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Copper Levels in the Third Trimester of Pregnancy

by

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ABSTRACT

Copper concentrations were determined in maternal serum, placenta, and umbilical cord blood with the objective of elucidating the metabolism of copper in various conditions of pregnancy as compared to normal levels in the third trimester of pregnancy. Maternal serum copper was determined in 206 cases of normal pregnancy and 144 cases of different conditions including foetal growth retardation, toxaemia, diabetes, premature labour, and postmaturity. In cases of foetal growth retardation, a statistically significant decrease in serum copper values (mean; 183.44 µg %) was observed when compared to values found in normal pregnancy (mean; 261.29 µg %). In cases of toxaemia and diabetes higher than normal values were found, which demonstrate a trend possibly related to hepatic injury with release of copper stores into the plasma. In cases of premature labour, umbilical cord serum copper concentrations were lower than normal; placental copper levels in these cases were higher (mean; 10.15 µg Cu/gm dry weight) than normal (mean; 8.45 µg Cu/gm dry weight). This may indicate that the placenta is retaining unbound copper for transfer to the foetus after the 36th gestational week.

A significant inverse relationship was found between maternal serum copper and hemoglobin levels. It is suggested that the function of increased ceruloplasmin levels in pregnancy, reflected by higher ceruloplasmin-bound copper levels, may be to mobilize iron from storage organs and to maintain a supply of transferrin for transplacental transfer to the foetus as well as to meet an increased usage by maternal hematopoietic organs.

RESUME

En vue d'expliquer le métabolisme du cuivre pendant le dernier trimestre d'une grossesse normale comparativement à d'autres et avec des conditions différentes, on a déterminé des concentrations de cuivre dans le sérum maternel, le placenta et le sang du cordon ombilical. On a ainsi mesuré le cuivre dans le sérum maternel dans 206 cas de grossesse normale et 144 cas de conditions différentes, y compris des cas de retardement de croissance du foetus, de toxémie, de diabète, d'accouchement prématuré et de post-maturité. Dans les cas de retardement de croissance du foetus, une dimunition statistiquement significative de la quantité de cuivre dans le sérum (moyenne: 183.44 µg %) a été constatée comparativement aux quantités en cours de grossesse normale (moyenne: 261.29 ug %). Dans les cas de toxémie et de diabète, on a pu observer des valeurs supérieures à la normale dénotant une tendance attribuable à une lésion hépatique, avec libération des réserves de cuivre dans le plasma. Dans le cas d'accouchement prématuré, les concentrations de cuivre dans le sérum du cordon ombilical étaient inférieures à la normale; les niveaux de cuivre placentaire dans ces cas étaient supérieurs (moyenne: 10.15 µg Cu/gm poids sec) à la normale (moyenne: 8.45 µg Cu/gm poids sec). Ce résultat peut indiquer que le placenta retient le cuivre non lié afin de le transférer massivement au foetus, après la 36e semaine de gestation.

Une relation significative inverse a été relevée entre le niveau de cuivre du sérum maternel et le niveau d'hémoglobine. On est porté à penser que l'action des concentrations accrues de céruloplasmine pendant la grossesse, tel que l'indiquent les niveaux plus élevés de céruloplasmine - cuivre lié, pourraient servir à mobiliser le fer provenant des organes d'approvisionnement et à maintenir une quantité de transferrine pour transfert par voie transplacentaire au foetus et pour utilisation accrue par les organes hématopoiétiques maternels.

FORWARD

As an undergraduate student at McGill University, I read a paper from the Gastrointestinal Research Laboratory describing the effects of macromolecular compounds on the absorption of trace elements. Subsequently I became interested in the field of trace element research, and after my graduation I applied for a speciality which would deal with the subject. I was informed that the only department where this investigation is carried out is Experimental Surgery. I consider myself fortunate to have been able to join a group specializing in this area of research.

Dr. Stanley C. Skoryna, Director of the Gastrointestinal Research Laboratory, offered me to work on a project involving estimation of copper levels in pregnancy because of the possible significance of this element in various complications. I found this project very stimulating.

One thing I have learned as the work on this project proceeded is that progress in research is made when actual data become available, which determine further course of investigation. This refers to our findings on placental copper levels on which no literature was available. Subsequently we were able to correlate maternal serum copper levels with foetal levels and thus postulate a possible explanation for the maternal transplacental copper transfer to the foetus. Significant variations of maternal serum copper levels in certain conditions of pregnancy also revealed the diagnostic

value of these measurements. We were also able to postulate a possible functional role for ceruloplasmin in pregnancy, relating to the maintenance of sufficient iron concentrations in gestation. This area of research is specially appealing to me since copper is involved in many clinical conditions, including leukemias, Hodgkin's disease, and bacterial infections.

I found my work in both the Donner Building for Medical Research and at St. Mary's Hospital very pleasant. I wish to sincerely thank Drs. S. C. Skoryna, Y. Tanaka, A. Hurlburt, as well as Mrs. M. Fuska, Ms. A. Pawloska, and other members of the laboratory staff for their constant encouragement and cooperation throughout the entire project. I especially wish to express my gratitude to Dr. Y. Tanaka for having taught me both the theoretical and analytical aspects of atomic absorption spectrophotometry using the heated graphite atomizer. Dr. A. G. Thompson, Surgeon-in-Chief of the Montreal General Hospital and Dr. L. D. MacLean, Surgeon-in-Chief of the Royal Victoria Hospital, whose seminars in Experimental Surgery I have attended have helped me to learn and appreciate the numerous ongoing projects that are carried out in their departments. Dr. F. Naftolin, Chairman of the Department of Obstetrics and Gynecology of McGill University whose grand rounds I have attended have helped me to understand various clinical aspects of Obstetrics. Dr. J. Gordon, Director of the Division of Surgical Research

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Finally, I wish to thank Mr. C. Hatter, medical photographer, for his excellent photographic reproductions accompanying this thesis; case room head nurse, Mrs. L. Blouin, who was always willing to lend a helping hand in various problems related to collection of specimens; and special thanks is due to Mrs. C. Couture for having carefully reviewed and diligently typed the final manuscript.

Trace elements in the seventies are like vitamins in the thirties. Continuous further research will surely reveal a greater importance of these microelements with reference as causitive factors of many diseases. When trace elements became better known, the main question asked referred

to the effects of deficiency and toxicity. The current studies indicate a much wider scope including significance of variation in intake and utilization as related to pathological conditions.

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CHAPTER 1

INTRODUCTION

1. General Comments

As life evolved, many of the minerals present on the earth and in the seas became incorporated into biological systems, including mammals. Some of these elements have been maintained throughout the biological evolutionary course and occur in living tissues in such small amounts that they were referred to by early workers as "trace elements". At that time the knowledge for measuring their concentrations with any great accuracy did not exist, and their presence in tissues was assumed to be a result of environmental contamination. Underwood[151] has categorized the trace elements into three groups; (a) those essential for higher animals and plants, (b) those possibly essential, and (c) the nonessential. An "essential" trace element, according to Cotzias[30], may be classified as such if it meets the following criteria: (a) all healthy viable organisms possess the element in all of their tissues, (b) a constant concentration of the element is seen between different animals, (c) its withdrawal from the body induces the same structural and physiological abnormalities regardless of the species in question, (d) the addition of the element can prevent or reverse the observed abnormalities, (e) specific biochemical changes are always associated with the

abnormalities, and (f) the abnormalities may be prevented or reversed when the deficiency state of an animal with respect to the element is alleviated. Every trace element exhibits a whole spectrum of actions, depending upon the dose given and the nutritional state of the animal or system in question. Venchikov[156] has elaborated this concept into a dose response curve. The "biological action" of the element is expressed in the first part of the curve, which shows an increasing biological effect with increasing concentrations of an element until a plateau is reached. With further increasing doses, the element enters the "pharmacological action" phase with concomitant irritation and stimulation of some biological function, and acts as a drug independent of a deficiency state. At still higher doses, the appearance of toxicity and perhaps death represents the "toxic action" of the element. With some trace elements, the margin between doses or intakes expressing the biological and toxic actions can be exceedingly small. For the "essential" elements, an internal homeostatic control mechanism has been postulated which maintains these elements within very narrow concentration ranges obligatory for their proper functioning.[151] The nonessential elements differ because their levels in various tissues are solely a reflection of environmental contamination.

The transition element copper, atomic number 29, is an "essential" trace element. It is of vital functional necessity and is indispensible in maintaining cellular integrity, growth, reproduction, and various specific physiological and biochemical

processes within the animal body. Copper was one of the first metals known and worked upon by ancient man some 7000 to 8000 years ago. Remnants of metallurgical artifacts dating back to the sixth and fifth millennia B. C. are still being uncovered today[133]. Copper is ubiquitous in nature and may be found in its native or elemental form as well as in the carbonate, oxide, and most commonly sulfide form[28]. The word "copper" originates from the Greek word Cyprus (Kyprios), which was distinguished in ancient times by the Romans for its rich stores and mines of the reddish-brown element. The aes cyprium (ore of Cyprus) was thereafter shortened to cyprium and later again modified to cuprum from which the modern English name is derived[28,122,133]. Copper's physical properties have been exploited throughout the ages by craftsmen, and its distinctive properties of electrical and heat conductivity, ranking second to that of silver, ductility, as well as its toxicity for certain microbial species are still being profited today by industrialized society.

The occurrence of copper in organisms was recognized some 120 years ago[11]. After the conclusive identification of copper in plant and animal tissues, the shortcomings of the analytical methodologies available at that time as well as a failure to recognize and relate the importance of the element to cellular metabolism placed copper, along with the other trace elements at that time, as an accidental "scientific curiousity"[151]. McHargue[100] was the first to provide suggestive evidence of the value of copper in the diet of rats, from studies of hemoglobin

regeneration in rats suffering from "milk anemia". In 1928, Hart[62] finally elucidated a specific physiological role for copper in the rat by demonstrating that it was necessary, in addition to iron, for hemoglobin formation. Subsequent studies demonstrated a much wider range of copper involvement in biological processes in addition to its role in hematopoiesis. It was shown that copper played a vital role in connective tissue metabolism, and was also associated with certain diseases induced by dietary copper deficiencies, such as "salt sick" cattle in Florida, "Lechsucht" occuring in parts of Holland, and enzootic Neonatal Ataxia in parts of Western Australia[151]. The importance and "essentiality" of copper as a micronutrient manifesting itself through a wide spectrum of actions was thus confirmed.

Copper is widely distributed in nature, and may be found in the soil, atmosphere, and water. Intricate soil-plant-animal interrelations exist in copper transfer, as is true with other trace elements. The concept that a deficiency or toxicity state in the animal body is a reflection of the copper status of the soil is thus an oversimplification. The amount of copper in the <u>soil</u> is firstly influenced by the nature of the parent rocks from which the soil was derived. Different parent rocks contain not only differing amounts of the trace element, but also contain different chemical forms which confer its stability[151]. The mean concentration of copper in crustal rocks has been estimated as 45 ppm. Climatic conditions can also influence copper levels

in the soil. Highly leached (podzolized) soils have usually lost a significant proportion of their original mineral content, in the case of copper by as much as 10 - 30 ppm, and soil copper concentrations less than 10 ppm may produce wide areas of deficiency when the leaching is severe. Kubota[81] has shown that a wet climate soil favours the uptake of cobalt and molybdenum by pasture plants but has no such effect upon copper. However, when copper is strongly bound to decaying matter, as in muck, its availability is considerably reduced for plant and animal uptake[133]. The industrial use of soil amendments, such as fertilization, irrigation, and aeration can coordinatively act as powerful factors in quantitatively modifying the trace element distribution in the soil as well as qualitatively changing their available forms for plant and animal uptake. Applications of copper to copper deficient soils usually raise both herbage yields and herbage copper concentrations, but the ability of most plant species to respond to such applications with high copper concentrations is much less with this element than with most other trace elements[151]. Other factors present in the soil, such as molybdenum and inorganic sulfate, are known to be copper antagonists in the gastro-intestinal tract and can also modify the availability of copper for plant uptake. As is the case with other trace elements, it is the soil, via the plants, and not the water or atmosphere that supplies the great part of the total daily intake of copper by animals and man[151].

The mean concentration of copper in the upper lithosphere has been estimated to range between 70 - 100 ppm. Tabor and Warren[143] have reported a variable distribution of copper in the air of twenty United States cities, with Boston suburbs containing the most (0.27 - 2.40 $\mu g/m^3$) and the Southern cities containing the least (0.09 $\mu g/m^3$). This curious distribution may in part be a reflection of environmental contamination by industry. Assuming a daily respiratory volume of $20m^3$ and the calculated daily pulmonary copper intake as 1.0 $\mu g/m^3$, it can be estimated that the average human subject receives approximately 20 µg of copper per day, which represents a minor fraction of the amount ingested with food and water.

In sea water, where life originated, the concentration of copper is of the order of 1 - 25 ppb and has varied insignificantly in geological time because of the continuous precipitation occuring in sea water. [133]Fresh running river water usually contains 1 - 7 ppb, but as a result of industrial contamination, values as high as 50 - 100 ppb have been reported. Regional and municipal water supplies range in their copper concentration from 0.6 - 250 ppb, with a mean of 8.3 ppb. However, with the advent of copper plumbing and in localities dependent upon a supply of soft water (which easily facilitates the corrosion of copper), the concentration of copper in drinking water may be considerably raised[39,133].

2. The Copper Content of Foods

The knowledge concerning the content and form of trace elements in foods, including copper, remains partial and incomplete, particularly for those elements that have only recently been classified as "essential". Generally, foods of animal origin represent a far better source than plant foods because the concentration of copper is higher than in the edible portion of plants. The amount of the metal ingested with meats tends to be made greater.[118] It is probable that copper in foods is bound in biological complexes which can rearrange themselves and subsequently affect the availability and absorption of the metal in the gastrointestinal tract. This was shown to be true in meadow grass, which contains copper bound in neutral or anionic complexes[151].

The different classes of human foods vary greatly in their copper content, with considerable individual variations occuring within particular food items. Tissues rich in phospholipids contain especially high concentrations of copper[133,151]. Crustaceans and shellfish are the richest sources of copper, with organ meats, nuts, dried legumes, dried vine, and cocoa following. Dairy products contain the least amount of copper, rarely more than 0.5 ppm[118,151].

Cereal refinement provides an excellent example of the civilized modification of the soil-plant-animal cycle. Copper is unevenly distributed in wheat grain, with the highest proportion being found in the outer layers and germ[118]. Wheat flour

technology removes these portions by a 72% extraction procedure in the manufacture of white flour, which results in a significant decrease in copper content (from 5.3 ppm to 1.7 ppm). Contamination with adventitious copper during processing, storage, treatment with copper fungicides as well as the domestic treatment of foods all contribute to modify the copper transfer from the soil to final animal ingestion[151].

