CHAPTER III

RESULTS

1 Sensitivity and Interference of Graphite Furnace Atomic Absorption Spectrophotometer

In the determination of Pb and Cu concentration, the Graphite Furnace Atomic Absorption Spectrophotometer (F-AAS) was commonly used at wavelength 283.3 and 324.8 nm, respectively (Sunderman, 1967). The F-AAS was thus used for the metal determination throughout this study. F-AAS was checked for its sensitivity and specificity. Instrument was shown to be quite sensitive, with minimum volume of 16 µl, Pb at ppb level could be detected in a reliable range (Figure 2(a)). The standard curve of Pb (0-40 ppb) as measured at the wavelength of 283.3 nm showed the increase in the absorption with Pb concentration with slight deviation from the linearity at high Pb concentration. The absorption was specific to Pb, since Cu (up to 1500 ppb) could cause little absorption (Figure 2(b)). The F-AAS (at wavelength 283.3 nm) was thus suitable for Pb detection. The same finding was also observed on Cu (0-20 ppb) at 324.8 nm as shown in Figures 3(a) and (b).

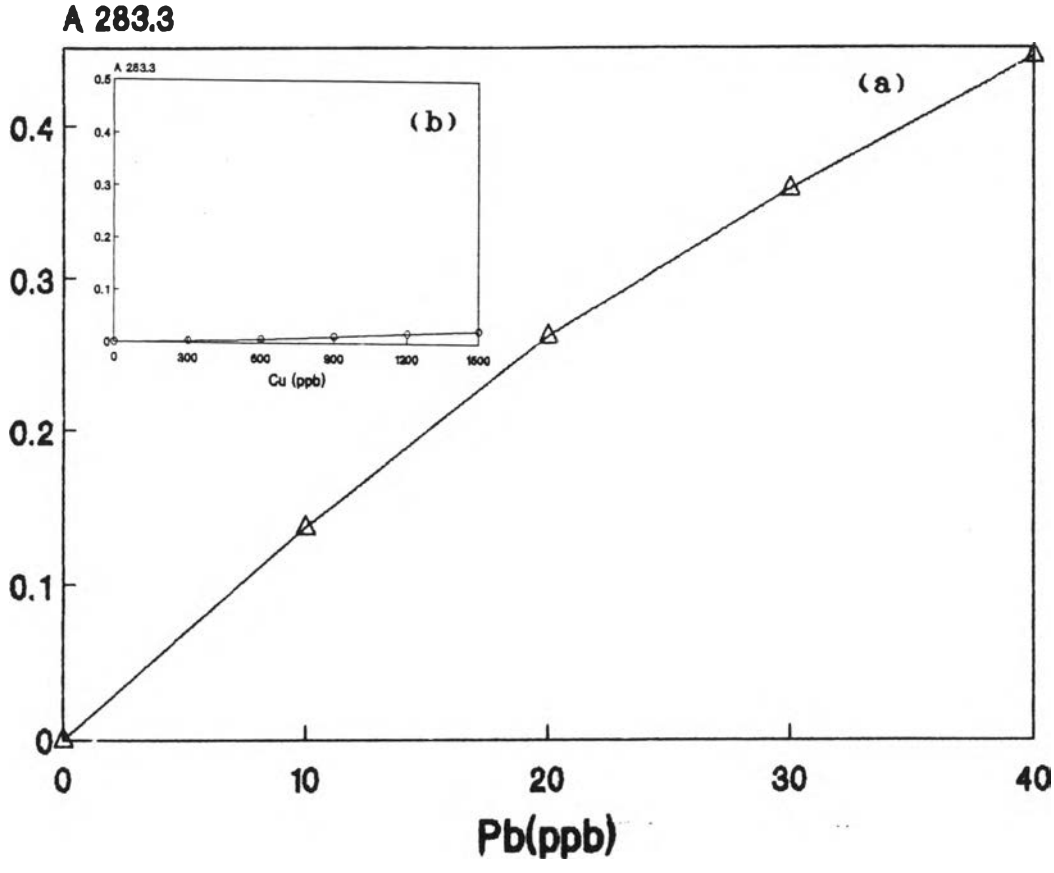

Figure 2 Standard curve of Pb determined by F-AAS

Various concentrations of Pb (a) and Cu (b) standards were prepared in distilled water; 16 μl of each concentration was directly injected to F-AAS and the absorptions at 283.3 nm was measured. Other conditions were as described in the Methods.

