CHAPTER I

INTRODUCTION

The studies of serum proteins by various methods have long been established in clinical laboratories. The studies were in the form of total protein, albumin globulin ratio and quantitation by electrophoresis. In the last few years, the characteristic changes in the concentration of various serum proteins in certain diseases have been demonstrated by the immunological technics. Approximately 30 different plasma proteins in human serum can be detected by immunoelectrophoresis.

These components differ in their electrical charge, size, and composition. They can be detected by immunodiffusion method of Oudin, double diffusion of Ouchterlony, and immunoelectrophoresis. They can be quantitated by RID. All of these methods contributed greatly to the study of serum protein components. This progress depend so much to the purification of these proteins, followed by the production of monospecific antisera.

The demand for monospecific antibody to human serum protein components are increasing in correspondence with the interests in research work. Antisera which are commercially available are expensive and not readily available in this country. Therefore, it is desirable to produce these antisera in locally to reduce expenses and encourage more research work in protein chemistry. The experience gained will be used in the study of protein chemistry in any area.

In this study, purification of haptoglobin and ceruloplasmin followed by the production of monospecific antibody is described. Quantitation of these serum proteins in normal and abnormal specimens were made by using the antisera produced.

Literature Review

a. Ceruloplasmin.

There are metal carrier proteins which was isolated by Koechlin. Among these are β_1-metal binding globulins, which are capable of combining with iron, copper, or zinc; α_1-globulin and α_2-globulin that bind with copper and zinc (67).

Following repeated precipitation of the Cohn fraction IV-4, the metal-binding globulin is obtained in a purity of 90-95% and a yield of 0.42 g% per liter of plasma is realised (29). This is accomplished by the precipitation of impurities at -5°C, pH 4.3-4.5, ionic strength 0.10 and 25% ethanol. By electrophoresis it moves in the β_1-globulin region. It contain about 5.5% carbohydrate which is consisted of N-acetylneuraminic acid, mannose, galactose and N- acetyl glucosamine. The iron binding capacity at pH 7.4 is 1.25. In the presence of carbon dioxide it binds 2 atoms of ferric iron (Fe^{3+}) per mol. of protein (126). This fraction was termed transferrin, with M.W. of about 85,000.

Alpha-globulin also contains a copper binding protein and this fraction was named ceruloplasmin.

Trace amounts of copper in body fluids and tissues are essential to life. Copper serves as an oxygen carrier in the hemolymph of mollusca and arthropods and as catalytic oxidant in the enzymatic formation of melanin pigment and insect cuticle (43, 50). In mammals it may play a role in heme synthesis, bone development,

and the normal functioning of the CNS (2, 109).

In normal subjects it was found that there are 4 fractions of copper in blood, two in erythrocyte and the others in serum. Total erythrocyte copper in normal is about 115 mcg/100 ml packed red cell. With 30-60 per cent of these amounts is called labile fraction which can exchange between erythrocyte and plasma (22). Mann and Keilin found the pale blue copper protein, in ox red blood cell, which contains 0.34% copper with a M.W. of about 35,000; but isolation and purification could not be done at that time. Isolation of this fraction from human red cell was accomplished recently, and it was named erythrocuprein. It is believed that erythrocuprein supports the integrity of adult erythrocytes. Experimental animals develop severe anemia when they lack of this copper protein fraction and their red blood cells have a markedly shortened life span (111). Total serum copper in normal subject is about 114 mcg/100 ml. It was demonstrated that there is a small fraction of the plasma copper (about 0-17%) which reacts directly with sodium diethyl dithiocarbamate, the copper colorimetric reagent (42, 49). This fraction has been referred to as the "direct-reacting fraction" of plasma copper and evidence has been shown that this fraction is actively concerned in the transportation of copper. The remainder of the copper in plasma does not react directly with the carbamate reagent and has been referred to as the "indirect-reacting fraction" of plasma copper (49). It will react directly with the carbamate

