CHAPTER IV

DISCUSSION

1 Sensitivity and Interference of Graphite Furnace Atomic Absorption Spectrophotometer

It was reported that the spectrophotometric method by now is the most suitable, reliable and acceptable technique for determining trace metal concentrations, especially in biological samples (Whitehouse *et al.*, 1982). As reported by Sunderman (1967), the F-AAS is sensitive enough to detect lead levels less than 0.2 ppm in an aqueous solution and the limit of sensitivity for the detection of copper in serum was 0.08 ppm . This method is rapid and requires only small amounts of samples.

In this study, with minimum volume of 16 μl, in ppb level both Pb and Cu could be detected in a reliable range with linear relationship at the wavelength of 283.3 and 324.8 nm as shown in Figures 2(a) and 3(a).

Since Cu and Pb were always present in the same sample, the interference of these two metals was also checked. As shown in Figure 2(b) and 3(b), Cu and Pb (up to 1,500 ppb) hardly interfere each other in F-AAS system.

Thus F-AAS was used for determining the concentration

of the metal in this study because F-AAS could be used with samples of very low metal concentration, such as those from chromatographic profile, and F-AAS had high potential to determine the concentration of each metal in the same containing both of Cu and Pb.

2 Lead-binding Ability of Serum Proteins

Mangkalee (1994) reported that when Pb was uptaken into the blood cells, a constant amount of Pb still remained in the serum. The remaining was proposed to be bound with serum metal-binding proteins, and transferrin (Tf) was proved to be one of them.

2.1 The Replacement of Pb to Cu

It is proposed in this study that Ceruloplasmin (Cp), the serum Cu-binding protein, may serve as another Pb-carrier. Since 90% of Cu in blood plasma is bound to Cp molecules (Oosthuizen, 1985), the first indirect experiment on Pb-Cp binding was set up in this study by measuring Cu level on serum proteins.

Berman (1966) reported that, blood lead level above 0.6 ppm caused lead-intoxication. A little higher concentration of Pb, in the range of 1.8-28.8 ppm, was used in this study. As shown in Table 2 and Figure 4, Cu was released from serum protein upon Pb-binding, from the normal

value of 1.39 ppm Cu, corresponded to that the value of 1.27 ppm reported by Goyer (1991) using 1.20-1.45 ppm of Pb. The result suggested the replacement of Pb to Cu on the Cp molecule. However, at 1.8 ppm of Pb utilized, the replacement was not complete. Although the concentrations in ppm of Pb increased and of Cu decreased were equal (0.12 ppm), the difference in atomic weight of Cu (63.57) and Pb (207.19) pointed out that the molar replacement was not 1:1. The calculation suggests the ratio of 0.31 atom of Pb bound to 1 atom of Cu released. It is important to note here that the mole ratio of Pb bound : Cu released was not constant upon the increase of Pb concentration. From literature survey, this replacement has not been reported elsewhere.

2.2 The Decrease of Oxidase Activity

It was reviewed that Cp also possessed oxidase activity (Cousins, 1985) and this activity was proportional to the Cu content on Cp molecule (Holmberg, 1951 and Michaelis, 1957). One assumption was set up in this study, stating that Pb-bound could not enhance the Cp's oxidase activity as Cu did. And if so, the decrease in oxidase activity may be used as another probe for Pb-binding on the Cp molecule. The oxidase activity was measured on *para*-phenylenediamine (PPD) oxidation (Holmberg, 1951), and the result from Fig. 5 supported this notion, the oxidase activity decreased concurrently with Pb binding. Since

the results from Fig.4 and 5 were from the same incubations, the results could be directly compared. Surprisingly, the patterns and degrees of Cu decreased (by Pb binding) and of oxidase decreased were similar. At the highest concentration of Pb utilized (28.8 ppm), the degree of Cu released and oxidase inhibition were the same (about 48 %).

From these results, it could be concluded that Pb could bind on Cp molecule, probably by replacing the Cu on Cp molecule and the replacement led to the decrease in Cp oxidase activity.

To confirm this, the experiments were further carried out on purified Cp as discussed in 4.

3 Characterization of Pb-binding proteins

Since Cu content and oxidase activity decreased with Pb-binding, Sephadex G-200 column chromatography which based on the molecular weight of the proteins and Non-denaturing Polyacrylamide Gel Electrophoresis (ND-PAGE) which based on molecular weight and charge, were also used as other tools to confirm Pb-binding on the Cp.

It was confirmed with the elution profiles from Fig. 6-8 that the high molecular weight Pb-binding proteins in both Pb-treated serum and Pb-toxicated patient serum were respected to be the Cp.

Moreover, there were two other peaks of Pb from

lead-toxicated patient column (peak II and III, Fig. 6 and 7). Peak II corresponded to the transferrin protein studied by Mangkalee (1994), and another peak indicated that there were other Pb-binding proteins with molecular weight lower than ceruloplasmin and transferrin but these proteins have not yet been characterized any further.