3. Biological Mode of Action of Copper

As is the case with most other trace elements, copper in the body serves host to a variety of functions, depending upon its chemical form or combination and its location in the body tissues and fluids. To maintain the structural integrity of the tissues, the functional form of copper as well as its specific concentration within the body must be carefully regulated and kept well within specific narrow limits. Primarily, copper functions as a catalyst in various intracellular and extracellular enzyme systems in tissue cells. As Green stated, "enzymic catalysis is the only rational explanation of how a trace of some substance can produce profound biologic effects".[52] According to Frieden, the importance of copper in living organisms is more profound than of other trace elements since "no metal ion surpasses copper salts in their versatility as catalysts for an impressive variety of reactions".[48] Of the four known electron transfer oxidases, which catalyze the reduction of molecular oxygen to water in the respiratory chain, all are copper containing enzymes. Since no other

alternative pathway exists by which the cell can catalytically reduce molecular oxygen to water, the uniqueness and importance of copper in biological systems is thus maintained[133]. The catalytic effects of copper in enzyme systems ranges from weak ionic strength effects to highly specific associations with enzymes, the metallo-enzymes, where there is a specified fixed number of metal atoms attached in a highly specific manner to a protein molecule. The significance of the high degree of metal-enzyme specificity in the metallo-enzymes is in the enhancement of the enzymatic catalytic activity. Substitutions for the naturally occuring metal may be achieved by dialysis against a chelating agent, but full activity cannot be reachieved due to the partial denaturation that results from the temporary absence of the stabilizing effects of the metal[118,151]. Harper[61] has summarized the proposed mechanisms by which trace elements, including copper, exert their effect in enzyme systems: (a) by direct participation in catalysis, (b) in combination with substrate to form a metal-substrate complex upon which the enzyme acts, (c) the formation of a metallo-enzyme that binds substrates in an enzyme-metal-substrate (or enzyme-metal-coenzyme-substrate) complex (d) the combination of a metal with a reaction product to alter equilibrium, and (e) the maintenance of quaternary structure.

Copper exists in three valence states[133]. The tetrahedral form (Copper I: Cuprous) possesses a high affinity for sulfur and nitrogen ligands and can only exist

in water solution in a complex bound form. Copper II (Cupric) usually forms planar, less stable chelates with nitrogen and oxygen ligands. Copper III most probably does not occur in the natural state. The copper present in the oxygen carrying enzymes, or in metallo-enzymes which directly react with molecular oxygen, is in the cuprous state. In the other oxidases, such as phenolases, the copper appears in the cupric form. A great number of copper containing enzymes, many of which were found to possess oxidative functions, have been isolated from living tissues. Tyrosinase, laccase, ascorbic acid oxidase, cytochrome oxidase, uricase, monoamine oxidase, δ amino-levulinic acid dehydrase, ceruloplasmin, and dopamine-B-hydroxylase were identified as copper containing enzymes. The manifestations of copper deficiency in animals were shown to be related to decreased tissue concentrations of certain of these enzymes, thus revealing the underlying biochemical defects[151].

CHAPTER 2

COPPER METABOLISM

The physicochemical properties of copper are such that free ionic copper is almost nonexistant, or occurs only in negligible amounts in the human body. Since ionic copper has a great affinity for organic substances, it readily forms tightly bound complexes with proteins, purines, pyrimidines, nucleotides, DNA, RNA, amino acids, and with a variety of other organic substances[50,123]. The existance of most of the body copper is thus in the form of these organic complexes. An exception is the stomach, where a relatively low and constant pH allows the dissolution of free copper ions[122].

1. Gastrointestinal Absorption

A) Absorptive Mechanisms

The daily adult dietary requirements for copper in human subjects has been estimated to range between 2 to 5 mg[17,20,58]. Several authors[2,14,75] have demonstrated that the oral and intravenous administration of a single dose of radioactive Cu^{64} results in an initial rapid fall of plasma activity, which is thereafter followed by a significant and sustained rise in total plasma activity (figure 1). The initial rise that occurs during the first few hours after administration represents the exclusive binding of Cu^{64} to plasma albumin, the "direct" reacting fraction

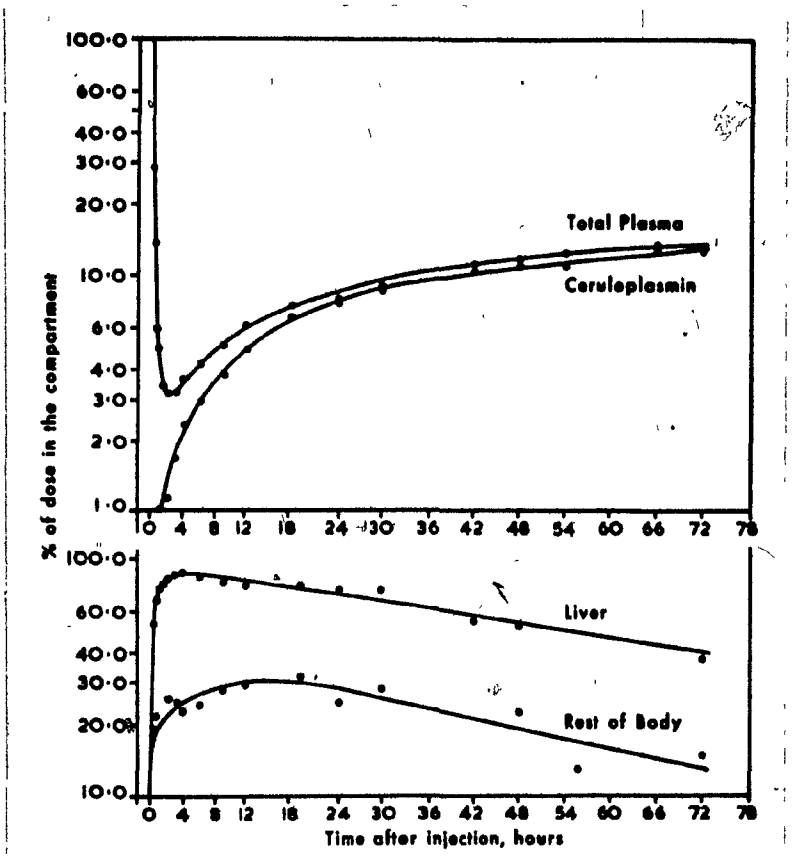

Figure 1 -- Distribution of a single intravenous dose of Cu^{64} in man (after Sass-Kortsak[122]).

of plasma copper. The secondary rise of activity can be accounted for by the incorporation of Cu^{64} into the α_2 globulin plasma protein, ceruloplasmin[2]. These experiments, in part, demonstrate that ingested copper is rapidly absorbed following its oral administration and that the absorption site for copper must therefore lie in the upper alimentary tract, probably in the duodenum in man.[149] Ingested copper is absorbed mainly from the stomach and upper jejunum by at least two mechanisms[13]. One, an energy dependent process, is facilitated by amino acids and probably represents the absorption of copper complexes of amino acids. Since copper ions have a high affinity for organic ligands, small stable complexes are formed which are superior for absorption than if copper cations were alone available for absorption. In the case of single amino acids as ligands, the rate of copper absorption depends upon the type of amino acid, its configuration, and the degree of polymerization.[151] The importance of this energy dependent mechanism for absorbing organic complexes was shown by Davis[35] who demonstrated that phytate can reduce the assimilation of this element. Other authors[154] showed that a diet high in ascorbic acid significantly depressed Cu^{64} absorption when the acid was placed into a ligated segment of the rat's intestine along with the radiocopper. The second absorptive mechanism accounts for a much greater percentage of copper absorption and is associated with two protein fractions in the intestinal mucosa. One of these proteins that copper binds with has recently been identified as the copper enzyme, superoxide

dismutase[118]. Superoxide dismutases are potentially toxic copper containing proteins that have already been isolated from erythrocytes (erythrocuprein), brain (cerebrocuprein), and liver (hepatocuprein). It was in 1969 that all were shown to be identical substances.[16] The function of the dismutase is in the conversion of superoxide free radical anions into oxygen and hydrogen peroxide as follows:[99]

$$2H^+ + O_2^- - O_2^- = O_2 + H_2O_2$$

The second of these proteins involved in copper absorption is rich in sulfhydryl groups and possesses similiar characteristics to the protein metallothionein. The postulated function of this yet unnamed protein is to provide binding sites for copper absorbed from the gut lumen which is then slowly released into the plasma for transport[41].

B) Factors Interfering With Copper Absorption

The absorption and retention of ingested copper is markedly affected by the chemical structure in which the metal is ingested, by the dietary levels of several other minerals and organic substances; and by the acidity of the intestinal contents in the absorptive area[151]. Transition elements such as cadmium, mercury, silver, and zinc appear to compete for copper binding sites on the intestinal mucosa or on the "metallothionein" protein. The diversity of the physiological responses and interactions manifested by these four chemically similiar transition elements is surprising and is inexplicable at the

present time. High dietary levels of cadmium were found to reorganize and change the distribution of copper in tissues in addition to severly depressing copper absorption[153]. The relationship of silver and ingested mercury to copper metabolism still remains to be cleared. Hill[68] observed that silver tended to augment the effects of copper deficiency in chicks whereas mercury had an adverse effect on copper adequate chicks. On the other hand, Van Campen[153] found that silver had very little effect on the Cu^{64} uptake from the intestine of the rat while mercury produced a moderate, but not statistically significant lowering of Cu^{64} uptake. Other dietary ingredients present in the gastrointestinal tract, such as calcium carbonate, ferrous sulfide, and particularly molybdenum and the sulfate radical also alter the availability and utilization of copper for absorption. High dietary levels of calcium carbonate severely inhibit the absorption of copper in the intestine, presumably due to the raising of the pH on the absorptive surface. An increase in dietary ferrous sulfide results in the formation of an insoluble and nonabsorbable copper sulfide which is not suited for intestinal absorption[37]. In the case of molybdenum and the sulfate radical, it was shown that copper retention was dependent upon the molybdenum status of the diet, and the limiting effects of molybdenum was in turn dependent upon the inorganic sulfate status of the diet and of the animal[38]. Dick[37] believes that at least in sheep, both inorganic sulfate and molybdenum reduce copper retention by diminishing the absorption of ingested

copper and by increasing the urinary excretion of absorbed and stored copper, due in each case to interference with the membrane transport of copper.

Thus, the absorption of copper in the gastrointestinal tract is by no means a simple phenomenon but one which involves the intricate interplay of several mechanisms. The concomitant presence of organic and mineral substances in the gut lumen can markedly alter the availability of copper for both absorption and retention by exhibiting severe and reciprocal antagonism with copper. These include cadmium, mercury, silver, zinc, molybdenum, inorganic sulfate, and calcium carbonate.

2. Copper Distribution In Blood

In normal human subjects, orally administered copper is better absorbed than in most species studied. Of the 2 to 5 mg of copper ingested daily, 0.6 to 1.6 mg, or about 32% is absorbed[21]. The copper absorbed from the intestinal mucosa enters the bloodstream and reaches the tissues and the liver by way of the portal vein. The copper present in blood is found in several different forms in both plasma and erythrocytes[58,85]. As yet there is no data concerning the copper content of leucocytes or platelets. Copper in plasma is present in two distinct forms, one firmly and one loosely bound. The firmly bound copper consists of the α 2 globulin

copper protein, ceruloplasmin.

A) Ceruloplasmin - Bound Copper

Ceruloplasmin is the major copper containing glycoprotein in plasma. It was first isolated by Holmberg and Laurell in 1948 who crystallized, purified, and characterized this compound. Some of the characteristics of ceruloplasmin known to date are summarized in Table 1. As the name suggests, ceruloplasmin is deep blue in appearance. It contains hexosamine, hexose, neuraminic acid, has a molecular weight of 151,000, and contains approximately 0.3% copper, which corresponds to eight atoms of copper present as four cuprous-cupric pairs per molecule[79]. Since its initial identification, ceruloplasmin has been subjected to a great deal of intensive research. But as Holmberg states, "although much has been learned about its structure and the factors regulating its concentration in the blood, its true role in the body's economy remains as mysterious as ever".[70] The initial finding of Holmberg and Laurell that ceruloplasmin is the only oxidase in human plasma has since been confirmed by the finding of a complete lack of oxidase activity in plasma void of ceruloplasmin, demonstrated by the complete lack of oxidase activity towards its preferred substrate, paraphenylenediamine. Ceruloplasmin's oxidase activity in plasma is not specific for only paraphenylenediamine. It also exhibits oxidase activity towards certain polyphenols and polyamines, such as benzidine, dihydroxyphenylalanine (DOPA), serotonin and epinephrine[72,73,87]. The copper-protein bonds

1) SOURCE: HUMAN PLASMA

2) MOLECULAR WEIGHT: 151,000

3) SEDIMENTATION CONSTANT: 7.29 ($S_{20,W} \times 10^{-12}$)

4) DIFFUSION CONSTANT: 4.5 ($D_{20,W} \times 10^{-7} cm^2/sec$)

5) ISOELECTRIC POINT: 4.4

6) ELECTROPHORETIC MOBILITY:

$\mu cm^2/volt \cdot sec. \times 10^{-5}$	pH
-3.46	7.0
-5.32	8.6

7) COPPER CONTENT: 0.34%

8) COLOUR: BLUE

9) ABSORPTION SPECTRUM:

$E^{1\%}_{1\ cm}$	λ
0.68	605
5.50	280

10) ENZYMATIC ACTIVITY: DEMONSTRATED

11) COPPER REMOVED FROM PROTEIN BY:

pH	CYANIDE	DTC
3.0	YES	NO

12) REVERSIBILITY OF COPPER PROTEIN BOND: DEMONSTRATED

13) IMMUNOCHEMICAL SPECIFICITY: DEMONSTRATED

14) PHYSIOLOGICAL ROLE: UNKNOWN

Table 1 Some physical and chemical properties of ceruloplasmin (compiled from Scheinberg[128]).

of ceruloplasmin are reversible, at least in vitro, if the copper is first reduced by ascorbic acid to give a white, copper-free protein apoceruloplasmin[126]. Ceruloplasmin can once again be formed by treating apoceruloplasmin with cuprous ions in the presence of ascorbate. The experiments which first illustrated the reversibility of the copper-protein bond in ceruloplasmin also indicated that out of the eight copper atoms present in each ceruloplasmin molecule, four appeared to be more readily exchangeable than the remaining four.[126] This was further confirmed by demonstrating that in ceruloplasmin subjected to chymotrypsin digestion, only one-half of the protein bound copper could be dialyzed.[33] Preliminary acid-base titrations and electrophoretic analytical studies on ceruloplasmin and apoceruloplasmin suggested that each copper atom may be bound to two negatively charged carboxyl groups in the intact protein, and that the firm copper-protein linkage could be regarded as the coordination of copper with four groups on parallel polypeptide chains[125]. All normal individuals studied were shown to possess at least two ceruloplasmins, which have been distinguished chromatographically and electrophoretically[104]. The relevance of ceruloplasmins chemical heterogeneity is still unclear, and it has not yet been shown that this heterogeneity is genetically determined, as is the heterogeneity of hemoglobin, albumin, haptoglobin, and transferrin.[104]

