CHAPTER IV

DISCUSSION

4.1 Methods for Metal Determination

Throughout this study , the concentrations of Fe and Pb were always determined in blood and serum samples, so, experiments in the first part of this thesis were on testing and selecting the most appropriate quantitation methods for Pb and Fe. Mori (1981) and Varley (1976) have reviewed on the chemical methods for Fe and Pb determination which are accepted as a convenient way to determine the metal concentration. However, the sensitivity of these chemical methods are quite low , both the Ferrozine method for Fe and King's method for Pb measurement provide the reliable detection range of mg/l (Mori , 1981 ; Varley , 1976 and Figure 1). Moreover, sample volume for the detection becomes the limiting factor, at least 1 ml of serum is required per assay.

Ferrozine method is a common chemical method for serum Fe determination in clinical diagnosis , in which the color reagent- Ferrozine is used. At acid pH and in the presence of a reducing agent, ascorbic acid , Fe reacts with Ferrozine and forms a magenta complex which can be determined by the absorbance at 592 nm. The result in

Figure 4 shows that among various buffers tested, acetate buffer pH 4.15 is most suitable for measuring Fe concentration by Ferrozine method , it gives the highest standard curve and does not interfere the iron-determination system , Pb (250-2,000 µg/l) hardly interferes this system (Figure 5). The specificity test in every method utilized is of importance for metal determination in this study, since both Fe and Pb were always present in the same sample. Moreover, Pb binding was highest at pH 4.15 , as compared to other pH's (Table 3). Fe determination by Ferrozine method, however, was utilized in this study only in some cases, when the metal concentration and sample volume were large enough. Chemical method was not suitable for Pb measurement in this thesis since its concentration in the sample was in µg/l range which is beyond the sensitivity of the method, and sample volumes were always small.

Another method for the determination of metals is radioactive technique which gives high sensitivity and precision (Talwar , 1980) but is difficult to handle. Moreover, Pb isotope is very expensive. This technique was, therefore, not considered in this study.

The spectrophotometric method by now is the most suitable , reliable and acceptable technique for determining trace metal concentration , especially in biological sample (Whitehouse *et al.* , 1982). Graphite Furnace Atomic Absorption Spectrophotometry (F-AAS) and Inductively Coupled

Plasma - Emission Spectrometry (ICPS) are two of the techniques widely used for the determination of metals .

There are many reviews that compare F-AAS with other analytical techniques for biological samples (Slavin, 1988), Morrison (1979) found that F-AAS offers the greatest accuracy among the available methods.

The simple concept of F-AAS was proposed by L'vov in 1969 stating that taking only a small sample and quickly heated to a particular high temperature (within an electric furnace)(Figure 24), all of the sample including the analyte will be converted to an atomic vapor. The analyte will absorb a portion of the light from a lamp containing a pure element. In this situation, the integrated absorbance , at the element resonance line will be proportional to the mass of element in the sample (Slavin, 1988).

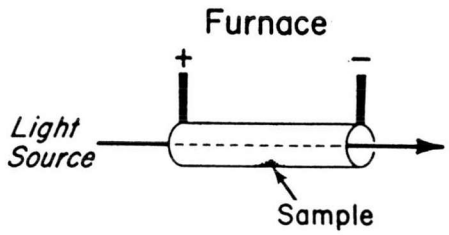

Figure 24 L'vov Concept (Slavin, 1988)

ICPS is a technique which is well - known for elemental analysis of metals with wide dynamic range and high sensitivity, $\mu g/l$ detection limits. It is claimed that it enables simultaneous or rapid sequential multielement determination of major , minor , and trace element sample constituents without changing experimental parameters , possessing a lower degree from matrix interference and a minimum of sample pretreatment (Wolnix, 1988).

In Inductively Coupled Plasma Spectrophotometer, the sample is usually transported into the instrument as a stream of liquid sample. Inside the instrument, the liquid is converted into an aerosol by nebulizer. The sample aerosol is then transported to the plasma by carrier gas (argon in this study). Inside the plasma , the aerosol is desolvated , vaporized , atomized , exited and ionized. The excited atoms and ions emit their characteristic radiation which is collected by a device that sorts the radiation by the wavelength. The radiation is detected and turned into electronic signals that are converted into concentration information for the analyte (Boss and Fredeen , 1989). A representation of the layout of a typical ICPS instrument is shown in Figure 25.

