tanaka, *Radioisotopes* (Tokyo), **19**, 184 (1970).

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The absorption spectra obtained by the reaction with sodium chromate and the chelating agents containing sulfhydryl group are characterized as those of the octahedral chromium (III) complexes from both absorption maxima and extinction coefficient. Therefore, it is reasonably assumed that the chelating agents containing sulfhydryl group form stable chromium (III) complexes by the reductive chelate formation with sodium chromate. The result of the measurements of the absorbance at 371 mμ, indicates that this reaction is complete when the molar ratio of penicillamine to sodium chromate is 6:1, as shown in Fig. 2.

On the basis of these results, the probable reaction scheme may be represented as the following equations.

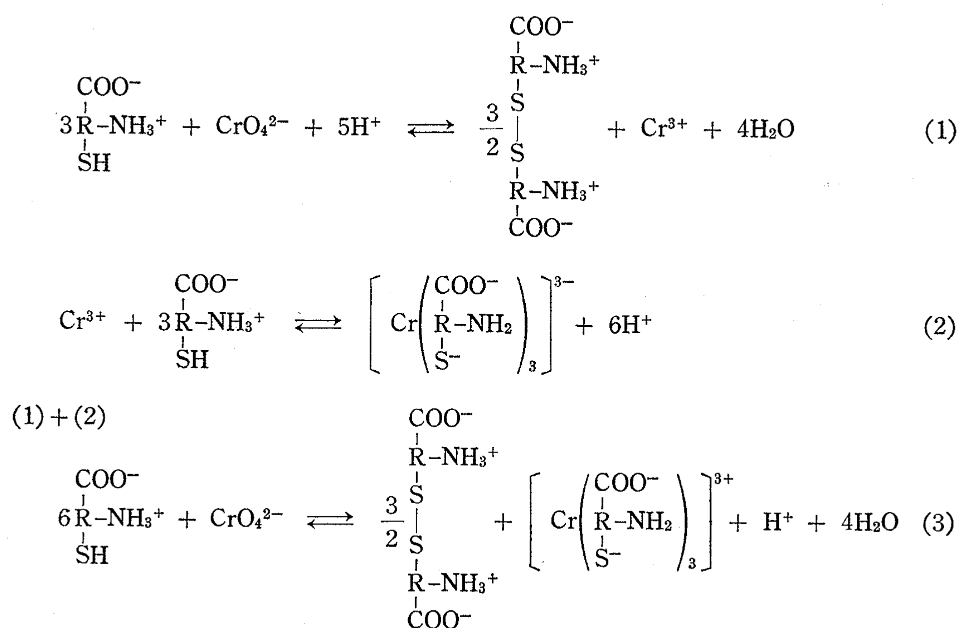

Fig. 3. Probable Reaction Mechanism

The spectral data of chromium (III) complexes with various chelating agents containing sulfhydryl group are noted in Table I, together with those of some amino acid complexes.

Table I. Spectral Data for Chromium Complexes[a)]

Ligand	d-d Bands $^4A_{2g}$-$^4T_{2g}$ ν_1 (cm^{-1})	d-d Bands $^4T_{2g}$-$^4T_{1g}$ ν_2 (cm^{-1})	Charge transfer band (cm^{-1})	$\nu_2-\nu_1$ (cm^{-1})	10Dq (cm^{-1})	B (cm^{-1})	Nephelauxetic ratio β_{35}
Valine	18590	24810	—	6220	18590	594	0.58
Methionine	18660	24880	—	6220	18660	592	0.57
Serine	18320	24570	—	6250	18320	599	0.58
Glycine	18250	24630	—	6380	18250	614	0.60
EDTA	18350	25510	—	7160	18350	712	0.69
DTPA	18180	25640	—	7460	18180	748	0.73
Thioglycolic acid	18020	22990	36170	4970	18020	459	0.45
Thiomalic acid	17540	23100	37880	5560	17540	522	0.51
Glutathione	17390	23150	37040	5760	17390	547	0.53
Cysteine methyl ester	18180	23810	37590	5630	18180	528	0.51
Penicillamine	18120	23810	37740	5690	18120	535	0.52
Cysteine	18220	24160	37740	5940	18220	562	0.55

a) All data were obtained by the reaction with chromium (III) and the ligands.