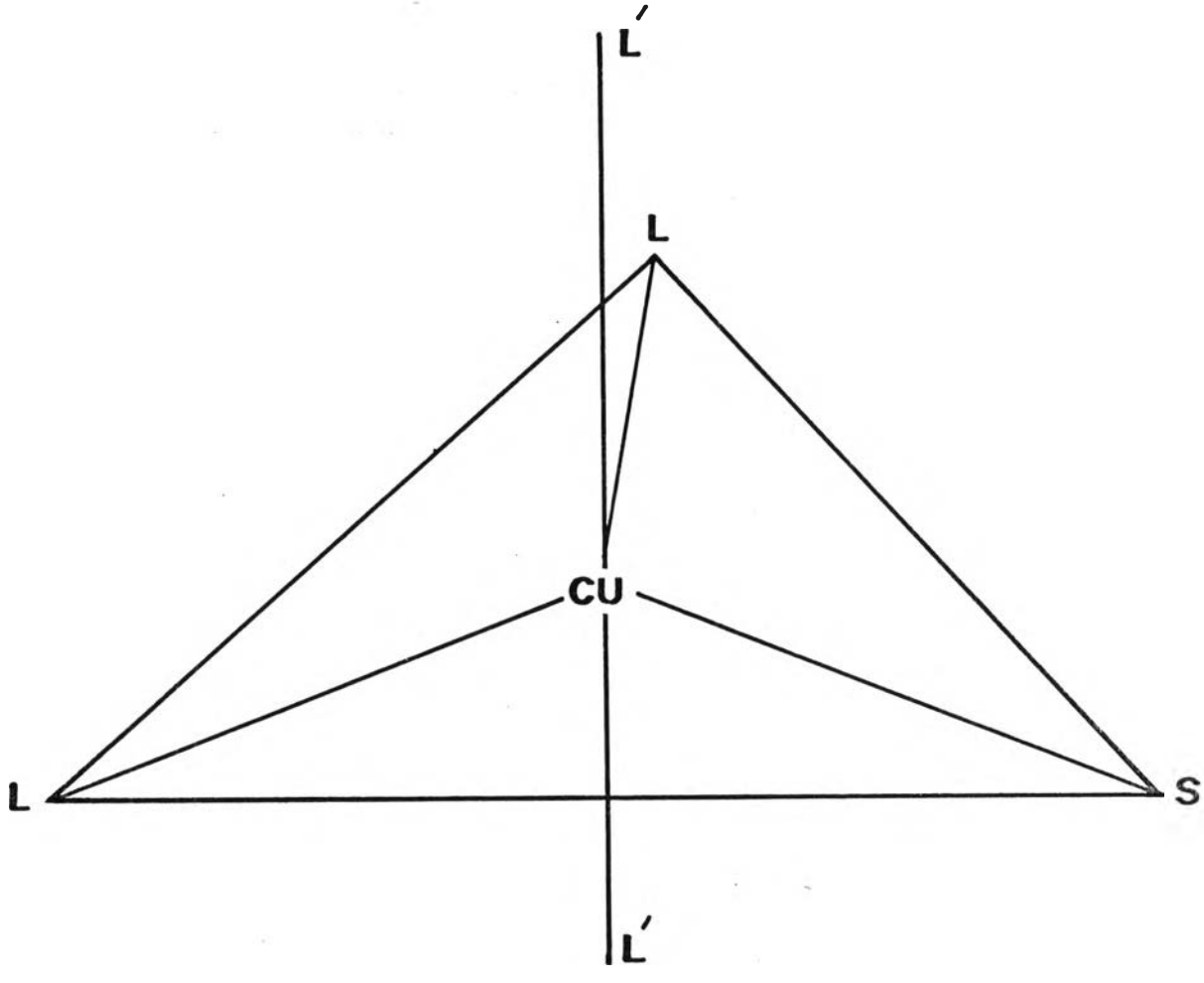
reagent after treatment with hydrochloric acid (83). Bearn and Kunkel nave shown that this fraction is bound to serum albumin, and it corresponds to ceruloplasmin (10).

Holmberg and Laurell were the first to isolate ceruloplasmin, the blue copper protein of plasma in 1948 (54). They characterized this protein as α_2-globulin with a copper content of approximately 0.34%, M.W. of about 151,000. It can bind 8 atoms of copper per mol. and it contains 7.5% of carbohydrate. The 8 atoms of copper are equally distributed between Cu^{+} and Cu^{2+} (1). Furthermore, the blue copper centers which found in the copper oxidase is a unique kind of mononuclear copper complex and its biological function appears to be intramolecular electron transfer (58). By using Pesonance Raman Spectroscopy it was found that ceruloplasmin do not have intense absorption near 600 nm (82), but fall in the frequency range of 350-470 cm^{-1} which is characteristic for the stretching of Cu-N or Cu-O (45), when the ligand atoms are not part of, or attached to, an aromatic ring. The ligand atoms could threfore be nitrogen from lysine or arginine side chains, oxygen from glutamate, aspartate, or serine side chains, or they could be nitrogen and/or oxygen from peptide units (91). Therefore the basic model for ceruloplasmin should be a trigonal plane with a cysteine mercaptide ligand sharing the equatorial plane with two strong field ligands (87)(Fig. 1, p.6).