Although the physiological role of ceruloplasmin has not been elucidated, several proposals as to its function

in vivo have been forwarded. As previously mentioned, ceruloplasmin exhibits weak oxidase activity for a number of polyphenols as well as such physiological substrates as adrenalin, DOPA, and serotonin. However, the slow rate of their oxidation by ceruloplasmin and the pH at which these oxidations occur has no physiological significance[34]. In vitro, ceruloplasmin has been shown to accept electrons in the respiratory chain. This finding however, appears difficult to relate to the in vivo condition where it is not plausible that an extracellular enzyme like ceruloplasmin could be of value to the intracellular mitochondrial enzymes for the purpose of respiration[10]. The inability of ceruloplasmin to exchange copper in vivo would seem to limit its usefulness as a transport protein for the metal, unless it functioned as a carrier of a copper containing prosthetic group which would serve as an oxygen activating unit for cytochrome C oxidase[9]. Perhaps the most widely accepted suggestion that has so far been proposed as to the possible in vivo role of ceruloplasmin pertains to the mobilization of body iron stores[49]. The function of ceruloplasmin in iron metabolism suggested is as follows. Iron is stored within the protein shell of ferritin in the form of a ferric salt. The mobilization of iron from the protein shell to the tissues and bone marrow requires an initial reduction of iron to the ferrous state, which allows it to detach itself and leave the ferritin molecule. The detached ferrous cation is subsequently reoxidized to the ferric state in order to attach

itself to plasma transferrin for transport to the bone marrow, where hematopoiesis occurs. Ceruloplasmin, or ferroxidase as it is commonly called, has been postulated as the enzyme required for the reoxidation step of ferrous to ferric iron in the plasma. In support of this hypothesis, it was shown that a link exists between experimentally induced copper deficiency and the concomitant hypochromic anemia produced[98]. Osaki and Johnson[111] also support this view on the function of ceruloplasmin by showing that livers of dogs and rats perfused with ceruloplasmin released a considerable quantity of body iron stores. Evans and Abraham[43] recently observed that hemoglobin and ceruloplasmin levels in the blood parallel one another during copper deprivation and repletion in the growing rat, which indicates a relationship of copper metabolism and iron availability. Contrary to this theory, evidence exists that the normal plasma concentration of ceruloplasmin may be too large to exert any regulatory influence on iron mobilization[119]. Impairment in iron mobilization and utilisation could only occur at such low plasma ceruloplasmin concentrations as could only be manifested in extreme cases of copper deficiency. The theory is also incompatible with the observation that during the early postnatal period, liver iron stores are mobilized while there is still very little ceruloplasmin in the neonates plasma[90]. Thus the role of ceruloplasmin in metabolism still remains unresolved and Broman[9] has summed up ceruloplasmin's position as "just an ineffective oxidase in the wrong place, lacking a suitable

substrate".

B) "Direct-Reacting" Plasma Albumin Copper

The plasma copper not related to ceruloplasmin accounts for a small, yet important fraction of total blood copper and is commonly referred to as "direct-reacting" copper. It will react directly with diethyldithiocarbamate, is nondialyzable, and is loosely bound to protein, probably plasma albumin[7,151,160]. Both sexes possess an equivalent plasma concentration of the "direct-reacting" pool of copper. Although this albumin bound copper accounts for approximately seven percent of the total plasma copper, its importance should not be overlooked since it is believed to constitute the true transport form of copper.[151] Studies utilising radioactive Cu^{64} have demonstrated that following the ingestion of Cu^{64}, there is a rapid increase in plasma radioactivity which is then followed by a sharp and rapid fall of activity.[2,14,75] Bearn and Kunkel[2] have observed that during the early postingestive stages Cu^{64} was bound exclusively to plasma albumin, giving support to the fact that the true immediate transport form of copper was not the large ceruloplasmin fraction but the smaller plasma albumin pool. The secondary rise of plasma activity was that due to the increasing appearance of radioactive copper in the newly synthesized ceruloplasmin that had been discharged from the liver. Thus, the loosely bound cupric ions passing the intestinal mucosa become incorporated into the "direct-reacting" pool of copper, which rapidly comes to equilibrium with tissue copper. That ceruloplasmin does not

fulfill an analogous role to that of transferrin in the transport of the metal was further shown by Sternlieb[141], who demonstrated that in living organisms the exchange between ceruloplasmin bound copper and ionic copper did not occur. Copper is tightly incorporated into ceruloplasmin only when the apoceruloplasmin protein is formed in the hepatic microsomes and is released from ceruloplasmin only when the protein is degraded.

C) Plasma Amino Acid Copper

Recently, a third fraciton of plasma copper in equilibrium with the "direct" albumin bound copper was shown to exist[106]. This fraction compares small even with the albumin-bound fraction and the copper in it appears to be complexed with amino acids. However, by virtue of its small molecular size, it has been proposed that it may have an important function in the transport of copper through cellular membranes. Of the amino acids present in human blood, histidine most effectively competes with albumin for the binding of copper. This evidence led to the suggestion that the true transport form of copper may be a complex formed by two different amino acids and one atom of copper, but as yet such a complex or complexes have not been isolated nor chemically characterized. In model experiments of Neumann and Sass-Kortsak[106] with dialyzing membranes, it was shown that when albumin was present on both sides of a membrane and Cu^{64} was added to one side, the presence of amino acids greatly facilitates the transfer of copper through

the membrane. However, there still remains a great deal to be learned about the actual transport mechanisms of copper from the blood to tissue cells and thereafter through the plasma membrane into the cells. Whether amino acid copper complexes act as carriers facilitating the transport of copper to the cell membrane where high affinity acceptor sites bind the copper, or whether the amino acid copper complex is actually transported through the membrane remains to be resolved.

D) Erythrocyte Copper

The copper in whole blood not accounted for by the plasma is found in the erythrocytes.[84] The amount of copper in the individual human red blood cell has been estimated as 65 ± 10.8 μμμg, and the amount of copper contained in 100 ml of packed erythrocytes has been estimated as 89 μg.[151] A nearly colourless copper protein, erythrocuprein (superoxide dismutase), has been isolated from human erythrocytes[95]. Superoxide dismutase has a molecular weight of 31,000, contains 3.4 μg Cu/mg protein, and accounts for approximately 60 percent of the total red cell copper[151]. This enzyme, which also occurs in the brain and liver, has the unique function of protecting cells from the injurious effects of the superoxide radical, which inhibits cytochrome C oxidase in the electron transport system[99]. The mean erythrocuprein concentration has been estimated as 16 (10 - 22) mg per 100 ml of packed red blood cells and there is no existent human sex difference in its concentration. Erythrocuprein remains extremely stable and

constant under a variety of conditions, even in patients with hypocupremia or hypercupremia. Unlike the ceruloplasmin concentration, normal erythrocuprein values have been observed in pregnant women.[22, 134]

The second component of erythrocytic copper is considerably more labile than erythrocuprein and is believed to be loosely bound to amino acid complexes or as yet unidentified proteins[13, 151]. It is believed that this freely dialyzable minute portion is in diffusion - equilibrium with plasma albumin bound copper and amino acid copper complexes, which in turn provide the copper that becomes tightly bound into erythrocuprein[15]. The erythrocyte copper concentrations are not influenced by the total plasma copper or ceruloplasmin concentrations. In humans, as well as in other animal species, the concentration of copper is higher in plasma than it is in the erythrocytes.[18, 22] Plasma copper, being more labile than corpuscular copper, is thus a more sensitive and reliable indicator of the copper status of an animal than is the whole blood copper.

E) Sex Differences

There exists a sex difference between both the serum ceruloplasmin and serum copper concentrations in humans, as seen in Table 2: normal men having 31 mg ceruloplasmin/100 ml serum and women 36 mg ceruloplasmin/100 ml serum. The amount of copper in the ceruloplasmin fraction can be calculated since the copper content of ceruloplasmin is known to be

3.2 µg/mg of protein.[69] The mean value of ceruloplasmin for both sexes, determined as 33 mg per 100 ml serum, multiplied by the copper content of ceruloplasmin (3.2 µg/mg protein), yields a value of 106 µg/100 ml serum. Thus approximately 93 percent of the copper in serum is bound to the ceruloplasmin enzyme. The distribution of copper in the serum and in the erythrocytes is represented in figure 2. Studies have also shown that a high correlation exists between the concentration of ceruloplasmin present and the level of copper in the serum of healthy human subjects (figure 3)[129]. In effect, the serum copper concentration is interchangeable to its ceruloplasmin concentration in that both serum copper and ceruloplasmin always vary in the same direction and either is a good index of the other.

F) Factors Affecting Serum Copper Levels

The normal range of copper concentrations in the blood of normal healthy animals is of similiar magnitude in all higher animals, lying in the range of 0.5 - 1.5 µg/ml[3, 151]. Variations within species can be accounted for more by individual differences than by diurnal variations. Although in man there appeared to be no cyclic serum copper variations, recent findings by Henkin[65] indicate that there is a circadian variation in the total serum copper concentration at different intervals in a twenty-four hour period, the serum copper concentration being significantly higher than the mean from 10:00 A.M. to 2:00 P.M. Serum ceruloplasmin concentrations showed a similar but less

Normal Values

Determination	No. of Subjects	Mean	95% Limits
Serum copper (μg./100 ml.)			
Men	135	109	81–137
Women	100	120	87–153
Total	235	114	81–147
Direct - reacting copper (μg./100 ml.)	30	7	0–20
Ceruloplasmin (mg./100 ml.)			
Men	15	31	25–37
Women	15	36	25–47
Total	30	33	25–43

Table 2 Total serum copper, direct-reacting copper, and ceruloplasmin concentrations in normal subjects. (after Cartwright et al[20])

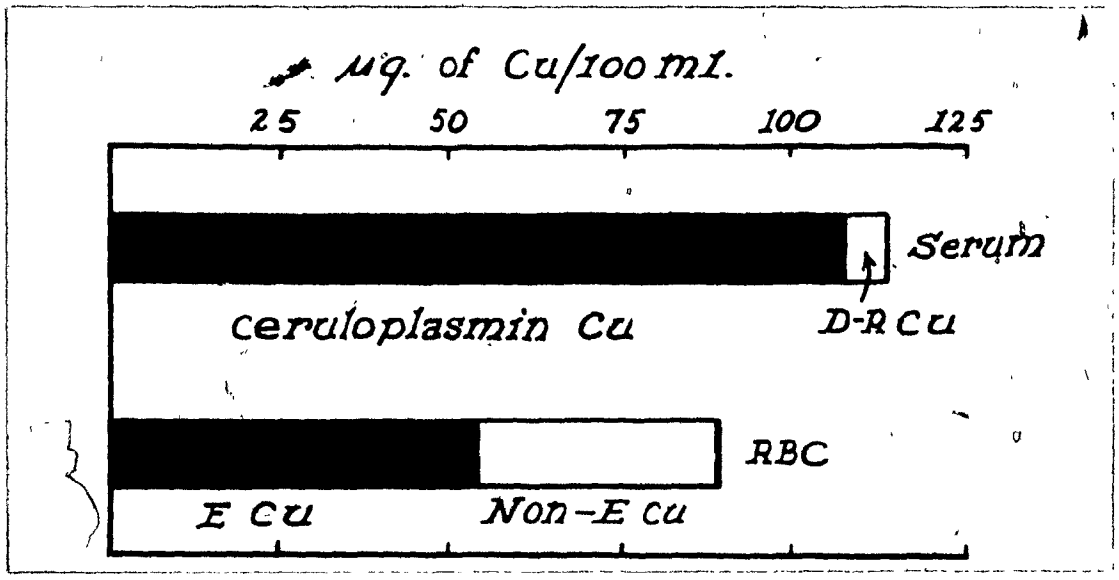

Figure 2 The distribution of copper in serum and erythrocytes, D-R Cu, direct reacting fraction of copper; E Cu, erythrocuprein copper; non-E Cu, nonerythrocuprein copper., (after Cartwright et al[20])

marked circadian pattern of variation, while urinary excretions of copper showed no obvious circadian variation pattern. Physical exertion, fasting, and the time period following meals do not affect the serum copper concentrations.[18] In most species, there are no significant differences in the concentrations of copper in whole blood and serum, but serum copper is higher in human females than in males. Several investigators have reported normal non-pregnant female serum copper concentrations to range between 1.14 µg Cu/ml serum to 1.24 µg Cu/ml serum.[18,26,60,107] No confirmative explanation for this sex difference has been advanced.

The metabolism of copper is altered in a wide variety of clinical conditions in man, most of which are associated with changes in the concentration of copper in the blood.[128] Bacterial infections, myocardial infarction, neurologic disorders, various anemias, hyperthroidism, portal cirrhosis, pellagra, chronic alcoholism, Hodgkin's disease, acute and chronic leukemia, and lymphomas are all associated with a marked and significant rise in the ceruloplasmin and serum copper concentration (Table 3). Perhaps the most striking observation is the rise in ceruloplasmin concentrations during pregnancy and after the administration of oral contraceptives, to be discussed later. As yet the etiological significance of these increases can only be speculated upon. Findings that Salmonella gallinarum as well as other stressors, including ACTH and hydrocortisone, produced a sixfold increase in ceruloplasmin activity in

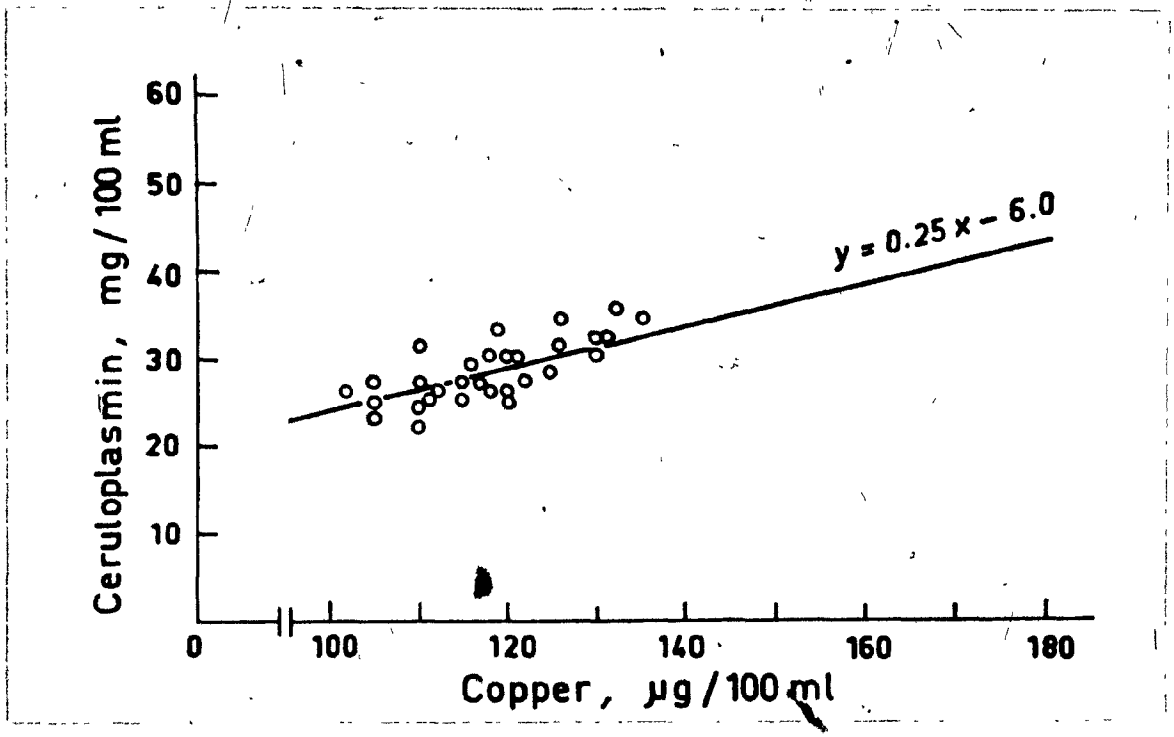

Figure 3 Correlation between serum copper and ceruloplasmin concentrations in man. (after Schenker[129]).

chicks led Starcher and Hill[140] to conclude that any stress, or any situation resulting in increased corticosteroid levels could increase ceruloplasmin concentrations. The value of these increases lies in the fact that they may be used as secondary aids along with other diagnostic data in the diagnosis of disease.