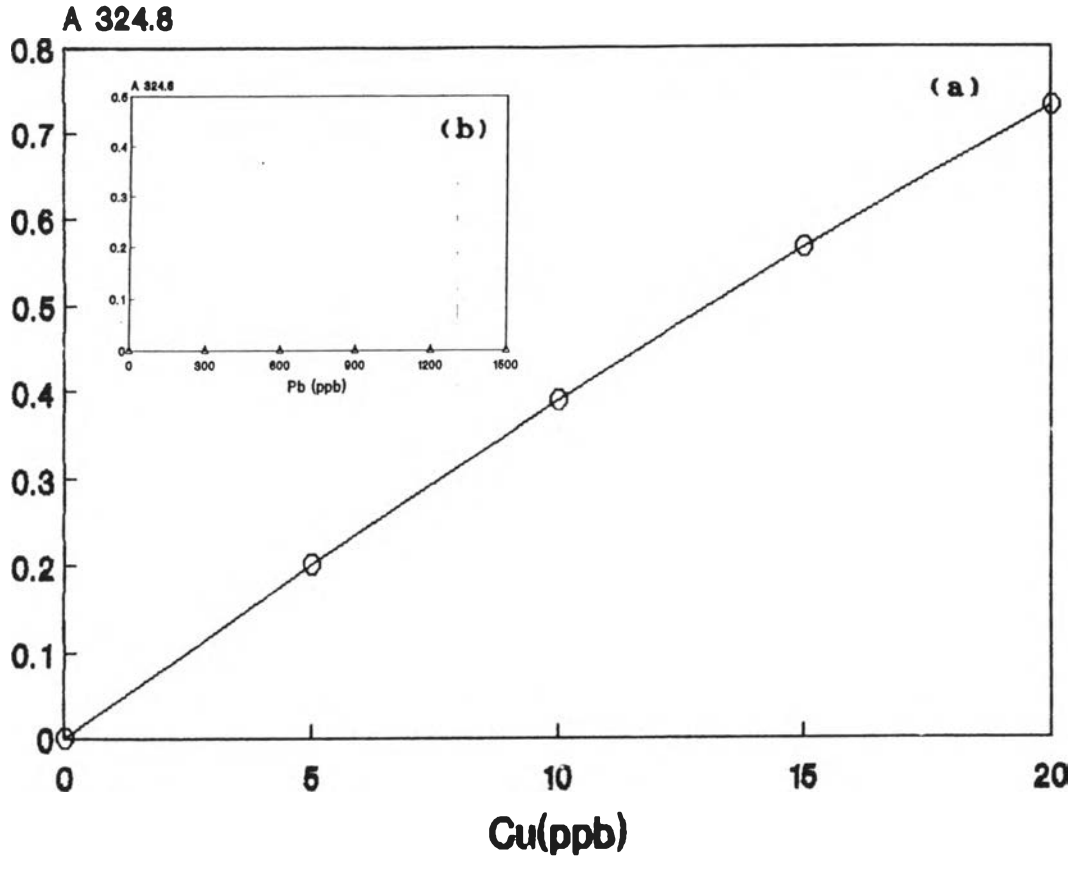

Figure 3 Standard curve of Cu determined by F-AAS

Various concentrations of Cu (a) and Pb (b) standards were prepared in distilled water; 16 μl of each concentration was directly injected to F-AAS and the absorptions at 324.8 nm was measured. Other conditions were as described in the Methods.

2 Lead-binding Ability of Serum Proteins

Mangkalee (1994) reported that Transferrin, the serum Fe-binding protein, could also bind to Pb. Ceruloplasmin (Cp) is another important metal-binding protein in serum and may serve as Pb-carrier when the metal enters the blood stream. For preliminary testing, the experiment was performed by incubating serum with Pb and measuring Pb and Cu retained on the high molecular weight biomolecules. The result shown in Table 2 indicated that both metals could be detected on high molecular weight biomolecule(s) but the bound Cu seemed to be released upon the Pb binding. At 1.8 ppm Pb used, Pb bound increased to 0.12 ppm while the same concentration of Cu was released. Although Pb binding could not replace all the Cu on the biomolecules, this result still suggested that Pb and Cu may compete for the same protein and since Cp served as the physiological Cu carrier, Cp may be the protein for this competition.

The result was more pronounced when Pb concentration was varied while keeping constant amount of serum (Fig.4). As the Pb concentration in the incubation was increased, the Cu decreased. However, Cu could not be completely eliminated at the highest concentration of Pb utilized (28.8 ppm). Technically, the concentration of Pb could not be increased any further due to its low solubility. It was found from the calculation of metal binding on serum proteins in Fig.4

that at the highest concentration of Pb utilized. For the loss of 2.58 atoms of bound Cu, there were an increase of 2.18 atoms of Pb bound to the proteins.

It is noteworthy to mention that Cp in serum also possessed oxidase activity (Cousins,1985). The activity had been proposed to function as ferroxidase that oxidized ferrous ion to ferric ion (Laurie, 1980) and the oxidation was an important step in heme synthesis. Holmberg (1951) reported that all eight atoms of Cu bound were required for the oxidase activity . This enzymatic activity served as another marker to confirm the binding of Pb on Cp molecule, since it decreased (Fig.5) concomitantly with Pb binding (Fig.4). Moreover, at 28.8 ppm of Pb utilized, the same degree of Cu released and oxidase inhibition were obtained (about 48%).

Table 2 The relationship between Pb binding and Cu released on serum protein(s)

One milliliter of normal serum was incubated with or without 1.8 ppm Pb (3.29 ppm lead acetate) in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min. The serum proteins were separated from free metals with Sephadex G-25 column chromatography and determined for metal concentration by F-AAS.

The data shown were means of 4 determinations.

Serum	Metal concentration (ppm)	
	Cu	Pb
Normal	1.39 ± 0.040	0.20 ± 0.001
Pb-treated	1.27 ± 0.010	0.32 ± 0.024
Difference	0.12 ± 0.030	0.12 ± 0.023