Ceruloplasmin has been shown to exhibit oxidase activity, with ascorbic acid, adrenalin, Dopa and paraphenylene diamine, and

Fig.1

The mononuclear copper complex of Ceruloplasmin molecule study by using Resonance Raman Spectroscopy.



L. might be nitrogen ligands (but not imidazole)
L′. might be nitrogen or oxygen ligands.

it is most active with paraphenylene diamine (56, 57). Some phenothiazine derivatives are known to be oxidized to free radicals by ceruloplasmin (80). The 10-position side chain of phenothiazine derivatives plays a major role in binding to the active site on ceruloplasmin molecule (88). Phenothiazine derivatives also increase the rate of ceruloplasmin calalyzed oxidation of Calecholamines (6, 80) and the same as the most reactive substrates of ceruloplasmin do to dopamine (81).

Dialysis against a chelating agent removes four atoms of copper, leaving a colourless protein and prolonged dialysis yields the apoprotein free of copper (126). Reduction to Cu^{+} bleaches the color completely under anaerobic condition (87). The reaction is completely reversible in the presence of oxygen (126).

The ferroxidase activity of ceruloplasmin appears to be essential for the transport of iron from reticuloendothelial cells to transferrin (105, 127). The ferroxidase activity in rat was about 1/3rd that of human ceruloplasmin and 1/10th that of porcine ceruloplasmin. Based on the studies in vitro demonstrating that ceruloplasmin (ferroxidase) catalyzes the oxidation of iron (94) and on the observation that ferric but not ferrous ion is bound by apotransferrin (45), it was proposed that ceruloplasmin functions in iron metabolism by oxidizing iron, there by enhancing the rate of transferrin formation. But Bates and Schlabach have presented arguments against this concept (7). So the role of ceruloplasmin

in the movement of iron from reticuloendothelial cells to transferrin is still not definite. One explanation is that ferrous iron occupies specific iron binding sites on the membranes of reticuloendothelial cells and that ceruloplasmin is required to remove iron from these sites, first by entering into a reaction with the site itself and then by the formation of a ceruloplasmin-iron inter-mediate which then transfer iron to apotransferrin by a specific ligand exchange reaction (85, 95).

It is plausible that ceruloplasmin may not act as the membrane-plasma interface but may act indirectly by correcting the defficiency of some other copper-containing enzyme. In this regard, it has been shown that cytochrome oxidase, a copper-containing mitochondrial enzyme, is required for the intercellular reduction of iron (128). This enzyme will be decreased in copper deficiency (46, 77). Ceruloplasmin may function by regenerating cytochrome oxidase, and thereby enhances the formation of the membrane-bound ferrous iron pool which is available to transferrin (19).

Ceruloplasmin is exclusively synthesized by the liver. This has been shown by using Cu^{64} in an isolated rat liver perfusion, and by injecting the hepatotoxic preparation into a rabbit. These experiments reaffirm the evidence that the liver parenchymatous cells are the principal site of ceruloplasmin formation. Its normal concentration as calculated from serum copper concentration vary from 20-35 mg per 100 ml. (76, 99).

Ceruloplasmin is formed during intrauterine life, but the serum copper value (Cu/S) at birth is only 50 mcg per 100 ml (78). The level incerase to the adult level in 1 year of age and still remain stable until adult. Since the Cu/S reflects the level of the ceruloplasmin, the variation of the ceruloplasmin in various diseases can be concluded from data of the variation on the serum copper in physiological and pathological conditions (55, 83). The alteration of ceruloplasmin level can be observed in many diseases (24, 25, 71). It is elevated in patients with infections, malignant tumours (leukemias, Hodgkin's disease), necrosis, and after administration of estrogens, thyroxine and pyrogens. During pregnancy ceruloplasmin rises to a mixinum in the last trimester. It is depleted in patients with Wilson's disease and nephrotic syndrome (26, 37, 71). In Wilson's disease the low level of ceruloplasmin has received much attention in combination with an increased concentration of direct-reacting fraction of plasma copper (9, 11, 26, 108).

Methods for quantitative estimation of ceruloplasmin in serum have been based on its blue color (35), its copper content (27), its oxidase activity (1, 3, 51, 63, 101, 102, 120, 129), or its immunological properties (108). The most commonly used procedures are based on the oxidase activity of the protein on diamines, such as benzidine (1), N,N-dimethyl-p-phenylenediamine (3), p-phenylenediamine (51, 101, 102, 120, 129), or o-dianisidine dihydrochloride (63). However, these methods require special precautions and purification

of substrate owing to light-and metal ion-catalyzed oxidation of the substrate and instability of the product (51). Furthermore, benzidine is a powerful carcinogen for humans (1). Therefore, immunological method is one of the most suitable method to determine serum ceruloplasmin in clinical laboratory.

b. Haptoglobins.