Hypocupremia in human beings is associated with many naturally occuring clinical disorders. The concomitant hypocupremia observed in cases of iron deficiency, hypoproteinemia, kwashiorkor, tropical sprue, non-tropical sprue, nephrosis, celiac disease, cystic fibrosis, and in Wilson's disease (hepatolenticular degeneration) may result from several different mechanisms (Table 4)[23]. The inability to synthesize ceruloplasmin at a normal rate, low dietary copper intakes, poor and inadequate intestinal and/or cellular absorption, and increased excretion resulting in an excessive loss of ceruloplasmin from the body may all result in the lowering of serum copper concentrations. In Wilson's disease, which is an autosomal recessive genetic disorder of copper metabolism, the low serum copper levels are highly positively correlated with low ceruloplasmin concentrations[19]. Levels of urinary copper as high as 1500 µg daily may be reached concomitant with increased copper deposition in the tissues. If not treated, the disease may eventually lead to hemolytic jaundice, cirrhosis, and other manifestations of chronic copper toxicity. It is interesting to note that in the newborn

and in patients homozygous or heterozygous for the Wilson's disease gene, the hypocupremia is due to a specific inability to synthesize apoceruloplasmin at a normal rate and is not a result of an inadequate supply of copper. In all of the other conditions listed, the hypocupremia is associated with hypoproteinemia, resulting in a considerable loss of proteins in addition to the loss of ceruloplasmin from the body.

3. Copper Distribution in the Body

Plasma albumin bound copper is widely distributed to the tissues and also receives copper from the tissues. It also readily exchanges copper with the loosely bound labile copper fraction of erythrocytes[15]. As mentioned previously, the actual copper transport mechanisms through the cellular plasma membrane, whether they consist of the penetration of amino acid copper bound complexes through the membrane, or whether the amino acid copper bound complexes bind to high affinity cellular acceptor sites is not yet known.

A) Hepatic Copper

The copper reaching the key organ in its metabolism, the liver, is incorporated into the mitochondria, microsomes, nuclei, and the cytoplasm of parenchymal cells in a proportion that is a function of age, strain, the copper status of the animal[57,102]. In healthy human subjects, the majority of liver copper is localized within the acid phosphatase rich pericanilicular lysosomes. Of approximately 24 µg of

Condition	No.of Subjects	Whole Blood Cu(μg/%)	Plasma Cu(μg/%)	Plasma Fe(μg/%)
Normal	63	98±13	109±17	115±42
Infection	37	141	167	57
Acute Leukemia	19	195	236	171
Chronic Leukemia	21	119	148	113
Hodgkin's Disease	14	142	171	78
Pernicious Anemia	10	111	121	173
Iron Deficiency Anemia	9	114	132	26
Wilson's Disease	3	79	55	64
Nephrosis	3	70	80	62

Table 3 Whole blood and plasma copper concentrations in various clinical conditions in man (adapted from Underwood[151]).

Condition	Defect in Apoceruloplasmin Synthesis	Low Dietary Intake of Cu	Low Dietary Intake of Protein	Decreased Absorption of Cu	Loss of Ceruloplasmin into	
					Urine	Bowel
Newborn	X					
Wilson's disease	X					
Wilson's disease heterozygotes	X					
Protein-losing enteropathy						X
Nephrotic syndrome					X	?
Tropical sprue				?		?
Nontropical sprue				?		?
Celiac disease				?		?
Cystic fibrosis			X			?
Kwashiorkor		?	X			?
Hypocupremia in infants*		X				?

* With hypoproteinemia, edema and iron deficiency anemia.

Table 4 Mechanisms for the production of hypocupremia in various diseases in man. (after Cartwright[23])

copper in a gram of dry adult liver, a great deal is assumed to be "storage" copper. It has not yet been resolved whether this copper exists in combination with proteins or other hepatic constituents[127]. The liver also contains a relatively rich supply of the copper containing enzyme, cytochrome C oxidase, which also accounts for a fraction of the organs copper. A third distinguishable fraction of hepatic copper exists in the form of copper bound proteins, which have been isolated from many species[118]. In copper loading experiments in rats, hepatic copper distribution differed from the controls, with the mitochondria and nuclei holding most of the excess copper while the microsomes and cytoplasm accumulated the metal to a much lesser extent[91]. In normal adult rat liver, 64.3% of the total copper was found in the soluble fraction, 8.2% and 5.0% in the mitochondrial and microsomal fractions respectively, and 20.3% was localized in the fraction containing the nuclei and cell residue. In all, about 10% of the total copper of the human body is present in the liver.

B) Copper Constitution of Other Organs

The average normal 70 kg adult man is believed to contain between 100 - 150 mg copper in his body.[25] The distribution of total body copper amongst the tissues varies with the species, age, and copper status of the organism. The most comprehensive and complete study on the copper content of human tissues was done by Tipton and Cook[150], by the

quantitative emission spectrographic analysis of 24 trace elements in 29 human organs (Table 5). Tissues were obtained from 150 adults from various parts of the United States who died a violent death and were free from disease. As can be seen from Table 5, the liver, brain, heart muscle, and kidney have in decreasing order the highest concentration of copper. Skeletal muscle has a comparitively low copper content per unit body weight. Nevertheless, because of its large mass, striated muscle as a total body compartment contains approximately one third, or more than any other tissue of the total body copper. The same situation is applicable to bone (vertebra), which contains only 5 µg Cu/g ash. However, because of its extensive bulky mass, together with muscle it accounts for more than half of the total body copper content[118]. The human brain contains a variable amount of copper in different anatomical regions. The locus coeruleus, a small blue colored area in the brain, contains a surprisingly high amount of copper, 107 - 404 µg/gm dry weight. Gray matter also contains a high copper content. White matter, which was previously thought to be copper lacking was conclusively shown by novel and more sensitive histochemical methods to contain copper[40]. Exceedingly high concentrations of copper, the significance of which is not clear, occur in the pigmented parts of the eye, especially in the iris where levels as high as 105 ppm (dry basis) have been reported[145]. The copper is associated particularly with the melanins, and is largely bound in ionic form to

COPPER CONTENT OF HUMAN ORGANS AND TISSUES

Organs and tissues	(1) μg Cu/g ash[a] Median	(1) μg Cu/g ash[a] 80% range	(2) μg Cu/g dry weight Median	(2) μg Cu/g dry weight 80% range	(3) μg Cu/g wet weight[b] Median	(3) μg Cu/g wet weight[b] 80% range	(4) Mass[c] (g)	(5) mg Cu in whole organ[d] Median	(5) mg Cu in whole organ[d] 80% range
Brain	360	340–500	21.9	14.6–30.5	5.40	3.60–7.50[f]	1 500	8 10	5 40–11 25
Liver	510	330–1300	18.8	11 8–48.0	6 63	4.16–16 9	1.700	11.28	7 07–28 70
Kidney	260	190–340	11.9	8.75–15.6	2 86	2 09–3.74	0.300	0 86	0 63–1.11
Heart	350	250–460	14.0	10.0–18.4	3.85	2.75–5.06	0 300	1 16	0 83–1 52
Diaphragm	140	110–200	4.76	3.74–6.80	1.34	1 06–1.92	—	—	—
Muscle	74	50–120	3.11	2.10–5.05	0.89	0.60–1.44	30 000	26 70	18 00–43.20
Skin	100	80–150	1 70	1.36–2.55	0 70	0.56–1.05	2 000	1 40	1 12–2.10
Omentum	190	110–280	0.38	0.22–0.56	0.38	0.22–0 56	10.000[e]	3 80[e]	2 20–5.60[e]
Spleen	89	67–120	4.45	3.35–6.00	1.25	0.94–1.68	0 150	0.19	0 14–0.25
Pancreas	140	92–210	4.90	3.22–7.35	1.68	1 12–2 52	0 070	0.12	0 08–0.18
Lung	120	92–150	5.75	4.41–6.65	1.32	1 02–1.98	1 000	1.32	1 02–1 98
Larynx	33	14–150	3.07	1.30–13.9	0.99	0 42–4.50	—	—	—
Trachea	56	25–96	3.02	1.35–5.19	0 90	0 40–1 54	—	—	—
Esophagus	120	84–200	4.56	3.19–7.60	1 08	0.76–1 80	0.365	0.39	0 28–0.66
Stomach	230	150–320	7.36	4.80–10.2	1.84	1.20–2.56			
Gut									
Duodenum	260	160–430	10.5	6.25–16 4	2 06	1 28–3 36			
Jejunum	220	160–330	8.97	6 25–12.9	2.07	1 44–2 95	2 000	3 34[f]	1 13–2 55[f]
Ileum	240	170–400	8.16	5.78–13.6	1.75	1 29–3.04			
Cecum	220	150–280	4.40	3.00–5.60	1 32	0.90–1.68			
Sigmoid	210	150–340	5.25	3.75–7.00	1 47	1 05–2.38			
Rectum	170	110–270	5.27	3.41–8 38	1.19	0 77–1 89			
Adrenal	200	160–250	2.20	1.76–2.75	0 92	0 74–1 15	0 020	0 02	0 015–0 023
Thyroid	94	43–240	3.48	1.59–8.88	1.03	0.47–2 64	0 020	0 02	0 009–0 053
Testis	80	58–110	4.55	3.30–6.37	0 88	0 64–1 21	0 040	0 035	0 026–0 048
Prostate	100	57–140	4.50	2.56–6 30	1.10	0.63–1 54	0 020	0 022	0 013–0 031
Ovary	120	85–180	5.28	3 74–7.91	1.16	0 82–1.75	0 008	0 009	0 007–0 014
Uterus	98	73–130	4.21	3.14–5.59	0.96	0.72–1.27	—	—	—
Urinary bladder	110	76–180	2 53	1.75–4.15	0 88	0.61–1 44	0 150	0 132	0 091–0 216
Aorta	90	50–140	3.42	1.90–5.32	1.26	0.70–1 96	—	—	
Blood[g]	—	—	—	—	0 98	0.72–1 24	5 400	5 290	3.89–6.70
Bone (vertebra)[h]	5.0	2.0–5.0	1.45	0.58–1.45	0.78	0.30–0 75	7.000	5 250	2 1–5 25
Subcutaneous tissue, bone marrow, lymphoid tissue[i]	—	—	—	—	—	—	7.900	6.57	4 96–8.92
Estimated total body content:								76 0	50.0–120 0

Table 5 Copper content of organs and tissues in man.

(after Tipton and Cook[150])

protein. Relatively high copper concentrations of 15 - 30 ppm are also found in the enamel of human teeth, with no significant differences being observed in the inner and outer layers of enamel.[151] Other studies have also shown a similar copper distribution amongst the organs, with liver, brain, kidneys, heart, and hair containing relatively high copper concentrations; the spleen, pancreas, muscle, skin, and bones containing an intermediary amount of copper; and the glands (prostate, pituitary, thyroid, and thymus) as being organs of low copper content[32,136].

On the basis of this data, a lower whole body copper concentration of 75 mg (range: 50 - 120 mg) was estimated by Tipton and Cook[150]. The mean concentration of copper in the adult human body was estimated as 1.25 µg/gm fat free tissue (range: 0.83 - 2.0 µg/gm), and these figures are also lower than the previous determinations of 1.5 - 2.5 µg Cu/gm fat free tissue reported by Widdowson[159]. The etiology and significance of the high copper concentrations occuring in certain tissues resides in the fact that copper in tissues is invariably associated with a vast array of specific intracellular and extracellular proteins which play a role indispensible for life. Since copper exists in an integral relationship with these proteins, tissues containing large amounts of these proteins will obviously contain a higher copper concentration.

4. Excretion of Copper

A) Biliary Copper

The main route of copper excretion is via the biliary tract to the faeces.[152] Presumably both ionic copper and copper complexed to albumin and amino acids are responsible for the copper incorporation into hepatic parenchymal cells. Within the cells, the absorbed copper is complexed with a specific sulfhydryl rich metallothionein protein which is believed to serve as a temporary storage form of copper until it enters the hepatic lysosomes. In the lysosomes, copper is incorporated into the biosynthesis of ceruloplasmin and other copper containing enzymes, or it is excreted into the bile in association with the bile salt, taurochenodeoxycholate.[13,118] These lysosomes, along with the mitochondria, contain 20% of the intrahepatic copper and are responsible for much of the copper that is excreted in the biliary tract. The copper excreted in the bile is primarily bound to protein macromolecules, bile salts, and a fraction is excreted as copper amino acid complexes. Because of the large size of the macromolecular copper complexes, and since the enterohepatic circulation of copper is minimal, the copper excreted in the biliary tract is not reabsorbed[13]. The measurement of copper in the bile presents a definite problem since the bile is difficult to collect from normal human subjects, and the daily outflow varies intermittentingly, fluctuating from 250 to 1,100 ml daily[22]. Postmortem gallbladder bile examinations from six normal subjects yielded

a concentration of copper that ranged from 24 to 538 µg/100 ml bile, with a mean value of 329 µg/100 ml bile.[36] Other investigators have reported the biliary copper concentration as varying between 6 to 205 µg/100 ml bile[155].

B) Urinary Copper

The urine contains only traces of copper (5 - 25 µg/day), and values higher than 60 µg daily are not observed under normal physiological conditions[22]. Administration of radioactive Cu^{64} to human subjects resulted in an increased rate of urinary copper excretion, with the highest values being recorded in the first two hours after administration[2]. It appears probable that a definite relationship exists between the rate of urinary copper excretion and the serum concentration of nonglobulin, nonceruloplasmin radiocopper. This suggests that copper loosely bound to albumin, or which has been dissociated from the copper albumin complex during its passage through the kidneys, is the main source of urinary copper.[14,22,152] Ceruloplasmin has not been demonstrated to exist in the urine under normal circumstances[96]. Negligible amounts of copper are excreted in the sweat and an estimated 20 µg Cu/day is lost during the menstrual flow.[88]

In normal human subjects to whom radiocopper was administered orally, 72.4% was localized in the stools and only 0.1% of the radioactivity appeared in the urine[22]. Thus assuming an average daily consumption of 2.0 to 5.0 mg of copper by a normal human being, it can be calculated that

32% (0.6 to 1.6 mg) is absorbed, 0.5 to 1.3 mg is excreted in the bile, 0.1 to 0.3 mg passes directly into the bowel, and 0.01 to 0.06 mg appears in the urine. These metabolic interelationships are represented briefly in figure 4 and a more detailed account of the normal metabolism of copper in man is schematically represented in figure 5.