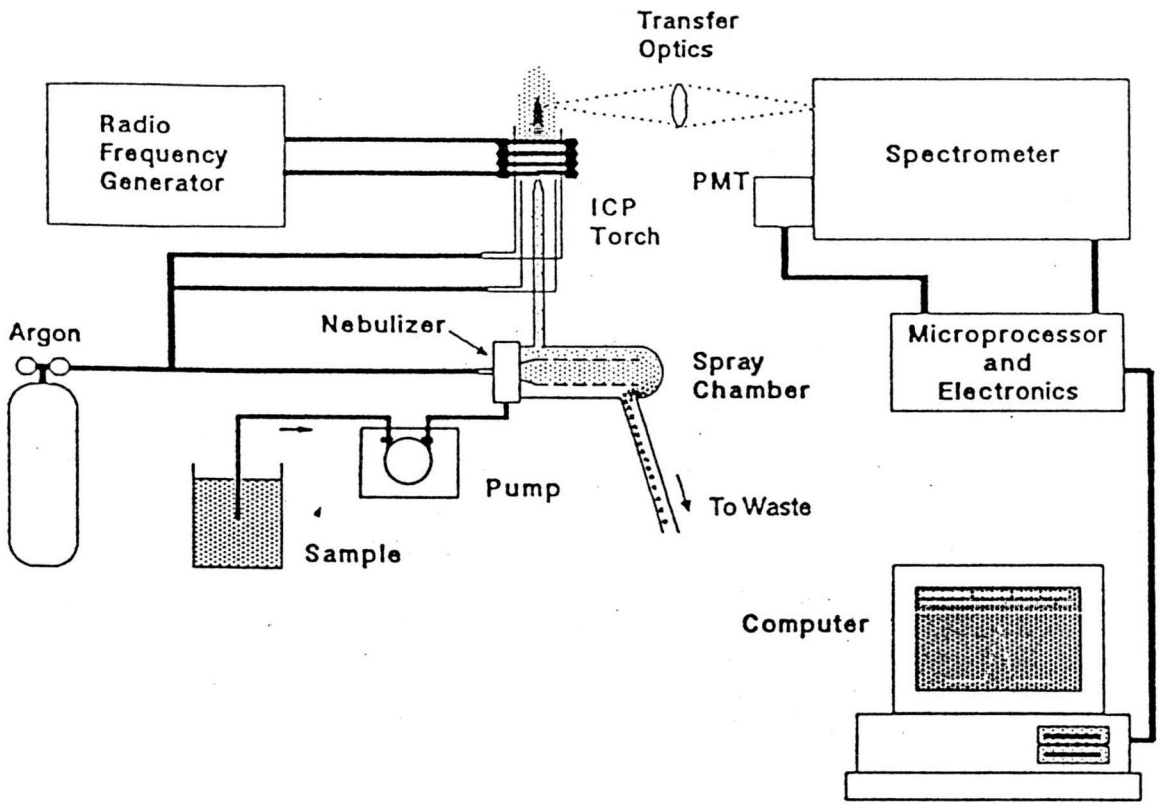

Figure 25 Major components and layout of ICPS

F-AAS and ICPS are methods of choice in this study with the following reasons. The preparation of samples for F-AAS and ICPS is very simple, only diluting with 0.02 M HNO_3. As shown in Figures 8-11, F-AAS provides many advantages , it is very sensitive , the detection limit for Pb and Fe are 10 and 5 $\mu g/l$, respectively. This allows possible measurement of metals in very dilute samples , such as those from chromatographic profile.

Although the system was interfered with serum and 0.1 M acetate buffer (Figure 8), these matrix interferences could be easily eliminated by diluting serum 30 folds and

lower the concentration of 0.1 M acetate buffer to 0.01 M. Futhermore, F-AAS is also specific for both Fe and Pb , the effect of spectral interference is very low (Figures 10, 11). F-AAS, therefore, has high potential in determining the concentration of each metal in the sample containing both of them.