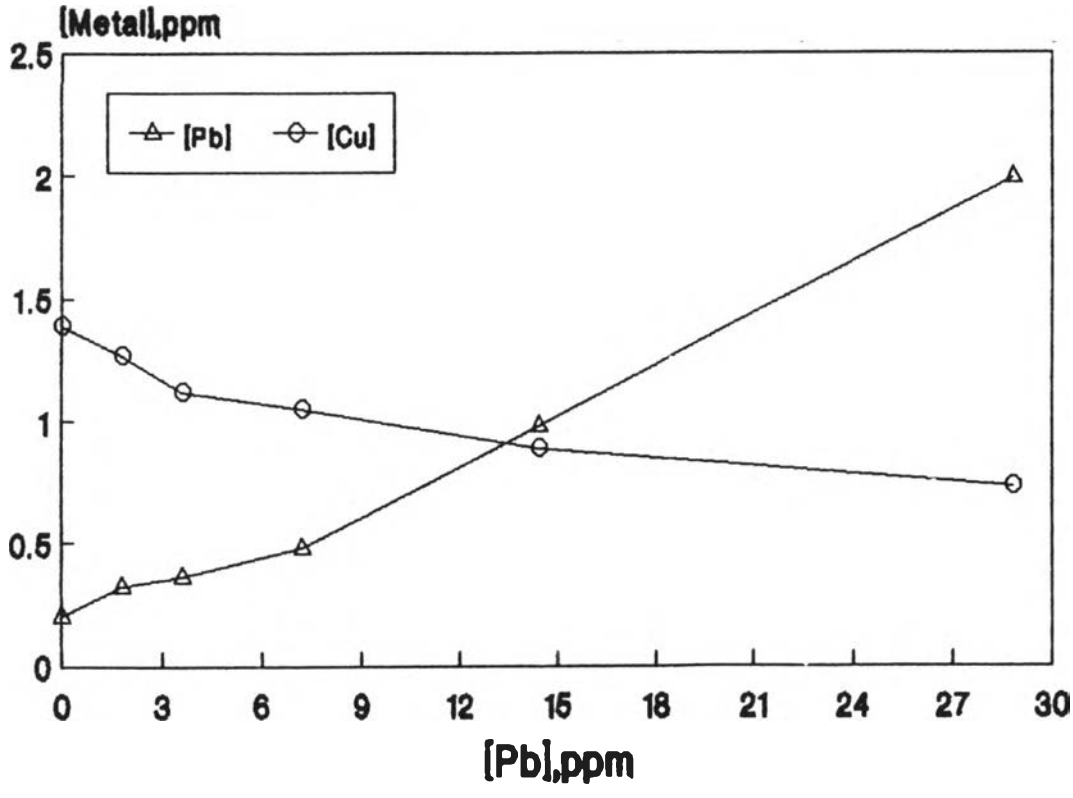

Figure 4 Effect of Pb on copper level on serum proteins

Serum was incubated with various concentrations of lead acetate in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min, afterwhich protein fractions were separated from free metals with Sephadex G-25 column chromatography and determined for metal concentration by F-AAS.

The data shown were means of 4 determinations.

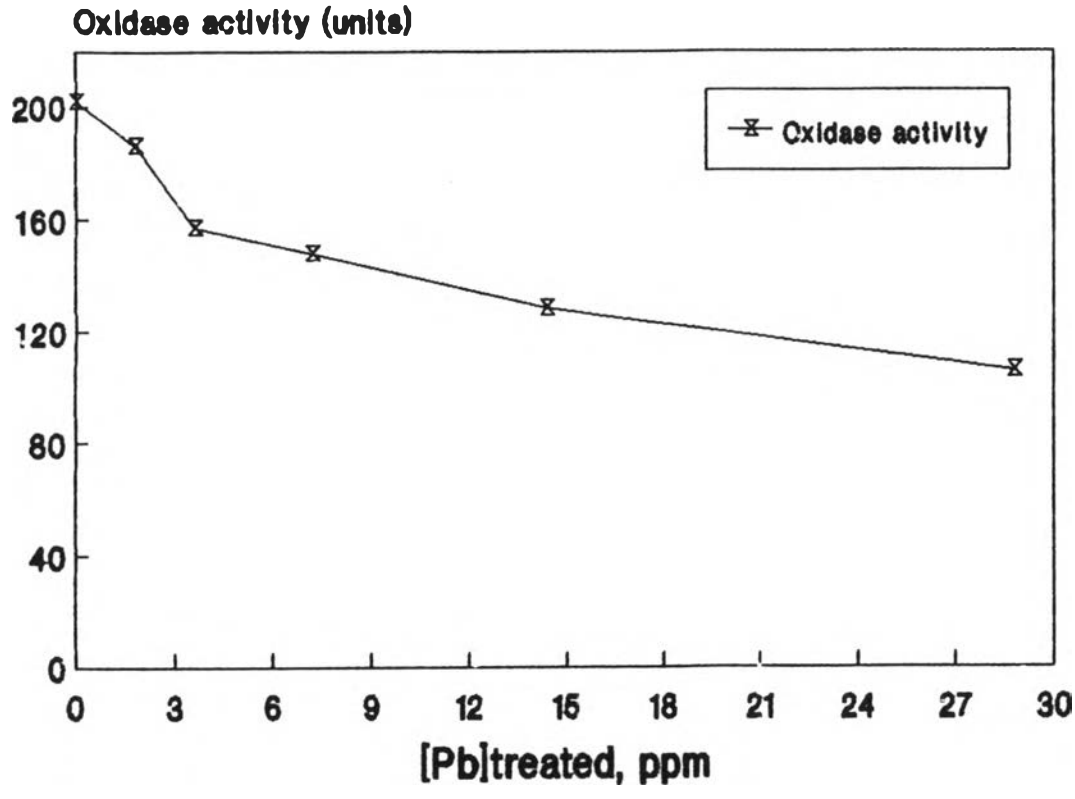

Figure 5 Effect of Pb on serum oxidase activity

The condition was as described in Fig.3 except the metal-free fractions were assayed for oxidase activity with PPD as substrate.

The data shown were means of 4 determinations.

3 Characterization of Pb-binding proteins

3.1 Sephadex G-200 column chromatography

Since most of the human serum proteins have been commonly characterized by their molecular weight with Sephadex G-200 column chromatography (Killander, 1964), this method was then used to confirm Pb-binding on Cp.

Figure 6 showed that when Pb-bound proteins were fractionated with Sephadex G-200 column chromatography, they were resolved into many peaks. To specify which of these peaks existed in the physiological condition, the serum from lead-toxicated patient was applied to the same column, the result was shown in Figure 7. Three distinct peaks of Pb-bound proteins were observed, namely peak I, II and III. The elution of these three peaks corresponded to those in Pb-treated serum. Moreover, peak I elution which possessed the highest amount of Pb, corresponded to Cu-binding protein in normal serum (Figure 8). These findings implied that when Pb entered the blood stream, it could bind to many serum proteins and one of the Pb-binding proteins was Cp.