5. Copper Deficiency

A) Copper Deficiency in Man

Under normal physiological conditions, a significant deficiency of copper has never been reported, although recent instances of copper dependent anemias with skeletal deformities have been described in children[78]. There are three main reasons why copper deficiency seldom, if ever occurs in humans.[128] Firstly, the ubiquitous distribution of copper in the environment makes deficiency in humans consuming even the most malnourished and mediocre diets highly unlikely. Secondly, normal diets in most parts of the world, especially in the Western countries, contain a large and sufficient amount of copper relative to needs. Finally, a specific regulatory mechanism appears to maintain this dietary surfeit of copper from reaching toxic levels.

B) Copper Deficiency in Animals

In contrast to human beings, naturally occuring copper deficiency in animals occurs with great frequency and manifests its effects in a vast array of biochemical abnormalities. Anemias, depressed growth, bone disorders,

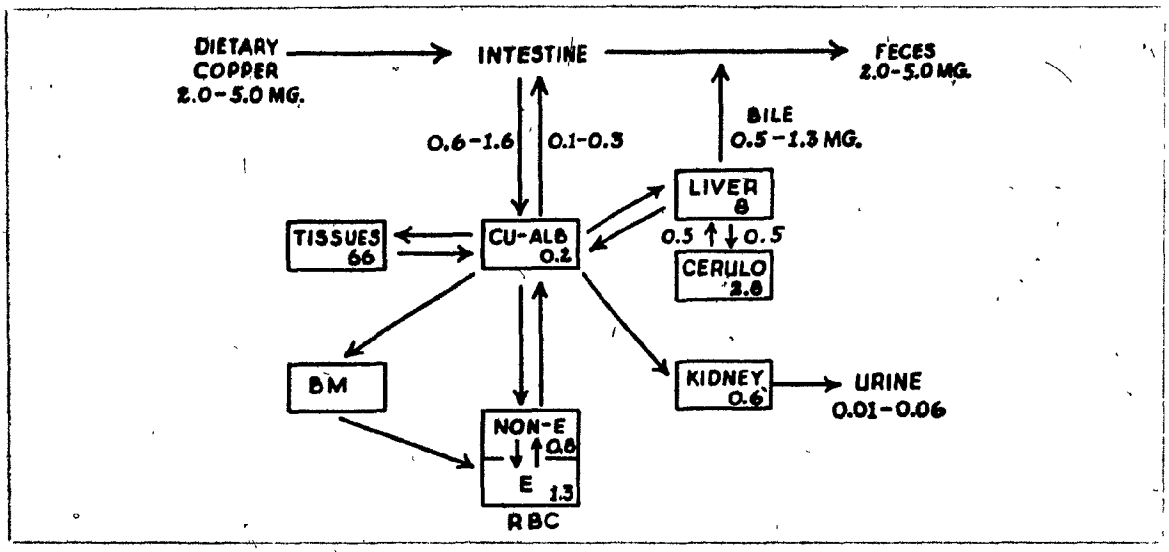

Figure 4 Schematic representation of some metabolic pathways of copper. The numbers refer to milligrams of copper. (after Cartwright[22])

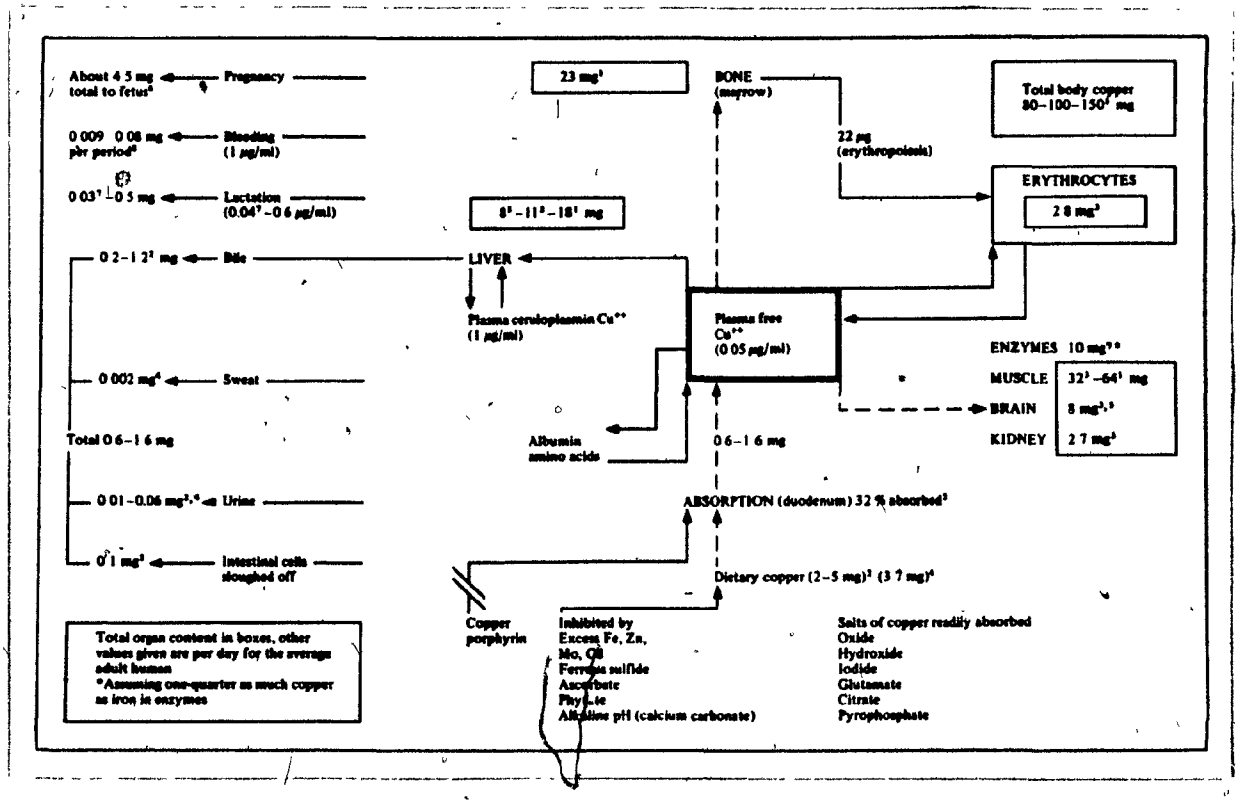

Figure 5 Copper metabolism in man. (after Linder and Munro[91])

depigmentation of hair and wool, abnormal wool growth, neonatal ataxia, impaired reproductive performance, heart failure, cardiovascular defects, and gastrointestinal disturbances have all been reported in animals with a dietary deficiency of copper.[151] The extent to which one or more of these disorders manifests itself is dependent upon the species in question, age, sex, environment, the duration and severity of copper deficiency, and the biochemical priority of the process in the organism. As an example of how a deficiency of copper results in the appearance of a naturally occuring disorder, a brief account of the extracellular copper containing enzyme, monoamine oxidase (MAO), and its involvement in cardiovascular defects follows.

It was observed in several laboratories that the elastin content of the aortas of copper deficient pigs and chicks was considerably decreased and contained an elevated lysine content significantly less desmosine and isodesmosine than did the controls[139,158]. Desmosine and its isomer, isodesmosine, are tetracarboxylic, tetramino acids formed by the condensation of two, or possibly four lysine residues, which together constitute the key cross-linkage groups in elastin[112]. This condensation reaction consists in the removal of the epsilon amino group of the lysine residues and the oxidation of the carbon to an aldehyde, a reaction catalyzed by the copper containing amine oxidases (MAO)[12,67]. The reduction in the plasma amine oxidase concentrations observed in these copper

deficient animals results in a reduction in the enzymatic activity of MAO. This in turn lessens the capacity for oxidatively deaminating the epsilon amino group of the lysine residues in elastin. The reduction in oxidative deamination results in less lysine being converted to desmosine, and thus in fewer cross linkages being formed. The elasticity of the aorta diminishes and becomes fragile. As a result, sudden deaths from ruptures of the malformed vessels occur.

6. Copper Toxicity

Despite the ubiquity of copper in nature and the widespread use of copper for plumbing, kitchen utensils, beer brewing kettles, whiskey stills, and the exposure of various types of workers to high concentrations of the metal, copper poisoning is rare in occurence.[24,28,29,128] Toxicity probably does not develop in man because the metal is excreted or incompletely absorbed. Under normal circumstances, copper is a "benign agent to man", but as Cohen states: "excessive concentrations of the metal in any of its forms may produce mild to serious and even life-threatening consequences".[28] Both the acute and chronic manifestations of copper poisoning in humans are dependent upon the mode of contact and the milieu in which this contact occurs. Toxic properties of metallic copper have been described and associated following topical exposure, inhalation, and ingestion of the metal in its various valence states. Acute copper poisoning due to ingestion of copper salts, particularly copper sulfate, is

rare because the emetic effect of copper limits its oral toxicity. Thus, the prompt ingestion of more than 10 to 15 mg of copper at one time results in vomiting, nausea, and perhaps cramps and diarrhea. As a result, very little copper is left in the intestinal lumen for further absorption.[5,28,74,128] Chronic copper toxicity, as would be expected from the widespread industrial-linked copper settings and applications, is also rare in occurence. Direct exposure to copper salts or dust may result in the development of a contact dermatitis, with the appearance of a greenish-black discoloration of the skin and hair. Turbidity, ulceration of the cornea, conjunctivitis, and edema of the eyelids have also been described resulting from direct eye contact with copper salts[142]. Workers chronically exposed to excessive concentrations of copper dust and fumes may develop congestion of the upper respiratory tract, particularly in the nasal mucous membranes and pharynx[8]. Repeated ingestion of elemental copper or its salt forms may also result in the development of hemoglobinuria, hematuria, acute and often massive hemolytic crisis, hypertension, and coma.[28]

In animals, acute and chronic copper poisoning occurs to a much greater degree than in man, and evidence suggests that copper can be more toxic in animals than in humans. Chronic copper poisoning may occur in animals under natural grazing conditions, from industrial and horticultural contamination of feeds and pastures, and as a result of the

use of copper supplements as growth stimulants. Hemolytic jaundice, diarrhea, necrosis, hemoglobinuria, lupinosis, as well as a vast number of other specific diseases have been described and attributed to chronic copper poisoning[151]. The greater susceptibility of animals to the toxicity of copper may be due in part to the lack of specific metabolic regulatory mechanisms which balance the body copper in a homeostatic equilibrium and prevent accumulations of the metal to toxic levels.

CHAPTER 3

MATERIALS AND METHODS

1. Maternal Serum Copper Levels at Delivery (Study I)

A) Trace Metal Analysis

Probably the most difficult aspect of trace metal analysis is the problem of environmental contamination. Dust particules from flaking laboratory paint and plaster, corrosion products from metals, improper analytical handling, the use of insufficiently pure reagents, improperly washed laboratoryware, and even the method of labelling the samples (since ink in glass fibre pens contains a high concentration of copper and sodium) may all introduce erroneous error in the final determination of the metal concentration in a sample. Extreme caution was followed throughout the technical and analytical phases of the present experiments to avoid the possible contamination of samples with extraneous copper. The water utilised in all stages of the experiments, including that employed for dilution purposes, was tap water passed through synthetic ion exchange columns which was further distilled to give an almost metal free solution. Unless otherwise specified, the word "water" utilised in any context of this thesis refers to this high purity deionized distilled water. All glassware (test tubes, volumetric and pasteur

pipettes, volumetric flasks, erlenmeyer flasks) were rinsed with tap water immediately after use, boiled in 1NHCl for one hour, rinsed with running tap water and subsequently rinsed three times with deionized water before drying. Disposable Eppendorf micropipet tips and disposable polypropylene test tubes, polypropylene known to contain a low copper content, were used in addition to the standard laboratory glassware.

The serum copper concentrations were quantitatively determined at delivery in women with normal pregnancies as well as in prenancies where various conditions were listed in order to ascertain a possible relationship between the serum copper concentrations with respect to these pregnancies. In normal control pregnancies, the serum copper content of umbilical cord blood was also determined.

B) Subjects of Study

The serum copper determinations were performed from a random sampling of 100 pregnant women at term with a normal gestational course. The delivery was single, vaginal, and at term. Serum copper determinations were also determined in 100 cases of pregnancy with various conditions where differences in the serum copper levels would be expected. These conditions included; 9 cases of intrauterine foetal growth retardation with a small foetal size for the due gestational date; 9 cases of maternal gestational diabetes with delivery between the 38th - 40th week of pregnancy; 24 patients suffering from Toxaemia (pre-eclampsia and eclampsia); and 58 postmaturity cases with

labour and delivery occuring after 40 weeks of gestation.

C) Methodology of Procurement of Specimens

At delivery, 10 ml of maternal venous blood and 5 ml of umbilical cord blood were drawn into a Vacutainer red stoppered glass tube. The blood was then allowed to clot and the serum was subsequently separated from the erythrocytic fraction by centrifugation at 2800 rpm. One ml of serum was drawn by a 1000 µl Eppendorf micropipet and this was diluted to 10 ml with deionized water. The same dilution was performed with the calibration reference solution (Dade Cation - Cal^{TM}) which was used as one of the control standards. The copper content of both maternal and umbilical cord serum was determined by atomic absorption spectrophotometry.

D) Methodology of Atomic Absorption Spectrophotometry

The Perkin-Elmer (Model 107) Atomic Absorption Spectrophotometer with a 3 slot burner head and Intensitron copper hollow cathode lamp was used for the serum copper analysis. Glycerol copper standard solutions to approximately match the viscosity of the diluted serum. A 5% glycerol water solution served as the blank solution. Two working standards of differing copper concentrations, 100 µg% (W/V) and 200 µg% (W/V) were prepared in a 5% glycerol water solution. These standards fell within the known linear working range for copper, approximately 500 µg% Cu taken as the allowable maximum upper limit. A liquid plasma protein based calibration reference, consisting of a 6% bovine albumin base to which pure chemicals had been

added (Dade Cation - Cal ™) containing a copper concentration of 200 µg% was used as an additional control standard. The instrumental settings on the spectrophotometer were adjusted as follows. The lamp selector was placed at position 3 with the lamp current being adjusted at 6 milliamperes. The spectral slit was opened to a width of 7 Angstrom units and the supply valve of air, the oxidant, was set at a pressure of 70 psi (4.9 kg/sq. cm). The oxidant air flow and the acetylene fuel flow rates were adjusted to the instrumental settings of 11.5 and 13.5 respectively to provide a lean blue oxidizing flame. The wavelength was first coarsely set at a value of 280 nm, with the maximum wavelength peak then being carefully adjusted using the fine wavelength control to 282.3 nm. This optimal wavelength corresponded to a maximal needle deflection on the energy meter. The blank solution was aspirated into the air-acetylene flame and the digital readout was zeroed to the point where aspiration of the blank solution resulted in a reading of zero absorbance units. The 100 µg% (w/v) and 200 µg% (w/v) copper standard solutions were subsequently aspirated into the air-acetylene flame to give absorbance readings of 0.04 and 0.08 units respectively. Aspirant flow rate as well as the vertical and horizontal burner head alignment were so adjusted as to give the maximum absorbance readings with the two standard solutions. The diluted control standard calibration reference solution, containing a copper concentration of 200 µg%, was subsequently aspirated into the

flame to verify the validity of the previously set conditions. The absorbance readings of the standard solutions were interchangeably converted to the corresponding concentration (µg%) using the expansion calibration on the spectrophotometer. Thus sample readout values were now calibrated to represent the concentration of the solution and not its absorbance. These conditions set the proper operating parameters for sample analysis to commence. The diluted serum sample was aspirated into the air-acetylene flame and the concentration of the sample was obtained. When stabilization of the readings to fairly constant values occured, ten consecutive readings were recorded from each of the samples. Each sample analysis was preceded by aspirating water for approximately ten seconds through the nebulizer and burner head. The zero position and the expansion calibration were also verified before the analysis of each sample to assure that erroneous drifts did not occur. The mean of ten consecutive readings for each sample was determined and multiplication of this figure by the appropriate dilution factor yielded the serum copper concentration.