In contrast, even though ICPS is free from matrix interference, its spectral interference is quite significant (Table 3). Fe cannot interfere Pb determination by ICPS but Pb emission could also be detected at Fe wavelength. Thus, ICPS cannot be used for measuring Fe concentration in the sample containing Pb. Moreover, the determination of metal concentration by this technique is limited by the following factors : the detection limit of Pb is rather high (50 μg/l, see Figure 7) and sample volume for the detection is quite large (at least 3 ml). Therefore , it is not suitable for sample containing low amount of Pb or sample volume lower than 3 ml. However, this technique can be used for measuring Pb concentration in some cases of this study, when the metal concentration and sample volume are appropriate.

4.2 Distribution of Lead in Whole Blood

Even though heme synthesis occurs mainly in nucleated erythrocytes of bone marrow , the process also takes place in developing blood cell of blood stream (Guyton, 1981). Since the latter can be obtained more easily, it has

always served as a better source for studying heme synthesis (Simons, 1986). Various reports on the effect of Pb on heme synthesis were from experiments with blood cells. Therefore, they were used in this investigation as the model to study Pb transportation. The distribution of Pb between serum and erythrocytes may reflect the transport mechanism, whether or not it requires carrier(s). The understanding may finally lead to the prevention and/or treatment of lead poisoning.

When red blood cells were incubated with the concentration of Pb reported to cause anemia (Goyer, 1991). The result in Figure 12, which corresponds to Simons' report, shows that Pb was taken up rapidly by the erythrocytes. When the blood concentration was kept constant , the rate of Pb uptake depended on the concentration of Pb treated. However, the uptake was not complete, a certain amount of Pb still remained in the serum. It is important to note that they were equal in both cases (600 and 1,800 μg/l Pb utilized). This result suggests that the transportation of Pb across cell membrane may not be with simple diffusion but require some factor(s) in plasma.

When Pb uptake to erythrocytes was compared in the presence and absence of blood plasma, the result in Figure 13 confirms the requirement of some plasma factor(s) for Pb uptake. In the absence of plasma, the taken up at saturation was only 30 % of that in the presence. Moreover, the rate of uptake was slower. In this study, the experiment

in the absence of plasma was done in phosphate buffer where Pb had been reported to possess lower solubility (Simons, 1986),and one may argue that this may lower the rate and percent uptake. However, the other results confirm the fomer idea. This study insists on the conclusion that the uptake of Pb requires some factor(s) in plasma.

4.3 Characterization of Pb-binding Protein in Human Serum

The Pb-binding factor(s) was further examined for its physiological property. The result comes up with the conclusion that it might be Fe-transporting protein. From Tables 4 and 5 , it cleary indicates that Fe was released from serum protein upon Pb-binding. The replacement was highest at pH 4.15 (Table 4) and its degree depended on the amount of Pb utilized (Table 5). It is important to note here that the mole ratio of Fe released : Pb bound was about 20:1. From literature survey , this binding has not been reported elsewhere.

As previously mentioned in section 1.4 that in physiological condition, Fe is almost totally transported by a glycoprotein (M.W. 90,000 Da) referred to as transferrin (Tf). Experiments were then set up to check whether Tf is the protein responsible for Pb-binding. The factor(s) which Pb was further characterized by two techniques ; Sephadex G-200 column chromatography, based on the molecular weight of the proteins, and Non-denaturing Polyacrylamide

Gel Electrophoresis (ND-PAGE), based on their molecular weight and charge.

The chromatographic results in Figures 14-17 indicate that Pb-binding protein was eluted at the same position of Tf. In addition, the positions of Pb peak (Pb peak II) and Fe peak in normal serum were the same. These results confirm that Pb and Fe bind to proteins of the same molecular weight about 90,000 dalton (Figure 18) which is close to Tf. These experiments on *in vitro* Pb-treated serum is further supported by experiment with Pb-toxic patient serum (Figure 17). The result is even clearer in the latter experiment , due to lower amount of free Pb in the sample. Most important, this finding not only confirms that Tf can bind to Pb in experimental condition, but the binding really exists *in vivo*.