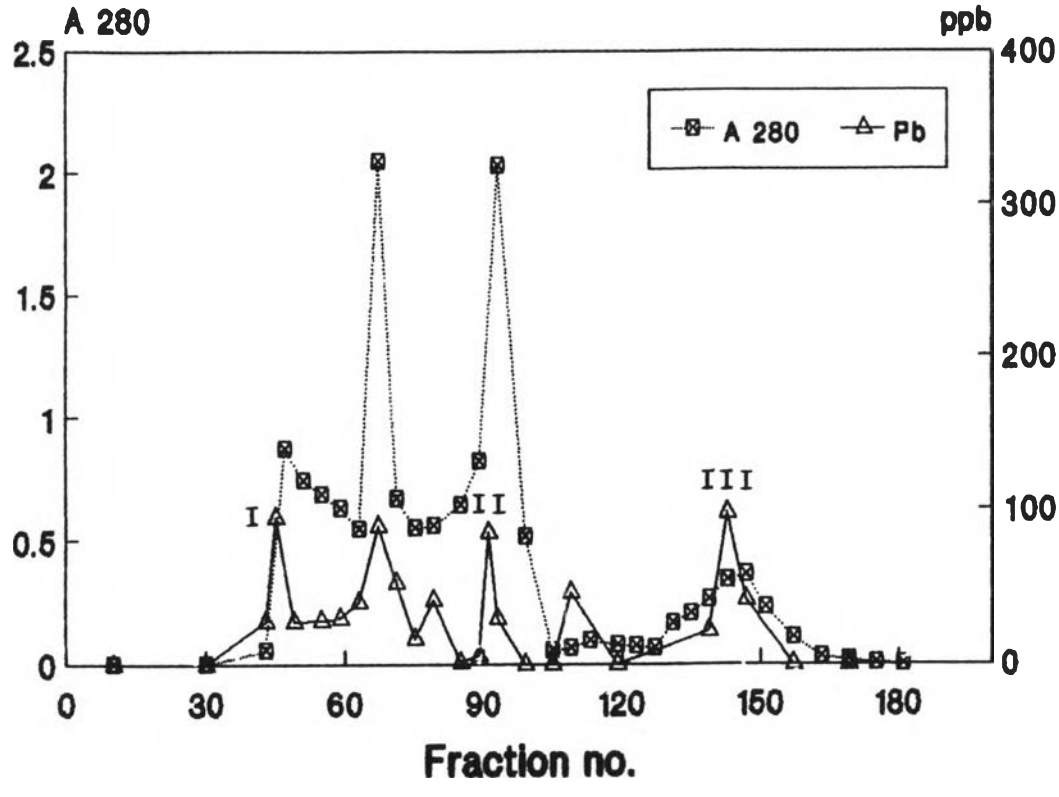

Figure 6 Sephadex G-200 column chromatography of Pb-treated serum

Two milliliters of serum was incubated with lead acetate (1,800 ppb of Pb) in 0.01 M acetate buffer, pH 6.0 at 37°C for 30 min. The sample was then loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated and then eluted with the same buffer while maintaining the flow rate at 20 ml/hr. Two-ml fractions were collected, protein concentration was monitored by the absorbancy at 280 nm. Pb concentration was determined by F-AAS.

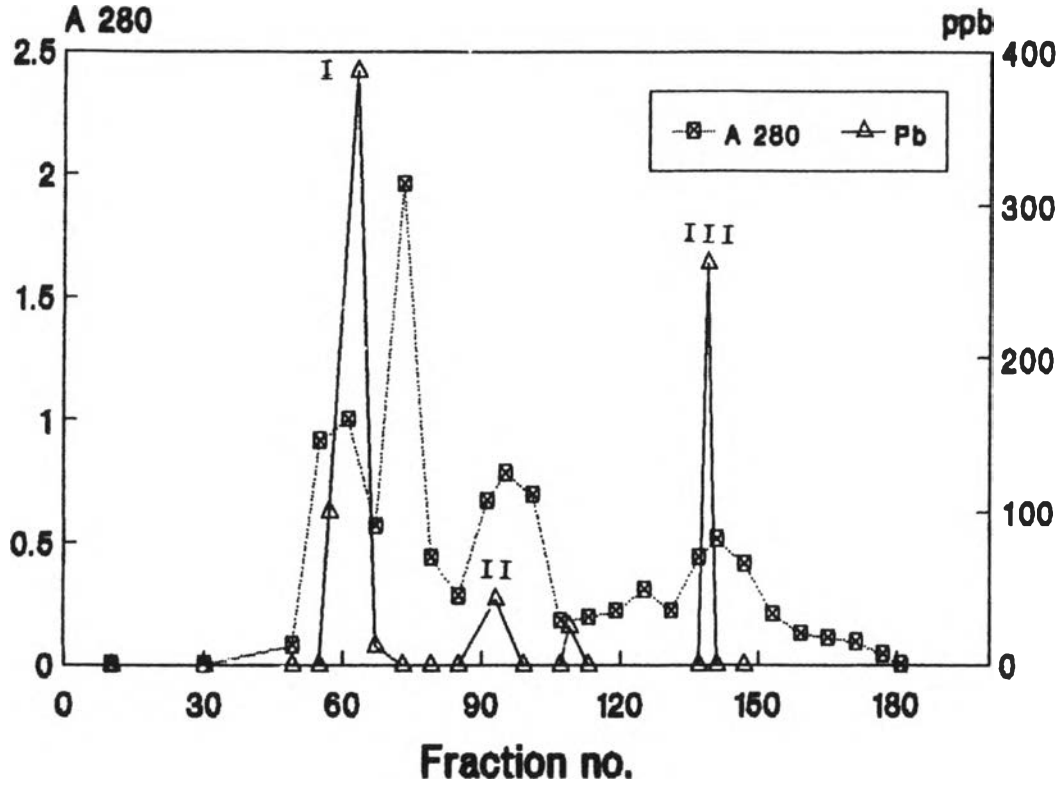

Figure 7 Sephadex G-200 column chromatography of Pb-toxicated patient serum

Two milliliters of patient serum was loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated with 0.01 M acetate buffer, pH 6.0. The column was then eluted with about 380 ml of the same buffer while maintaining the flow rate at 20 ml/hr. Two-ml fractions were collected, protein concentration was monitored by the absorbancy at 280 nm. Pb concentration was determined by F-AAS.