2. Maternal Serum Copper, Placental and Umbilical Cord Serum Copper Concentrations at Delivery (Study II)

A) Subjects of Study

The copper concentrations were determined in maternal serum, umbilical cord serum, and in placentas of 106 normal pregnant women as well as in 44 cases with various conditions

of pregnancy including the following; 7 cases of premature labour with delivery occuring between 33 - 36 weeks of gestation; 13 postmature labour cases with delivery after 40 weeks of gestation; 9 cases of maternal iron deficiency anemia with concomitant hemoglobin values of less then 10 G%; 9 cases with premature rupture of the membranes; and 6 cases of Toxaemia. These studies were carried out in order to ascertain any variations in the placental copper concentrations as well as to elucidate any relationship between the maternal serum copper concentrations with respect to the placental copper levels. The 44 cases with these conditions were compiled from a total number of 811 consecutive pregnancies. In all cases viable infants were delivered.

B) Methodology of Preparation of Placental Specimens For Copper Determination

Immediately following the delivery of the foetus, placentas were obtained from the case room. Two excisions of approximately 10 grams each were obtained from the maternal aspect of each placenta from the area located 3 centimeters laterally to the umbilical cord entry on the opposite (foetal) side. The blood content of the excision biopsy of placental tissue was not washed out. The excised portion of the placenta was weighed on a Mettler P163N semimicrobalance and the wet weight was recorded. The placental biopsy was then lyophilized for 24 hours in a Virtis freeze drying apparatus and the dry weight recorded. The freeze dried placental specimen was then

placed in a porcelain crucible and ashed at 400° C. for 72 hours in a muffle furnace. The resultant ash was dissolved in 10 ml of 0.1 N hydrochloric acid (Baker J. T. Chemicals, Ultrex Grade) and the solution was vigorously shaken to yield a homogenous distribution. The method of obtaining maternal and umbilical cord blood samples have been described previously.

C) Methodology of Atomic Absorption Spectrophotometry for Placental Copper Determinations

Analogous to serum copper determinations, the placental copper concentration measurements were performed on the Perkin - Elmer (Model 107) Atomic Absorption Spectrophotometer with a 3 slot burner head. The technical analysis as well as the spectrophotometer instrumental settings were the same as for serum copper determinations described previously. The only difference in the analysis was as follows. Two working standards of differing copper concentrations, 100 µg% (W/V) and 200 µg% (W/V), were prepared from a Certified Atomic Absorption Copper Reference Solution (Fisher Scientific Co.) and diluted with deionized water, not with a 5% glycerol water solution as in the case of serum copper determinations, since the viscosity (specific gravity) of the placental samples diluted in 0.1 N Ultrex hydrochloric acid was comparable to that of deionized water and not the specific gravity of serum. The liquid plasma protein - based calibration reference solution (Cade Cation - Cal™) was not used for the same reason. Ten consecutive concentration readings for each placental sample were taken and their mean

value was calculated. Taking into account the appropriate dilution factor and the original weight of the placental specimen, the copper concentration (µg Cu/gram placenta) was determined for both the wet and dry weight.

CHAPTER IV

RESULTS AND DISCUSSION

1. Maternal Serum Copper Levels in the Third Trimester of Pregnancy

A) Normal Pregnancy

In the one hundred normal uncomplicated pregnancies with delivery at term, the mean maternal serum copper concentration was found to be 261.29 µg% (S.D. ± 33.65), (figure 6). The maternal serum copper concentrations were found to range between 171-353 µg%. As can be seen from Table 6, 76% of the observed normal serum copper values fell within the limits of 200-300 µg%. In 17% the values were higher than 300 µg% and only 7% of the total normal maternal serum copper levels at delivery had values less than 200 µg%. The frequency distribution bar graph of maternal serum copper concentrations at term more specifically demonstrates the serum copper distribution (figure 7).

The mean maternal serum copper concentration observed in this study seems to agree with mean serum copper values reported by Schenker (275 µg% S.D. ± 39.70) [130], O'Leary (261 µg%, S.D. ± 73.9) [109], Henkin (221 ± 14 µg%) [65], and Ylostalo (258 µg%, S.D. ± 79.2) [161]. It can be seen from the standard deviations accompanying these values that considerable variations exist, although the values are significantly higher than those in nonpregnant women.

GROUP	NO. OF CASES	MEAN MATERNAL SERUM COPPER LEVEL(μgCu/100 ml SERUM)
NORMAL PREGNANCY	100	261.29±33.65
INTRAUTERINE FOETAL GROWTH RETARDATION	9	183.44±50.54
GESTATIONAL DIABETES	9	291.22±37.46
TOXAEMIA	24	276.18±49.71
POSTMATURITY	58	235.33±37.16

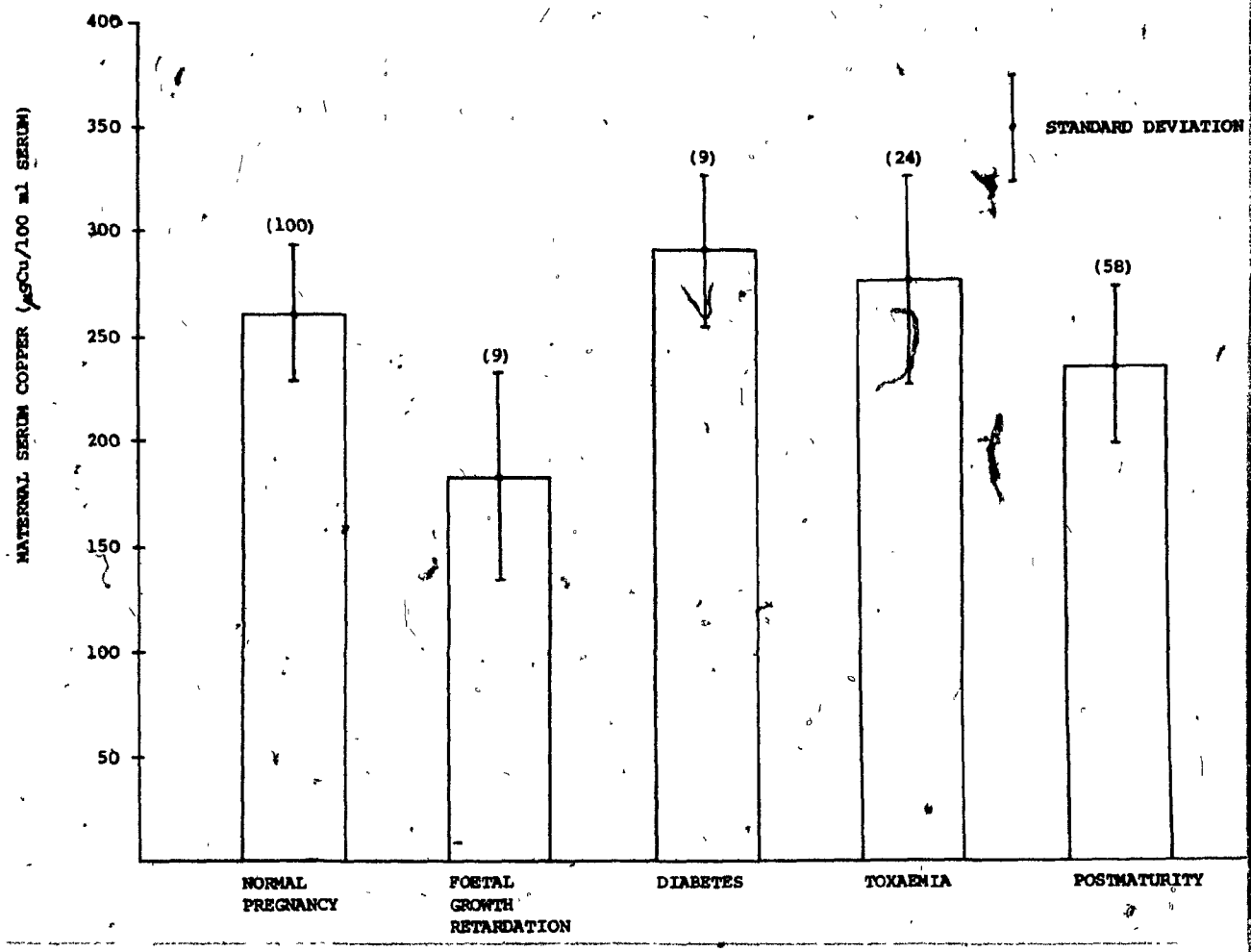

Figure 6 Mean Maternal serum copper concentrations at term (μg Cu/100 ml serum) in normal pregnancy and in various conditions.

CONDITION / SERUM COPPER LEVELS	NORMAL PREGNANCY	I.U.G.R.	DIABETES	TOXAEMIA	POSTMATURITY
	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES
LESS THAN 200µg%	7(7%)	5(56%)	0(0%)	0(0%)	12(21%)
200-249µg%	34(34%)	3(33%)	1(11%)	6(24%)	24(41%)
250-300µg%	42(42%)	1(11%)	5(56%)	13(54%)	17(29%)
OVER 300µg%	17(17%)	0(0%)	3(33%)	5(22%)	5(9%)
TOTAL	100	9	9	24	58

Table 6 Frequency distribution of Maternal serum copper levels (µg Cu/100 ml serum) in the third trimester of pregnancy.

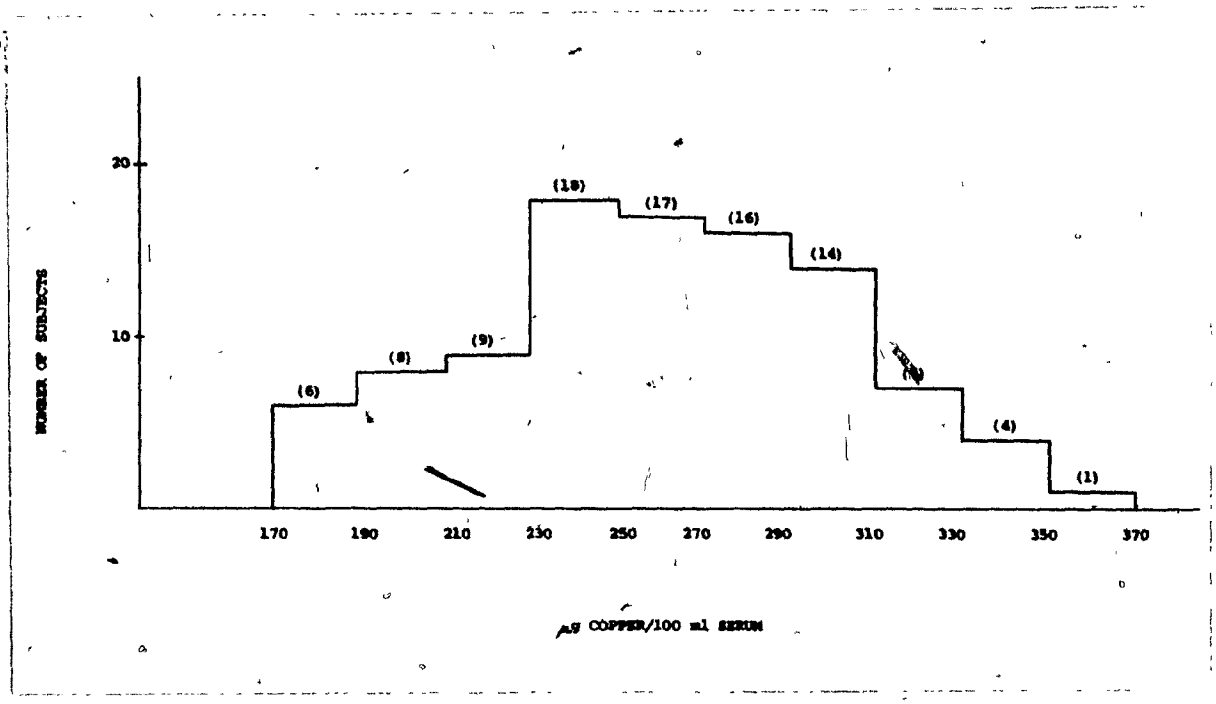

Figure 7 Frequency distribution of serum copper levels in the third trimester in one hundred cases of normal pregnancy.

GROUP	NO. OF CASES	MEAN MATERNAL SERUM COPPER LEVEL(μgCu/100 ml SERUM)
NORMAL PREGNANCY	106	257.91±40.69
TOXAEMIA	6	291.67±55.99
PREMATURE LABOUR	7	247.71±37.56
POSTMATURITY	13	247.85±37.58
PREMATURE RUPTURE OF THE MEMBRANES	9	247.78±31.87

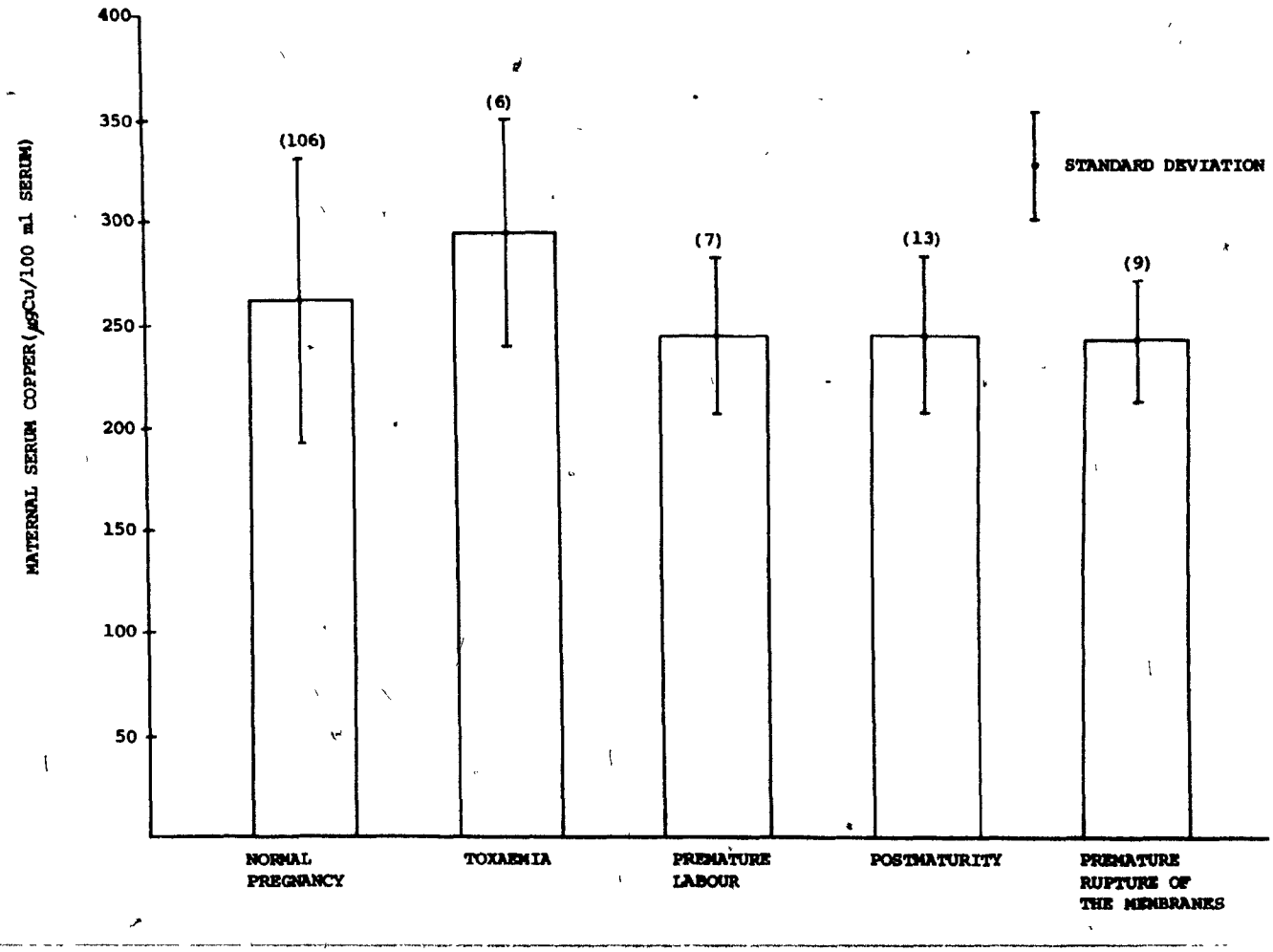

Figure 8 Mean Maternal serum copper concentrations at term (μg Cu/100 ml serum) in normal pregnancy and in various conditions.