Small amounts of hemoglobin (1-3 mg per 100 ml) are normally demonstrable in the plasma or serum from which erythrocytes have been separated (33). This circulating plasma hemoglobin is bound to haptoglobin, the hemoglobin binding plasma protein, which was first demonstrated by Jayle et al in 1939 (97). It is a glycoprotein containing 20% carbodydrate. It has α_2 electrophoretic mobility. It is normally present in serum of man and many mammals. The haptoglobin molecules in most species so far have been known to be composed of 2 different polypeptide chains, α and β chains, linked by disulfide bonds (32, 66, 112, 114). In rabbit the disulfide bridges is absent (76). The molecular structure closely resembles that of the γ-globulin which may reflect a common evolutionary origin (18).

Human haptoglobin exhibits genetic polymorphism determined by allelic genes. Smithies and his colleaques found that haptoglobin could be classified into 3 types by starch gel electrophoresis. This three types are 1-1, 2-1, and 2-2, which is genetically determined (6, 9, 113). The genetic variability of haptoglobin is based

on structural differences of α-polypeptides. The hypothesis was confirmed by Black and Dixon who showed that there are 3 common alleles of the haptoglobin locus Hp^{1F}, Hp^{1S} and Hp^{2}. The combination of alleles accounts for the presence of the six common haptoglobin phenotypes. They are, Hp 1F-1F, 1F-1S, 1S-1S, 1F-2, 1S-2 and 2-2 (15, 116). Allison et al found some cases with no detectable haptoglobin in their study and this substantiate the existence of an Hp^{0} allele (5). This type of haptoglobin may represent either defficient synthesis of haptoglobin or synthesis of an altered haptoglobin that no longer binds hemoglobin. The β chains of Hp, which are involved in the binding of hemoglobin are similar, possibly identical in all types (28, 32, 47, 48).

Human haptoglobin has been well characterized biochemically and genetically, although the physiological function has not been clearly established (121). Its significant biological property is its affinity for binding stoichiometrically with hemoglobin in vivo and in vitro forming a stable complexes (62). On complexing with haptoglobin, hemoglobin dissociates into α-β dimers (21, 75, 90, 113). One mole of haptoglobin binds 1 mole of hemoglobin, but it may vary with various Hp phenotype. The formation of the Hb-Hp complex is rapid and irreversible (89).

Hp appears to be synthesized exclusively in the liver (79, 96, 123) and is released directly into the plasma. The physiological role of haptoglobin depends on the formation of the Hb-Hp complex.

This is the first step in the metabolism of plasma hemoglobin (20). By complexing with hemoglobin, Hp prevents urinary loss of hemoglobin and iron. Most of hemoglobin liberated intravascularly is immediately complexed with Hp until Hp is saturated. Saturation of Hp is never reached under normal circumstances. Once bound, the Hp-Hb complex is rapidly and completely removed from the circulation by the reticuloendothelial system (13, 65). Regeneration of Hp is slow; the normal concentration is not reached again until 5-7 days after complete removal from serum (72). Its maximum half-life time is 5 days (93), which represents its normal catabolic rate plus the amount normally removed as the Hb-Hp complex.

Apparently, the capacity for producing Hp does not develop until sometimes after birth (115, 124). These proteins are not transmitted across the placenta, although the M.W. of Hp, type 1-1, is only 85, 000 (61) and the complex with Hb has a M.W. of 155,000 or about the same as that of γ-globulin which are readily transfered from mother to fetus (4, 5).

Serum Hp has a normal range of 100-300 mg%. Variations in levels occur in several pathologic conditions. Elevation of the serum level is characteristic, but nonspecific, finding in infections and inflammatory conditions. The concentration rises slowly with the onset of infection, and return slowly to normal after the clearing of infection. Depletion of Hp levels indicate recent acute intravascular hemolysis. Thus, Hp quantitations are highly

applicable for laboratory diagnosis of hemolytic crisis. These conditions are blood group incompatibility, and various forms of hemolytic anemia, and even in pernicious anemia (92).

Methods, based on various principles are currently in use to determine serum Hp levels. These include measurement of the peroxidase activity of the Hb-Hp complex (60, 122) which can be inhibited by certain serum factors (104), electrophoretic (41), gel filtration (79), column chromatography (100), and immunodiffusion technics (38, 66).