Moreover , Pb-associated peaks could also be eluted from the column at other positions (peak I and peak III, Figures 14, 15 and 17) , indicating the presence of other Pb-binding proteins with molecular weight higher and lower than Tf, respectively. However, these proteins have not yet been characterized any further.

The results of ND-PAGE in Figures 19-20 confirm the presence of Tf in Pb-rich fraction , even it was still contaminated with other proteins.

4.4 Binding of Pb on standard Tf

As depicted in Figures 19,20 and 22, the Pb-binding peaks were still contaminated with other serum proteins. Purified Tf , therefore , was used to confirm the Pb-binding to Tf and to calculate the mole ratio of Pb : Fe. It is expected that the result from such stoichiometric study may lead to the understanding of mechanism of Pb to Fe replacement , the result is shown in Table 7. Since the standard Tf used in this experiment was not saturated with Fe , the addition of ferric ammonium sulfate results in an increase of Fe bound to Tf molecule. The mole ratio of Fe:Tf rises from 0.77:1 to 1.73:1 which is close to that reported previously by Righetti (1990). The author reported that each molecule of Tf can bind to two molecules of the metal at saturated amount of Fe. The binding is specific to the hydrophobic pocket , with side chains of four amino acids , namely Asp 61 , Tyr 93 , Tyr 191 and His 252, as shown in Figure 26.

The result in Table 7 also indicates that Pb cannot completely replaces Fe , since the decrease in mole ratio of Fe:Tf molecule is from 1.73:1 to 0.56:1. This may be due to the concentration of Pb used was too low (the concentration used was only that reported to cause anemia by Goyer (1991). In contrast, the increase of mole ratio of Pb:Tf was very low(From 0:1 to 0.03:1). The interpretation is Pb binding may weaken the binding of Fe to Tf and cause the release of

Fe from Tf molecule. However, Pb:Tf ratio cannot be calculated conclusively in this experiment because Pb concentration utilized here is what reported to cause anemia, much lower than the saturation level. The only conclusion up to now is " at Pb concentration which causes anemia - 1 mole of Tf binds to 0.03 mole of Pb ". In order to study the stoichiometry of replacement, the higher concentration of Pb must be employed. The kinetic study of Pb binding may also be important.