In a second set of experiements (Study II), the mean maternal serum copper concentration at term was 257.91 µg% (S.D. ± 40.69), (figure 8). This value is in close agreement with the serum copper levels reported by other authors[65,109,130,161] and with the value observed in our previous study. Table 7 demonstrates the frequency distribution of serum copper values in these cases. The majority (64%) of values were observed to range between 200 - 300 µg%. Twelve percent of the values were less than 200 µg% as compared to 7% in the previous study. Similarily 24% of the observed values were higher than 300 µg% (as compared to 17% in the first study).

According to O'Leary[109], there is normally a continuous increase in maternal serum copper until term (figure 9). The degree of maternal serum copper increase seems to be dependent upon the stage of gestation. A significant increase is seen at the end of the first trimester corresponding to the formation of the placenta. Changes in the level of serum copper may be detected as early as the fourth week of gestation, but a significant rise can be observed by the tenth week when values of approximately 200 µg% are recorded. The plateau is then continued until the twenty fourth week when an increase occurs, eventually reaching levels around 300 µg% at term. The serum copper levels decline with the onset of labour and gradually decrease during the puerperium to pre-pregnancy levels (figure 10).

B) Intrauterine Foetal Growth Retardation (I.U.G.R.)

Infants weighing less than 2500 grams at birth and small for their gestational age are not necessarily premature. Inadequate intrauterine nutrition, congenital abnormalities, or intrauterine infection may contribute to chronic retardation of growth. Although the malnourished infant will appear undersized, the growth of the head and brain are often unaffected. Clinically, such infants appear initially more mature than those of similiar weight, but learning problems and neurological symptoms may occur later. In addition, these infants rarely achieve normal growth and continue to lag behind throughout life[101]. Thus, the earliest possible detection of foetal growth retardation is of vital importance.

In our survey, the 9 cases of pregnancy with I.U.G.R. concomitant with a small foetal size for the due gestational date had a mean maternal serum copper concentration of 183.44 µg% (S.D. $\pm$ 50.54) at delivery (figure 6). This is significantly lower than the serum copper concentrations for the same gestational period in normal pregnancy. In only 3 cases the serum copper concentration was between 200 - 300 µg% and only 1 case had a serum copper value exceeding 250 µg% (Table 6).

The severe pregnancy hypocupremia observed in these cases is highly significant, even at a S.D. of 1 in comparison to normal pregnancy. At delivery, the expected percentage of normal mothers possessing serum copper concentrations less

SERUM COPPER LEVELS \ CONDITION	NORMAL PREGNANCY	TOXAEMIA	PREMATURE LABOUR	POSTMATURITY	P.R.M.
	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES
LESS THAN 200µg%	13(12%)	0(0%)	1(14%)	1(8%)	1(11%)
200-249µg%	32(30%)	1(17%)	3(43%)	6(46%)	4(45%)
250-300µg%	35(34%)	3(50%)	3(43%)	4(31%)	3(33%)
OVER 300µg%	26(24%)	2(33%)	0(0%)	2(15%)	1(11%)
TOTAL	106	6	7	13	9

Table 7 Frequency distribution of Maternal serum copper levels (µg Cu/100 ml serum) in the third trimester of pregnancy.

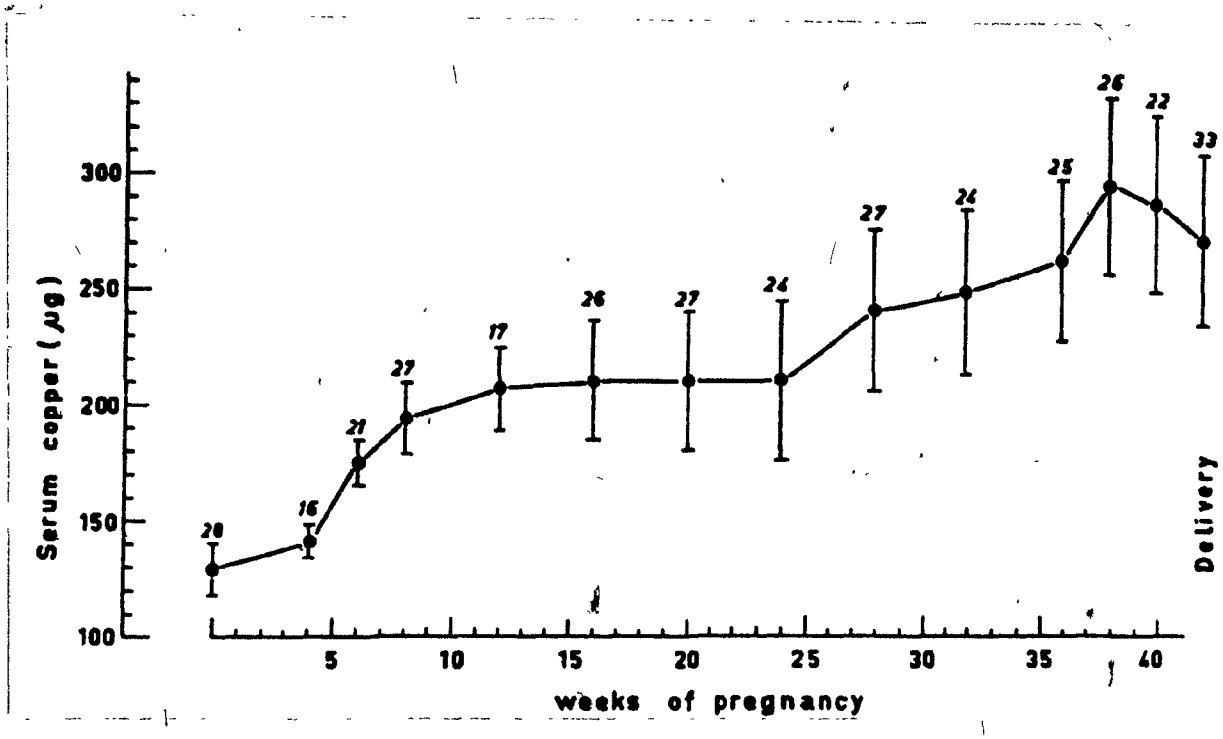

Figure 9 Mean Maternal serum copper (µg Cu/100 ml serum) at various stages of pregnancy.
(after O'Leary[109])

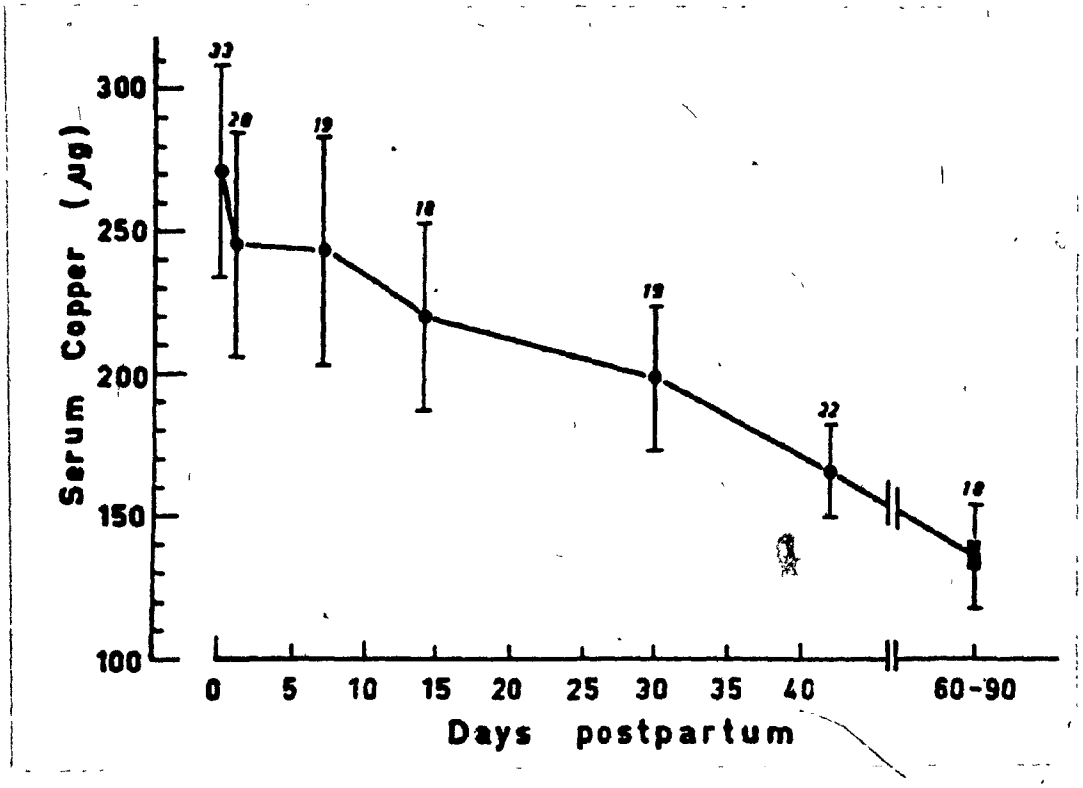

Figure 10 Mean Maternal serum copper (µg Cu/100 ml serum) during the puerperium.
(after O'Leary[109])

than 200 µg% is in the order of 7% (as compared to 56% observed in the cases of I.U.G.R.). Banerjea[1] studied the estriol excretion levels in 2 cases of foetal growth retardation diagnosed by the nonincreasing height of the uterus. With Case A, at 38½ weeks, the estriol titre began to fall and labour was induced. The patient delivered an infant weighing 3 pounds 8 ounces. Case B was admitted at 34 weeks of gestation on the diagnostic basis of "foetal growth failure" and the infant was delivered by Caesarean section, as there were signs of marked foetal stress. The estriol excretion concentrations were lower than normal in both cases. Banerjea's impression of poor foetal growth is that it is associated with "poor placental function". In the 9 cases of I.U.G.R. studied, none of the mothers had intrauterine infections nor were they malnourished. One would expect the I.U.G.R. developing as a result of infections would probably elevate the maternal serum copper concentrations, since it is well known that infections of various types markedly increase the serum copper content. Although the infants delivered were small and undernourished, they possessed no congenital abnormalities. Thus at least in the 9 cases studied, it would appear that "poor placental function" postulated by Banerjea may have been the factor associated with the I.U.G.R. The question arises as to whether copper plays a primary etiological role in the development of I.U.G.R. or whether it is a secondary indirect result of the I.U.G.R. It would seem

logical to assume that there was a substantial and sufficient amount of copper present in both the direct - reacting pool of plasma copper and in the ceruloplasmin fraction. Although pregnancy imposes a greater maternal demand for copper, even the most mediocre diet would supply sufficient copper to maintain normal physiological processes. It would also appear logical to assume that if it is not the lack of copper that is responsible for the pregnancy hypocupremia observed in the cases of I.U.G.R., other associative factors may vary the serum copper levels. Alterations in the foetal-placental-maternal complex may possibly disturb the estrogenic balance, estrogenic output being closely associated with the foetal-placental-maternal complex. The small placental size accompanying I.U.G.R. cases could invariably result in a quantitative decrease in the amount of physiologic estrogens synthesized and secreted by the placenta, thus lowering the induction rate for ceruloplasmin synthesis. These factors, solely, or in combination with each other, may be responsible for the significant hypocupremia observed in pregnancies with I.U.G.R.

These cases of pregnancy hypocupremia indicate that maternal serum copper concentrations may provide the obstetrician with an additional prognostic tool in conjunction with other diagnostic data. Women in the last trimester of pregnancy who show markedly depressed serum copper concentrations should be considered as "high risk" patients who have a greater than

normal probability of carrying a foetus whose growth is retarded. Prognosis as to the well being of the foetus may also be carried out with further serum copper determinations and in conjunction with the urinary estriol output. An increase in maternal serum copper levels in such cases may signify a favourable improvement in the foetus, while decreasing copper concentrations could indicate a less favourable prognosis with intrauterine foetal death becoming a reality. Some supportive evidence upholding the speculation on the validity of the prognostic value of serum copper determinations emerges from a study by O'Leary[109], who has monitored the total serum copper concentrations in two women with chronic hypertensive disease from the twenty eighth week of gestation (figure 11). An essentially normal gestational date vs serum copper curve was observed in both women until approximately the thirty sixth gestational week, when rapid and marked decreases in serum copper levels occurred. The continuous decreases persisted until term. Both women had intrauterine foetal deaths and delivered stillborn infants. Certainly serum copper determinations in such cases represent a much simpler test than estriol assays.

C) Gestational Diabetes

Metabolic abnormalities associated with diabetes mellitus may occur many years before the onset of actual clinical symptoms. This "prediabetic" state may develop into actual clinical diabetes by various stress factors, one of

which is pregnancy. Mothers already diabetic, or mothers who develop class A diabetes during pregnancy for the first time run a high risk of subjecting their offspring to metabolic abnormalities that increase both foetal and neonatal abnormalities. Although the advent of insulin has reduced the maternal mortality rate to a level similiar to that for nondiabetics, various complications concomitant with gestational diabetes have been described. Abortions, premature labours, intrauterine and foetal deaths, toxaemia, excessive sized infants, hydramnios, congenital malformations, and foetal anoxia have all been described in diabetic mothers[101].