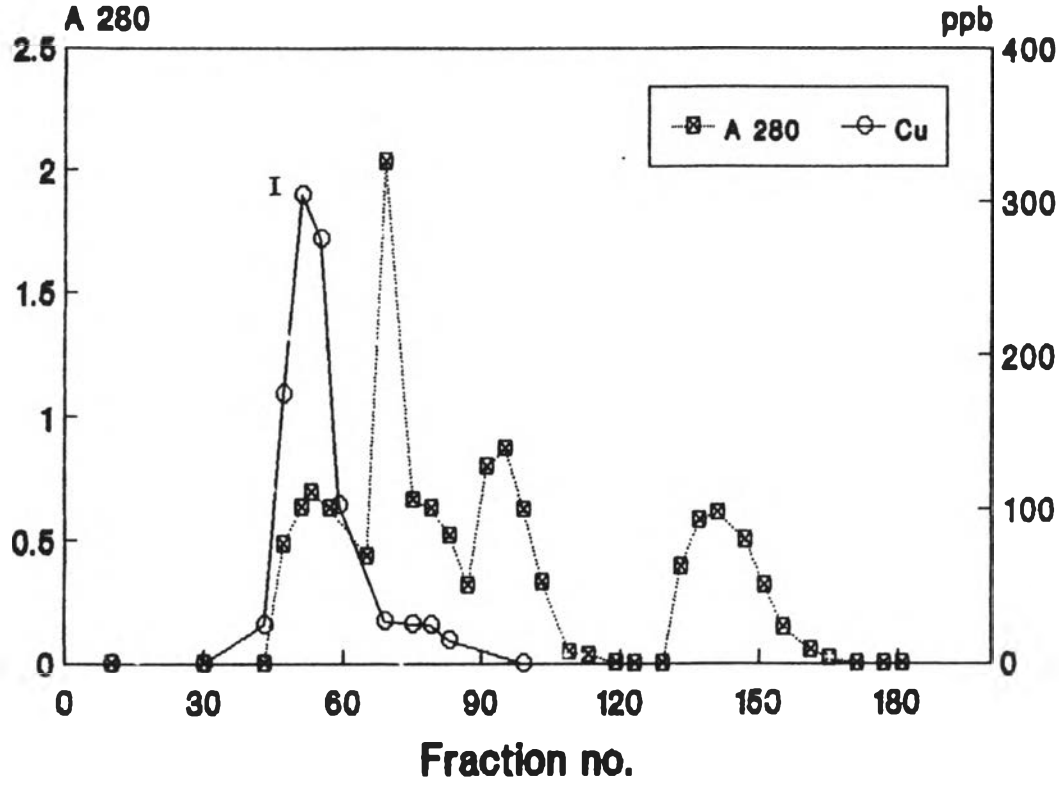

Figure 8 Sephadex G-200 column chromatography of normal serum

Two milliliters of normal serum was loaded onto Sephadex G-200 column (1.8 x 120 cm) equilibrated with 0.01 M acetate buffer, pH 6.0. The column was then eluted with about 380 ml of the same buffer while maintaining the flow rate at 20 ml/hr. Two-ml fractions were collected, protein concentration was monitored by the absorbancy at 280 nm. Cu concentration was determined by F-AAS.

3.2 Polyacrylamide Gel Electrophoresis

Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE) at pH 8.3 was used for further characterization of Pb-binding protein.

Peak I fraction from each Sephadex G-200 column were concentrated 10 folds by freeze drying and loaded to Disc-PAGE as described in the Method. The protein pattern in Figure 9, indicated that the peak I from Fig.7 , 6 and 8 (lane 2, 3 and 4), which possessed high Pb concentration, (or high Cu in normal case) contained similarly proteins patterns, of which one had the same electrophoretic mobility as standard Cp.

This result again suggested that although Sephadex G-200 chromatography could not fractionate Cp from other serum proteins, the presence of Cp in the fractions which possessed high Pb concentration implied that Cp may serve as Pb-binding protein.

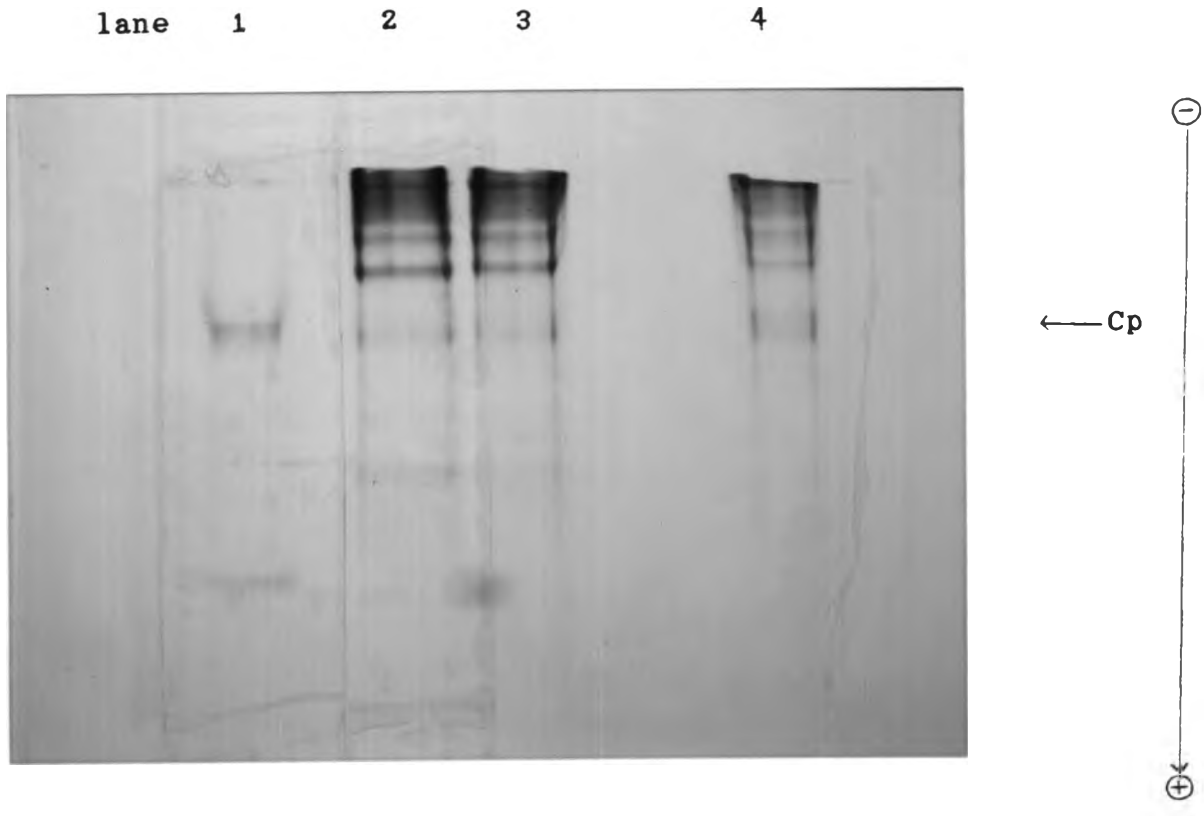

Figure 9 Disc-PAGE of Pb binding protein

Peak I from Sephadex G-200 Column Chromatography of Pb-toxicated patient serum (lane 2), Pb-treated serum (lane 3), normal serum (lane 4), and standard Cp (lane 1) were concentrated 10 folds by freeze-drying, 20 μg from each fraction were loaded on Disc-PAGE (non-denaturing gel), the electrophoretic condition were as described in the Methods.