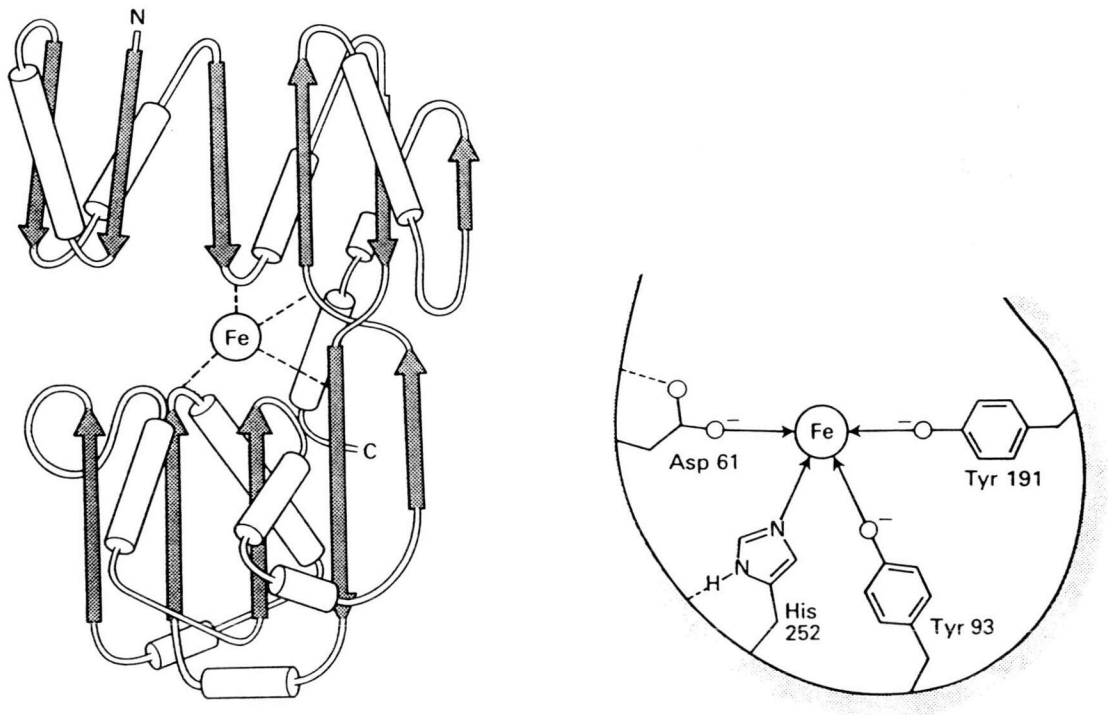

Figure 26 The binding of Fe to Tf molecule (modified from Frausto da Silva and William, 1991)

(a) The outline fold of Tf, a hinged protein

(b) Details of the binding site for Fe

To support the above notion, the investigation on shifting Tf bands in IEF-PAGE with metal binding was done. Righetti reported in 1990 that Tf exists in apo and holo forms , the former is Fe - poor Tf and the latter is Fe-saturated Tf. Addition of Fe to the apo form can turn it to holo form, shifting the mobility of Tf major band to more anodal site. This shifting is due mainly on the charge of metal bound to the protein molecule. The mobility of Tf shifted by Pb or Fe as shown in Figure 23 was identical, similar changes in charge on Tf molecule suggest that the same amount of Pb and Fe bind to Tf.

The noncorresponding results from this study and stoichiometric study in Table 7 make the interpretation on Pb to Fe replacement more difficult. To earn that decisive conclusion , the mole ratio of Pb:Fe must be experimented at their saturated levels.

4.5 Reduction of Tf-bound Pb with Some Chelators

Chisolm (1971) reported that the effect of Pb on heme synthesis is a reversible process and the anemia can be improved by the preventing of the patient from exposure to excessive amount of Pb. The author also introduced the treatment with drug containing chelating agents; the molecules that tend to bind metal atoms firmly, sequestering them and thus rendering them highly soluble. Selbst (1985) and Orapan Metadilogkul (1992) reported that

some chelators can, moreover, mobilize Pb from tissues or target organs and promote its excretion through the kidney and the liver. From this experiment, EDTA, Dimercaprol and D-penicillamine , the commonly used chelators at the concentrations used in clinical treatment, were tested for their ability in the removal of Pb from Tf molecule. The result in Table 6 indicates that all the chelators used can eliminate Pb bound to the protein almost or completely.

Gathering all the results in this study, together with others from recent reports (Clarkson and Kench, 1958 ; Simons, 1986) , one can propose the mechanism of Pb transportation in blood stream. When Pb enters the blood stream, its major portion may be taken up by red blood cells, the uptake requires Tf. Both red cells and Tf are responsible for the transport of Pb to other target organs specific to Tf. Not only red blood cells, but other organs, such as bone marrow, will also be affected by Pb.

The finding that Tf plays an important role in Pb transport may provide some clue to a new diagnostic method for lead poisoning. Since, there is a striking increase in Tf concentration in Fe-deficient anemia with decreased heme synthesis (White *et al*, 1968), the amount of Tf increased in anemia caused by Pb, may serve as a biochemical indicator for lead poisoning suffered by workers who have been occupationally exposed to Pb.

SUMMARY

1. F-AAS is tested and selected for determining Fe and Pb concentration in this study. ICPS with its lower sensitivity was also used with specific precaution on its spectral interference.

2. There are some factors in serum which can bind to Pb and involve in the rapid transportation of Pb to blood cells.

3. One of the Pb-binding factors in serum was characterized. Fe-binding property , molecular weight determination by Sephadex G-200 column chromatography and identification by Non-denaturing Polyacrylamide Gel Electrophoresis , identify the protein as transferrin.

4. $CaNa_2EDTA$, Dimercaprol and D-penicillamine are the chelators which can eliminate most or all Pb from Tf molecule.