The results of ND-PAGE in Fig.9 confirmed the presence of Cp in Pb-rich fraction, eventhough it was still contaminated with other proteins.

4 Conditions for Pb-Ceruloplasmin Binding

Prior to further study on the Cu replacement with Pb, the optimum condition for Pb-Cp binding was studied.

As shown in Figure 10, in controlling purified Cp at pH 6.0, there was 8 Cu atoms per Cp molecule. The result corresponded to that reported by Holmberg (1948) who measured the amount of Cu on Cu-binding protein in both human and pig serum by using the light absorption technique of Cu at 605 nm and reported that 8 Cu atoms bound on Cp molecule of normal mammalian blood. The amount were smaller at pH 5.0 and 7.0. Furthermore, the maximum replacement of the metal was also detected at pH 6.0 (0.63 atoms/Cp molecule).

Humoller (1958) reported that the optimum pH range for the oxidase activity to N,N-dimethyl-p-phenylenediamine of Cp was pH 5.5-6.0 and that at pH 6.0 seems to be well

justified. From Fig.10, pH 6.0 was selected for the study on Pb-binding and also on Pb-induced oxidase inactivation.

The stability of Pb binding on Cp was also checked. As shown in Figure 11, the longer time for Cp storage, the larger the decrease in Pb binding. And if necessary, the allowable time for the storage of Cp at 4°C was 3 days.

5 Binding Studies of Pb on Cp

To study the binding of Pb on the Cp, purified Cp was used in the following experiments. The metal-induced oxidase inactivation and metal replacement were compared.

The result in Figure 12 supported the close relation between these two parameters. The result corresponded very well with that reported by Holmberg (1951) and Michaelis (1957) stating that Cp activity was proportional to the Cu content on Cp molecule. Bearn (1952) observed a considerable decrease in serum oxidase activity observed in all patients (Wilson's Disease) with the low copper levels. While most of the research works pointed out that Pb-intoxication on heme synthesis enzymes caused anemia (Chisolm, 1971), the results from this study suggested the other explanation. Since Cu-bound Cp could oxidize ferrous to ferric iron, the essential component for heme synthesis, in physiological condition, the decrease in Cp oxidize activity caused by Pb may decrease the ferric iron concentration which finally lead to

anemia, the common symptom found in Pb-intoxicated patients.

Since the binding constant of Pb on the Cp was not determined in this study, it could not be compared with the binding constant of Cu. The result of this stoichiometric study indicated that the ratio of Pb binding : Cu released was lower than 1.

The mechanism of replacement may not by substituting Cu atoms in all binding sites. Messerschmidt (1990) reported that Cp molecule had 7 Cu binding sites. Fox (1995) reported that Cu atoms in Cp are classified by their coordination to up to four amino acid residues, and each type is distinguishable by optical absorption and by electron paramagnetic resonance spectra : type 1 Cu have a characteristic blue color and coordinated to the 4 amino acids specifically given in the sequence ; His-Cys-His-Met (Leu), type 2 Cu are generally coordinated to 3 histidines with a weak association and they do not have visible absorption spectra, and type 3 Cu are binuclear pairs with an absorbance peak at 330 nm which each Cu in the pair is coordinated to 3 histidine residues. Based on X-ray crystallographic structure, only the type 1 Cu may govern the Cp oxidase activity. It could be concluded that Pb may not directly inactivate oxidase activity, the inactivation may be due to the loss of Cu essential for oxidase from Cp molecule and Pb may selectively replace for oxidase-catalized Cu. Further study on the Pb binding kinetics on Cp

may confirm this explanation.

It can be suggested from this results that the decrease in Cp's oxidase activity may be used for the prognosis of Pb intoxication.

6 Effect of some Chelators on The Metal-Cp Binding

Goodman (1975) reported that the drugs possessing the common property of forming soluble complexes with heavy metals which are excreted in the urine, thereby preventing or reversing the binding of metallic cations to body ligands are called chelating agents. The product of such a reaction is a heterocyclic ring and a metal chelate is usually more stable than is the nonchelate complex of the same metal and one ligand. The following are many chelators which are used in the treatment of lead toxicity.