4 Conditions for Pb-Ceruloplasmin Binding

Before further study on the stoichiometry of Pb-Cp binding, optimum condition for the binding was characterized.

4.1 Effect of pH on Pb-Cp binding

Purified Cp was incubated at various pH with or without lead acetate (1.8 ppm of lead), and Cu and Pb bound to the protein were determined by F-AAS.

The result in Figure 10 showed that in the control (without Pb treated), the amount of Cu bound to the Cp at various pH were different. The highest amount of 8 Cu atoms per Cp molecule, corresponded to Holmberg's report (1948), was detected at pH 6.0. When treated with Pb, the metal could be detected on the Cp, while Cu was partially released. The replacement was highest at pH 6.0. The increase amount of Pb bound (0.63 atoms/Cp molecule) was slightly lower than the amount of Cu released (0.96 atoms/Cp molecule). Further study on Pb-binding was always performed at pH 6.0.

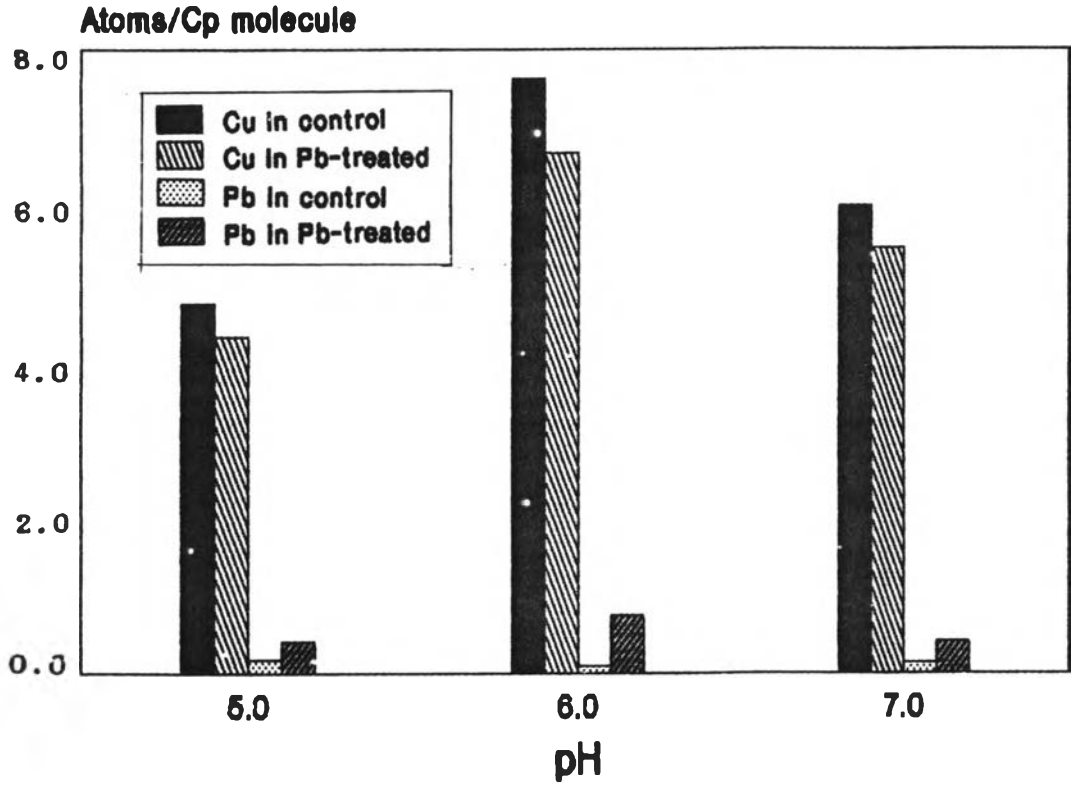

Figure 10 Effect of pH on metal binding on ceruloplasmin

One milliliter of purified Cp (0.6 mg/ml or 4 μmole/l) was incubated with or without lead acetate (1.8 ppm of lead) in 0.01 M acetate buffer pH 5,6 and in 0.01 M Tris-HCl buffer pH 7 at 37°C for 30 min. The protein fractions were separated from free metals as described in the Methods and determined for metal concentration by F-AAS.

4.2 Effect of storage on Pb bound Cp

Figure 11 showed that Pb bound to Cp was slowly released upon storage at 4°C, only 3.16% decrease was detected on day 3. Throughout this study, Pb bound to Cp was analyzed immediately after the binding or stored not more than 6 days, in unavoidable cases.