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With reference to the energy-level diagram of chromium (III) in an octahedral field,[8] in all cases the band at *ca.* 18000 cm^{-1} may be assigned to $^4A_{2g}$—$^4T_{2g}$ transition, and that at *ca.* 24000 cm^{-1} to $^4A_{2g}$—$^4T_{1g}$. On the basis of these assignments, the ligand field splitting parameter (10 Dq) and the Racah interelectron repulsion parameter (B) were calculated[9] and the results are listed in Table I. The 10 Dq values for the chelating agents containing sulfhydryl group are less than for the amino acids as expected from the spectrochemical series.[10] The decreasing in 10 Dq values indicate the decreasing in ligand field strength. In contrast with the results in 10 Dq value, the lower B values in complex than the value of B in free ion (1030 cm^{-1}), indicate that the charge cloud of the d electrons is most likely more spread out in the complex than in the free ion as a result of the covalent bond effects. Thus, as seen in the values of Table I, chelating agents containing sulfhydryl group clearly coordinate with chromium (III) ion through their sulfur atom, and the nephelauxetic effect in these complexes occurs to a larger extent with sulfur-containing ligands than the other ligands. In Pearson's acid-base theory[11] the coordination of mercaptide ion toward chromium (III) should not be favored, since chromium (III) ion is a hard acid and mercaptide ions are soft bases. However, it is recently reported[12] that the chromium (III) mercaptides are much more stable against hydrolysis and exchange reactions than their alkoxide analogues, and the coordination ability of sulfur donor for chromium ion has been reevaluated.[13]

While several chromium compounds, labelled with ^{51}Cr, are being used extensively for diagnostic or therapeutic purposes.[14] Particularly, it is known that chromium ion attaches to red blood cells with high affinity.[15] Hence, the effect of the sulfhydryl ligands on the uptake of chromium ion by protein and on the its removal from protein was investigated by the use of hemoglobin as a model protein.

In order to investigate the binding of chromium to protein and its removal from protein, interaction between chromic or chromate ion and hemoglobin was studied. The results are shown in Fig. 4.

The rate of the binding of chromate ion was very low, whereas, chromic ion was incorporated appreciably to hemoglobin. In the presence of penicillamine with the molar ratio of 6 to 1 to chromate ion, the rate of the binding of chromium to hemoglobin was very high, as well as in the case of the binding of chromic ion. The effect of penicillamine may be attributed to its ability of the reduction of chromium (VI) to chromium (III). However, the presence of penicillamine with the molar ratio of 20 to 1 to chromate ion, inhibits remarkably the binding of chromium. In this case, the effect of penicillamine may

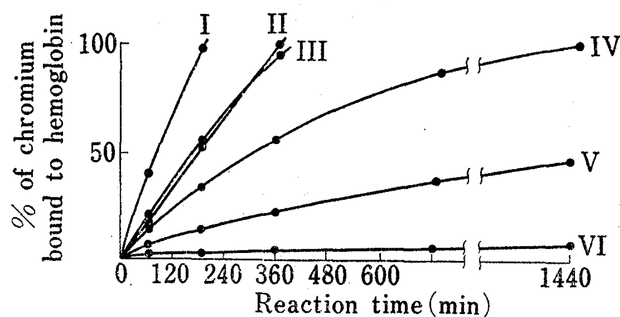

Fig. 4. Binding of Chromium to Hemoglobin

I: [hemoglobin]=[CrO_4^{2-}]=[Hg^{2+}]=5.0×10^{-4} M
II: [hemoglobin]=[Cr^{3+}]=5.0×10^{-4} M
III: [hemoglobin]=[CrO_4^{2-}]=5.0×10^{-4} M, [penicillamine]=3.0×10^{-3} M
IV: [hemoglobin]=[CrO_4^{2-}]=5.0×10^{-4} M
V: [hemoglobin]=[CrO_4^{2-}]=5.0×10^{-4} M, [penicillamine]=1.0×10^{-2} M
VI: [hemoglobin]=[CrO_4^{2-}]=5.0×10^{-4} M, [penicillamine]=5.0×10^{-2} M

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Table II. Effect of Metal Ion on Binding of Chromium to Hemoglobin at 180 min after Reaction[a)]

Metal ion	% of chromium bound to hemoglobin
—	48.4
Hg^{2+}	87.8
$C_2H_5Hg^+$	90.1
Cu^{2+}	89.9
Zn^{2+}	86.4
Ca^{2+}	82.8

a) All data were obtained under the following conditions: [hemoglobin]=[CrO_4^{2-}]=[metal ion]=5.0×10^{-4}M, pH 7.0

Table III. Binding of Chromium to Protein at 1440 min after Reaction[a)]

Protein	% of chromium bound to protein
Hemoglobin	94.1
Albumin	45.3
Myoglobin	1.0

a) All data were obtained under the following conditions: [Protein]=[CrO_4^{2-}]=5.0×10^{-4}M, pH 7.0

be attributed to its ability of the formation of stable chromium (III) chelate. The effects of the presence of various metal ions are shown in Table II.

Metal ions such as copper, zinc and calcium ions accelerate the rate of the binding of chromium to hemoglobin. Although the mechanism of the effect of these metal ions can not be explained from a few experimental results, the importance of the effect of foreign metal ions in the metal poisoning is suggested from this result.

As seen in Table III, the ability of binding of chromium to protein decreases in the order, hemoglobin<serum albumin<myoglobin.

The effects of the chelating agents containing sulfhydryl group on the removal of chromium bound to hemoglobin were compared with those of ascorbic acid and EDTA which have been proposed as the effective antidote in the poisoning of chromium.