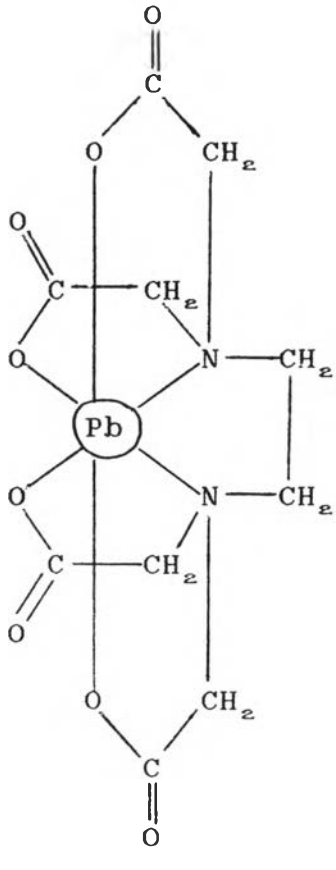
1) $CaNa_2EDTA$, compounds have found application as industrial and analytical reagents for a number of years owing to their property of chelating many divalent and trivalent metals. The successful use in the treatment of lead poisoning is due to the ability of lead to displace calcium from the chelate and $PbNa_2EDTA$ disappears exponentially from the cisculation with a half-life of 20-60 minutes, about 50 % is excreted in the urine in 1 hour and over 95 % in 24 hours.

2) BAL, SH-containing molecules which can form a

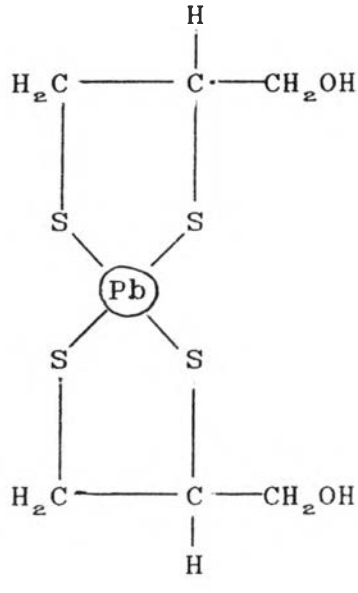
stable and relatively nontoxic chelate ring with the heavy metals, has the short half-life and metabolic degradation and excretion are essentially complete within 4 hours.

3) Penicillamine, an effective chelator of many heavy metals and promotes the excretion of metals in the urine. The chelate is well absorbed from the gastrointestinal tract and is rapidly excreted in the urine, therefore, has a decided advantage over other chelating agents.

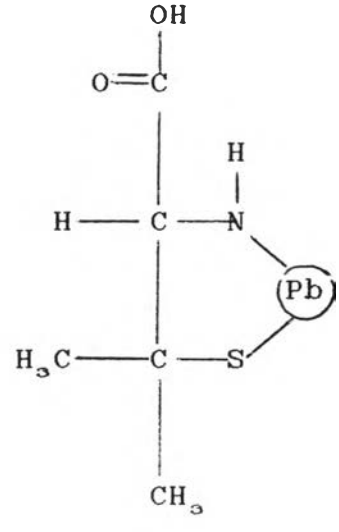
The review paper of Chisoim (1971) showed the diagrams of lead chelates formed by EDTA, BAL and D-penicillamine which was shown in the following.



Pb-EDTA



Pb-BAL



Pb-(D-penicillamine)

In this experiment, $CaNa_2EDTA$, BAL (Dimercaprol) and Pinicillamine, the commonly used chelators at the same concentrations used in clinical treatment, were tested for their abilities to remove Pb from Cp molecule. It is concluded from Table 3 that all chelators used could remove Pb bound on Cp molecule almost or completely. However, the replacement of Cu with Pb could not be detected on IEF-PAGE (Fig.13). It may be due to the low resolution of the IEF to resolve the small difference of pI's. This was confirmed by the results in lane 6, 7 and 8 (Fig.13) in which 3 standard proteins of moderate difference in the pI's showed little difference in electrophoretic mobility. The other possible explanation is the loss of the lossely metal-bound from the Cp molecules in electrophoretic field.

SUMMARY

1. F-AAS was tested for its sensitivity and specificity, and was selected for determining Cu and Pb concentration throughout this study.

2. Protein factors possessing Pb-binding activity was found in human serum. One of these factors was characterized by its Cu-binding activity with F-AAS, its molecular weight by Sephadex G-200 column chromatography and its electrophoretic mobility by non-denaturing anionic polyacrylamide gel electrophoresis. All the results showed that it was ceruloplasmin, the Cu-binding protein in serum. Preliminary study in human serum showed that Cu was removed from the serum protein upon Pb binding.

3. Pb-bound ceruloplasmin was also found in Pb-intoxicated human patient serum by Sephadex G-200 column chromatography.

4. Pb-binding study was also confirmed on purified ceruloplasmin. At saturation concentration of Pb utilized, 5.0 atoms of Cu was removed from a molecule of Cp while 3.2 atoms of Pb bound. Upon removal of bound Cu from the protein with Pb, its oxidase activity was decreased, both effects corresponded with each other.

5. The metal chelators : $CaNa_2EDTA$, dimercaprol and D-penicillamine could almost or totally remove Pb bound on the Cp molecule. However, IEF-PAGE (pI = 4-6), with the condition used in this study, could not fractionate the

metal-bound from metal-chelated Cp.