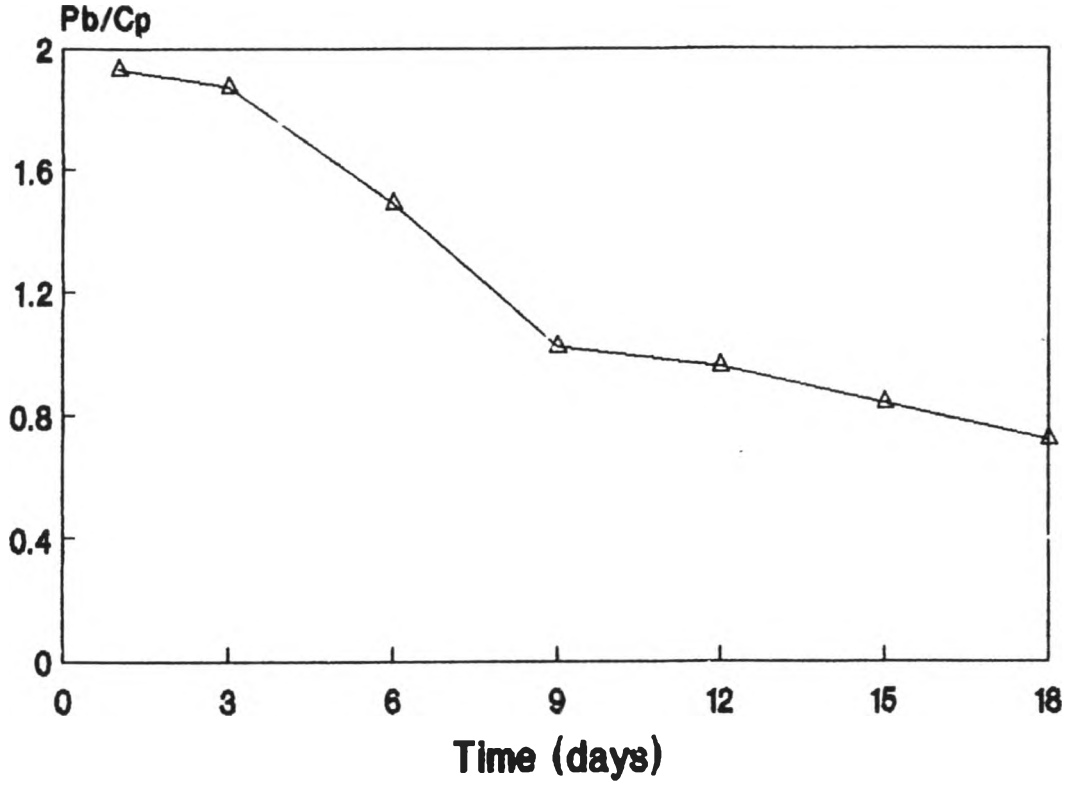

Figure 11 Storage of Pb bound Cp

One milliliter of purified Cp (0.6 mg/ml or 4μmole/l) was incubated with lead acetate (28.8 ppm of lead) in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min. Afterwhich protein fractions were separated from free metals and the aliquots were stored at 4°C. At the indicated times, 2 ml was withdrawn, Pb-Cp was again separated from free Pb, and finally determined for metal concentration by F-AAS.

The data shown were means of 4 determinations.

5 Binding Studies of Pb on Cp

5.1 Relationship between Pb binding and Oxidase Activity

To study the stoichiometry of Pb binding on Cp molecule and its relationship to the oxidase activity, the purified Cp was incubated with various concentration of Pb; up to 278 atoms Pb per Cp molecule was used to saturate the Cp, Pb and Cu bound to Cp and oxidase activity were measured after being separated from the metal. As shown in Figure 12, in the absence of Pb, 8 atoms Cu/mole of Cp were detected which the value closed to that reported by Holmberg (1948). Both Pb binding and Cu released were concentration dependent. Moreover, at the highest mole ratio of Pb treated/Cp utilized, only about 5.0 atoms of Cu were eliminated which was substituted by 3.2 atoms of Pb, so that the mole ratio of substitution of Pb to Cu on Cp molecule was not 1:1.

The decrease of oxidase activity of Cp paralleled with the release of Cu. At the highest mole ratio (278 atoms Pb treated/Cp), the same degree of Cu released and oxidase inhibition were obtained (about 63 %). This finding corresponded to the report of Holmberg (1951), stating that Cu atoms bound to Cp were required for the oxidase activity. Furthermore, the curve of binding and inhibition were hyperbola curve that were similary with the curve of the velocity of and enzyme-catalyzed reaction as a function of the substrate concentration and in this curve Cp was

likely to enzyme and Pb was likely to substrate, that at a constant concentration of enzyme (Cp) the reaction rate (the binding of metal on Cp) increased with increasing substrate (Pb) concentration until a maximal velocity is reached.

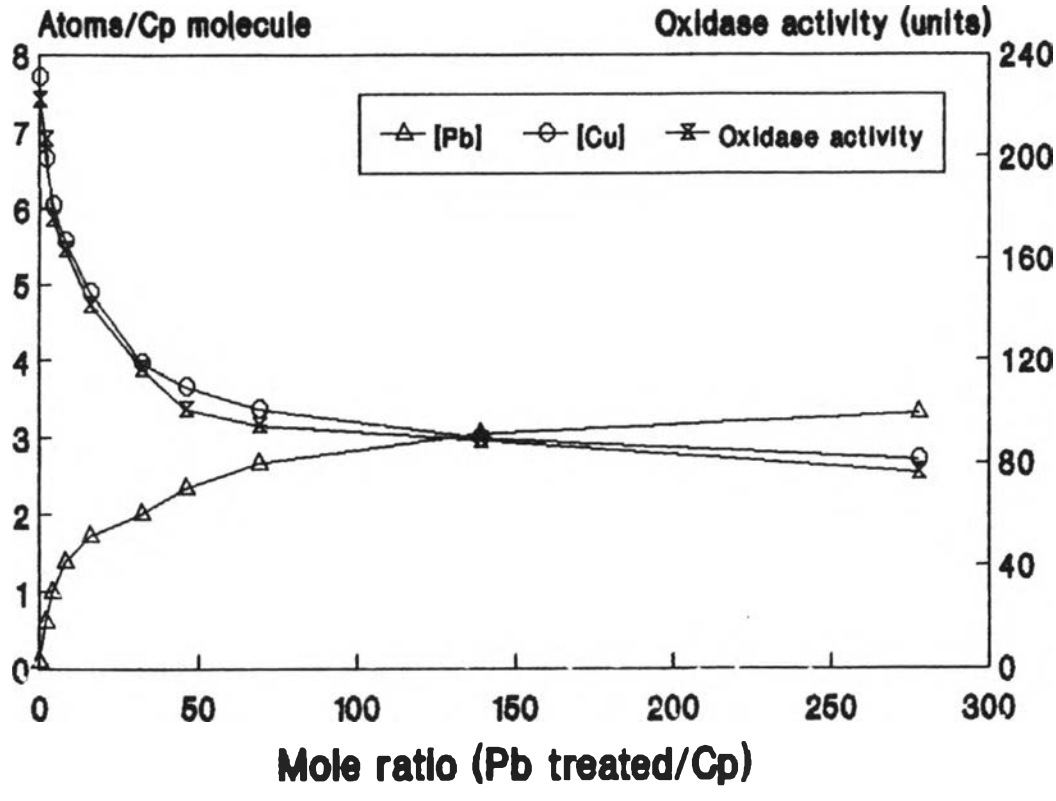

Figure 12 Relationship beteween metal binding and oxidase activity on purified ceruloplasmin

One milliliter of purified ceruloplasmin (0.6 mg/ml or 4 μmole/l) was incubated with various concentrations of lead acetate in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min, to give Pb/Cp mole ratio as indicated. Afterwhich protein fractions were separated from free metals then determined for metal concentration and oxidase activity as described in the Methods.