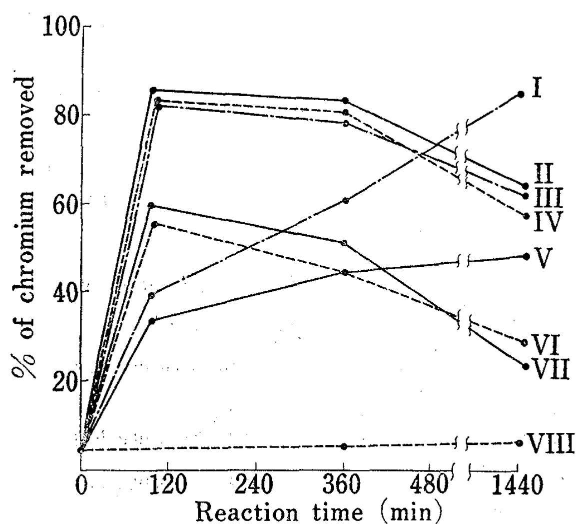

Fig. 5. Ability of Chelating Agents in Removal of Chromium Bound to Hemoglobin

concentration of chromium complex of hemoglobin: 5.0×10^{-4}M, concentration of chelating agent: 5.0×10^{-2}M

I: EDTA; II: penicillamine; III: penicillamine methyl ester; IV: cysteine; V: valine; VI: ascorbic acid; VII: N-acetylpenicillamine; VIII: none

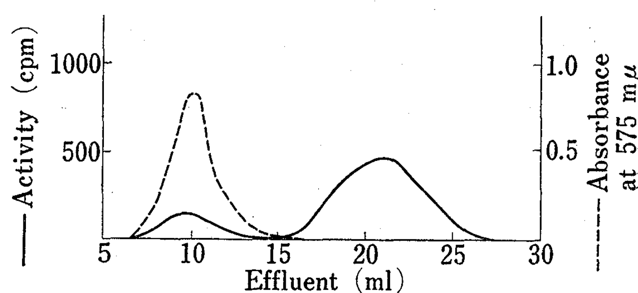

Fig. 6. Separation of Ternary Complex by Gel-filtration

concentration of chromium complex of hemoglobin: 2.5×10^{-4}M, concentration of cysteine-^{14}C: 2.5×10^{-3}M

As presented in Fig. 5, the chelating agents containing sulfhydryl group, such as penicillamine, cysteine and penicillamine methyl ester are superior to ascorbic acid and EDTA, in the rate and the amount of the removal of chromium. However, after the long time

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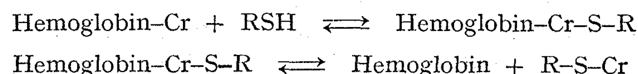
course, the effect of the removal decreased considerably in the cases of the chelating agents containing sulfhydryl group. This fact may be explained by the redistribution of chromium which may occur in long time course, between hemoglobin and the chelating agents. In EDTA, amount of chromium removed was high, but longer time was necessary to attain maximum amount in removal. In the chromium chelate of penicillamine, the coordination of sulfur-nitrogen type is reasonable, since the effect of penicillamine was very close to that of penicillamine methyl ester but not to that of N-acetylpenicillamine. In the study of various metal chelate of penicillamine, sulfur-nitrogen coordination in which carboxyl group does not participate in the coordination, was approved in the case of the metal ions whose ionic radius are small (0.7—0.8 Å).[6b] Sulfur-nitrogen coordination is regarded to be reasonable, since the ionic radius of chromium is 0.69 Å.

The ternary complex involving protein, metal and low-molecular weight substance has been proposed as an important intermediate in the transport of metal ions.[16] In the case of the reaction of chromium with protein in the presence of the chelating agents, the pattern of the radioactivity in the gel-filtration indicates the presence of a ternary complex involving hemoglobin, chromium and cysteine, since the radioactivity of carbon-14 of cysteine clearly appeared in the fraction of hemoglobin in the system containing hemoglobin, chromium and cysteine-C^{14}, but not in the system without chromium. The similar ternary complex was also approved to be present in the reaction of mercuric ion with albumin in the presence of cysteine.[17]

Accordingly, the process of the removal of chromium may be expressed by the following equations, a ternary complex being assumed as an intermediate.



$$\text{Hemoglobin–Cr} + \text{RSH} \rightleftharpoons \text{Hemoglobin–Cr–S–R}$$
$$\text{Hemoglobin–Cr–S–R} \rightleftharpoons \text{Hemoglobin} + \text{R–S–Cr}$$

In conclusion, hexavalent state of chromium may be reduced to tervalent state and binds to protein in physiological condition, and the chelating agents containing sulfhydryl group such as penicillamine and cysteine may be regarded as the effective antidote against chromium poisoning through their abilities of the removal of chromium bound to hemoglobin.

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