The data shown were means of 4 determinations.

5.2 Effect of some Chelators on The Metal-Cp Binding

Chisolm (1971) reported that Pb could be removed from the blood and tissues by some chelators such as EDTA, Dimercaprol and D-penicillamine, it is of interest to investigate the chelating property of these chemicals on Pb from Cp molecules. The result was shown in Table 3 . 100 mg/ml $CaNa_2EDTA$, or 100 mg/ml Dimercaprol and 1,000 mg/ml Penicillamine, the commonly used chelators at the concentrations used in clinical treatment, reduced the concentration of Pb bound on Cp molecules by 89.05, 96.52 and 99.01%, respectively. However, these chelators could not chelate Cu bound to Cp.

It is interesting to study the mobile property of Cp which had unequal metal-bound on molecules, on IEF-PAGE condition. The mobilities on IEF, pH 4.0-6.0 of Cp fully bound with Cu or Pb and metal-chelated Cp were compared, as shown in Figure 13. The shifting of the Cp band could not be detected in the different conditions. However standard serum albumin, β-lactoglobulin and transferrin, which are slightly different in their pI's (pI= 4.88, 5.10 and 6.10, respectively) showed little difference in the shift of protein bands, thus it was possible that if proteins (Cp fully bound with Cu or Pb and metal-chelated Cp) had little difference in the pI's, the difference in electrophoretic mobility on this IEF condition could not be detected.

Table 3 Effect of some chelators on metal-Cp binding

One milliliter of purified Cp (0.6 mg/ml or 4μmole/l) was incubated with lead acetate (28.8 ppm Pb) in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min. After separated from free metals, the protein (0.6 mg) was separately incubated with 5 μl of different chelators: 100 mg/ml of $CaNa_2EDTA$, or 100 mg/ml of Dimercaprol and 1,000 mg/ml of Penicillamine, at 37°C for 30 min. Free metals were removed from each incubation again as described in the Methods, the bound metal concentration was determined in each fraction by F-AAS.

Sample	Atoms / Cp molecule	
	Cu	Pb
Control Cp	7.71 0.67	0.09 0.01
Pb-Cp (28.8 ppm)	3.97 0.16	2.01 0.05
$CaNa_2EDTA$-(Pb-Cp)	4.40 0.01	0.21 0.00
Dimercaprol-(Pb-Cp)	4.37 0.16	0.07 0.00
Penicillamine-(Pb-Cp)	4.53 0.03	0.02 0.00

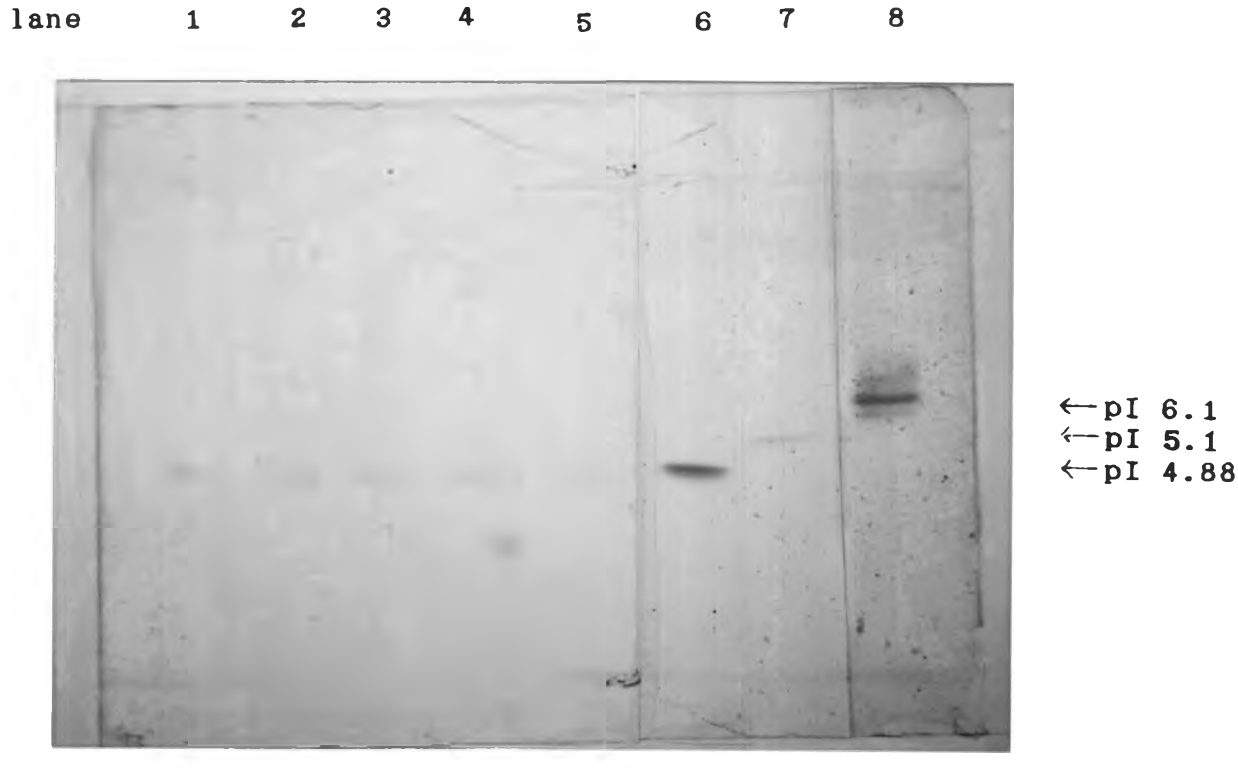

Figure 13 Effect of some chelators on IEF-pattern of Cp

The experiment was as described in Table 3, except after incubation with chelators the samples (10 μl) were run on IEF-PAGE (pH 4.0 - 6.0) , the running condition was as described in the Methods.

lane 1: Control Cp (pI=4.4) 2: Pb-Cp

3: $CaNa_2EDTA$-(Pb-Cp) 4: Dimercaprol-(Pb-Cp)

5: Penicillamine-(Pb-Cp) 6: Serum album (pI=4.88)

7: β-lactoglobulin (pI=5.1) 8: Transferrin (pI=6.1)