In the 9 cases of gestational diabetes studied, the mean maternal serum copper level of 291.22 µg% (S.D. $\pm$ 37.46) at delivery was higher than in the controls (figure 6). The serum copper concentrations in the diabetic pregnancies ranged between 249 - 350 µg% with 8 cases having values between 250 - 350 µg% (Table 6). The majority (5 cases) of the serum copper values fell within the limits of 250 - 300 µg% while no cases were observed to have values less than 200 µg%. Only 1 case was observed having a serum copper concentration within the 200 - 249 µg% bracket.

It appears that there is a greater incidence of diabetic maternal serum copper concentrations occuring above the 250 µg% mark. Only 11% of these cases had values below 249 µg%, in contrast to 41% below 249 µg% in normal pregnancy. Thus, a patient with a higher than normally

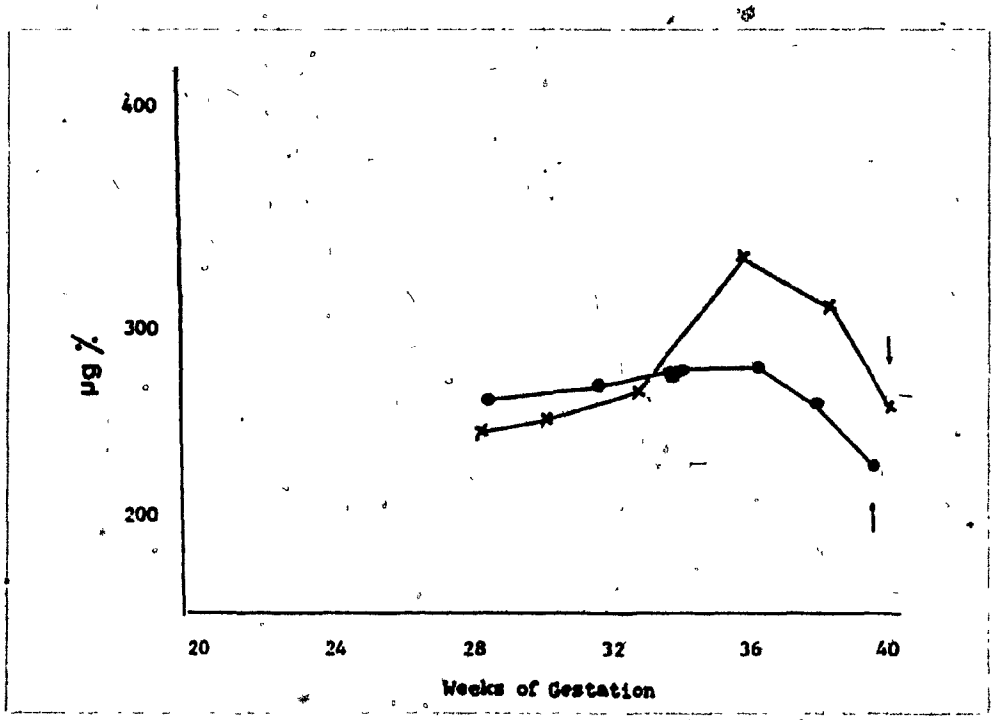

Figure 11 Total serum copper values in two patients in which foetal death occurred in utero. A very sharp decrease in serum copper was observed prior to abortion. (after O'Leary[109])

expected serum copper concentration in the third trimester of pregnancy should be carefully observed, since there may exist a greater than average probability that she is diabetic. The mean maternal serum copper concentration observed in this study is in agreement with the slight hypercupremia observed in gestational diabetes by other authors[130, 131, 161]. The etiology of hypercupremia in diabetic pregnancy still remains to be resolved, and only one explanation as to the factors responsible for the increase in maternal serum copper levels has so far been proposed. The hypercupremia of gestational diabetes may be related to hepatic metabolic changes associated with the disease, and to mild or severe toxaemia that usually accompanies and complicates diabetes. Toxaemia is postulated to result in subclinical hepatic damage, which in turn may result in the release of stored hepatic copper into the bloodstream[131].

In contrast to the constant hypercupremia observed in diabetic mothers, the available literature on their urinary estriol levels contains very conflicting reports, postulating nondeviating normal patterns of urinary extriol excretion[120], an elevation of urinary estriol excretion[148], and a decrease in urinary estriol excretion[113]. Perhaps the most intensive study dealing with the problem of urinary estriol excretion in diabetic mothers has been carried out by Greene[55], who studied a total of 88 pregnancies associated with diabetes mellitus. According to Greene, urinary estriol excretions

less than 4.0 mg/24 hours after the thirty-third week of gestation indicate intrauterine foetal death and call for the termination of pregnancy. In his study, 12 patients (14%) had estriol values less than 4.0 mg/24 hours and 6 of these pregnancies terminated in foetal death. Estriol excretion values ranging between 4.0 to 12.0 mg/24 hours were categorized with foetal distress and foetal jeopardy, leading to possible congenital anomalies and neonatal complications. A total of 39 patients (44%) were found in this group. Estriol excretion values above 12 mg/24 hours were associated with no perinatal mortalities and a normal gestational course. Thirty seven patients (42%) fell into this group. In normal uncomplicated pregnancies one would expect urinary estriol values to fall in the range of 16-17 mg/24 hours. Although Greene did not mention or elaborate upon whether his estriol values were low, normal, or high in comparison to those encountered in the controls, his results clearly indicate that more than half (58%) of his diabetic patients possessed estriol values in the "risk" zone of less than 12 mg/24 hours.

D) Toxaemia

The appearance of hypertension, proteinuria, and edema in the last trimester of pregnancy indicates preeclampsia in its incipient stage. Although the etiology of this disease is not known, the current "uterine ischemia theory" has so far received the greatest support. This hypothesis advances that mechanical or neurogenic factors, such as uterine size,

multiple pregnancy, hydramnios, and chronic hypertensive vascular disease may impair the uterine blood supply. The resultant placental hypoxia may release "protein substances" which produce both antidiuretic and hypertensive effects, as well as capillary thromboses and proteinuria. The factors responsible for these observed effects have not as yet been identified. In addition to what has just been said about preeclampsia, the patient who has progressed to the point of tonic convulsions is said to have eclampsia. Although eclampsia is a rare disorder, it is nevertheless a serious one manifested by initial convulsions before and after labour and during the puerperium. Prolonged coma without the recovery of consciousness culminating in death, although rare in occurence, may result after these convulsions[101]. Because of the small sample sizes encountered in the present studies, cases of preeclampsia and eclampsia have been grouped together under the heading of toxaemia, since the only differentiating factor between them is in the severity of the disease and the resultant onset of convulsions.

In our survey, the mean maternal serum copper concentration obtained in 24 cases of Toxaemia (Study I) was slightly higher (276.18 µg%, S.D. $\pm$ 49.71) in comparison with the serum copper concentrations in normal pregnancy at delivery (figure 6). Nineteen cases fell within the limits of 200 - 300 µg% (Table 6). No cases of Toxaemia were observed with serum copper values below 200 µg%, while 5 cases

possessed serum copper concentrations above 300 µg%. The majority of the values fell within the 250 - 300 µg% bracket while 24% of the cases of toxaemia as compared to 41% in normal subjects had values less than 249 µg%. Thus, the general tendency in the serum copper distribution of toxaemic patients is that a higher incidence of increased serum copper levels occur in comparison to normal pregnancies. Analagous to the previous study, the six cases of toxaemia encountered in the second study also possessed a slightly hypercupremic mean maternal serum copper concentration of 291.67 µg% (S.D. ± 55.99), (figure 8). The frequency distribution of the maternal serum copper concentrations agrees well with that of the first study (Table 7). The majority of the values ranged between 250 - 300 µg% with a lower than normal incidence of values falling below 249 µg% (17% vs 42% in the normal controls). Five cases had values exceeding 249 µg%.

The mean maternal serum copper concentrations observed in our toxaemic patients is in agreement with hypercupremic values of 340 µg% (S.D. ± 42.1)[131], 340 µg% (S.D. ± 38.3)[130], 286.8 µg% (S.D. ± 96.9)[161], and 390 µg%[109] reported by other authors. The elevation of maternal serum copper levels in toxaemic patients seems to be a paradoxical finding since Zondek[6] found low circulating estrogen levels in toxaemic patients. From these findings it appears that besides changes in estrogenic activity, other factors may play a role in affecting serum copper levels. As the liver

is frequently affected in toxaemic patients, the observed hypercupremia with a concomitant decrease in the amount of circulating estrogens could be attributed to subclinical hepatic damage. This subclinical hepatic damage may result either from intrinsic degenerative processes on the cellular level (infarction or unrecognized cellular degeneration) or on a subcellular enzymatic level[109]. Partial support of this hypothesis resides in the work done by Rasuli in toxaemic patients[117]. In women dying from severe eclampsia, he observed marked depletions of hepatic copper content, decreasing from the normal 800 µg Cu/gram to 40 µg Cu/gram. Mischel[103] ascribes a beneficial aspect of the hypercupremia in toxaemic pregnancies. He believes that copper ions act as a compensatory mechanism improving the utilisation of oxygen in tissues, increasing glycolysis in muscle, and inhibiting the inhibition of enzymes by heavy metals. Studies by Clemetson[27] on the metabolism of ascorbic acid in preeclampsia suggest that the resultant hypercupremia of toxaemia may be responsible for altering the ratio of reduced to oxidized ascorbic acid. The result is the accumulation of diketogulonic acid and ascorbone, which is a hypertensive agent in rats. Dehydroascorbic acid may be a predisposing factor involved with or resulting from toxaemia. It is unlikely that it is the primary chemical mediator in toxaemia, although additional studies are required to resolve this issue.

Thus maternal serum copper concentrations may be of importance in the clinical assessment of toxaemia. A patient manifesting increased serum copper concentrations and decreased urinary estrogen levels in the third trimester of pregnancy may be a "suspected" toxaemic case. Studies also reveal that there is a positive correlation between the clinical severity of the disease and the concentration of serum copper[109,130,131]. As such, the maternal serum copper levels may also be valuable in assessing the prognosis of the patient, with further increases in serum copper reflecting an increasing spread and severity of toxaemia.

E) Premature Labour

Spontaneous labour occuring after foetal viability is established and before foetal maturity is defined as premature labour. Foetuses weighing 1,000 to 2,499 grams (29 to 36 weeks of gestation) are considered as premature, and premature births are by far the most common cause of neonatal mortality. Chronic vascular diseases, toxaemia, placenta previa, foetal anomalies, multiple pregnancies, and maternal urinary tract infections are the most common causes of premature labour. However, in less than 50% of premature births can any definite cause by shown[101].

In 7 cases of prematurity included in our series, the mean maternal serum copper concentration of 247.71 µg% (S.D. $\pm$ 37.56) was recorded; this is slightly lower (statistically not significant) than levels found in the

controls (figure 8). Six cases fell within the 200-300 µg% bracket, with an equal number of cases being observed in the 200-249 µg% bracket and 250-300 µg % bracket (Table 7). Only 1 observed case of prematurity had a serum copper concentration less than 200 µg %, while no cases were observed to have values above 300 µg %.

The mean maternal serum copper concentration observed in this study is in agreement with the slight hypocupremic mean value of 250 µg % (S.D. $\pm$ 35.1) in premature births reported by Schenker[130]. It is logical to assume that since premature births occur before the thirty eighth week of gestation, at which time the serum copper concentrations reach their peak levels, determinations of copper levels several weeks before this date should yield lower values.

F) Postmaturity

The state of postmaturity is defined as a prolongation of pregnancy beyond 40 weeks of gestation, which may be detrimental to the foetus because of the limited life span of the placenta. Late gestational placental insufficiency, resulting in inadequate foetal nourishment may consequentially occur. Since reliable and accurate information relating to a patient's menstrual history, time of ovulation, and time of conception is sometimes lacking or is not well documented, the classification of a patient in many instances is an approximation[101].

A lower mean maternal serum copper concentration

of 235.33 µg % (S.D. ± 37.16) was observed in the 58 cases of postmaturity (figure 6). Twenty one percent of the observed postmature cases as compared to 7% in normal pregnancy had corresponding serum copper concentrations of less than 200 µg % (Table 6). Similiarly, 41% of the postmature values as compared to 34% in normal pregnancy had serum copper levels between 200 - 249 µg %. In the second study (Study II), the mean maternal serum copper was also lower than in the control subjects (figure 8).

The mean maternal serum copper values observed in these studies are in agreement with mean serum copper levels of 230 µg % (S.D. ± 24.38) and 240 µg % (S.D. ± 40.3) reported by Schenker et al[130,131]. Lundwall[92] has postulated that the low serum copper levels of postmaturity may be correlated to placental dysfunction. This hypothesis appears feasible due to several factors. Since the placenta has a limited life span in which time it can optimally function, the prolongation of pregnancy may result in the progressive dysfunction of the placenta. Since an intimate relationship exists between the maternal-placental-foetal complex with respect to estrogens and ceruloplasmin, progressive placental dysfunction may result in decreased estrogen production. Quantitative decreases of estrogens may decrease maternal serum ceruloplasmin concentrations since less estrogens would be available for "inducing" the synthesis of ceruloplasmin RNA templates. Another possible mechanism that may contribute

to the hypocupremia of postmature patients deals with the foetal estriol precursor isolated by Magendantz and Ryan[94], 16∝- hydroxydehydroepiandrosterone. This precursor may be decreased in activity with prolonged pregnancy either due to, (a) the sufficient maturity of the foetus which decreases the use of the foetal specific precursor with the commencement of induction of adult mechanisms or, (b) the progressive placental dysfunction may result in the insufficient ability to convert 16∝-hydroxydehydroepiandrosterone to estriol in the placenta, leading to decreased estriol synthesis and less estriol being excreted in the urine. The resultant decreased estriol levels may lead to lower ceruloplasmin concentrations since less estriol would be available for "inducing" RNA templates. The urinary excretion of estriol in postmaturity also remains a subject of controversy. The patients constituting the "postmaturity" group are a mixed and unhomogenous group whose diagnosis is based on subjective patient information. Indeed Lundwall[92] states that "there is no doubt that in many cases of postmaturity the pregnancy is not prolonged and the date of confinement is miscalculated". Postmaturity is often associated with other pathological conditions in pregnancy which ultimately lead to variability in the concentration of urinary estriol excretions. An intensive study by Lundwall[92] on the urinary excretion of estriol in postmaturity showed that out of a total of 171 postmature women investigated, 37 (22%) had

low excretion values of less than 16 mg/24 hours. Because of the "unhomogeneity" of the postmature group, Lundwall did not discuss his results in comparison to normal pregnancy estriol levels. Similiarly to Greene[55], he merely gave guidelines of certain estriol levels in relation to clinical applicability.

The consistent hypocupremia observed in postmaturity patients suggest that the maternal serum copper level should be used as an additional diagnostic factor to those presently employed, such as fundal height measurement, roentgen evaluation of foetal osseous development, ultrasound scanning, and amnioscopy for the assessment of foetal intrauterine status and maturity[101].

G) Premature Rupture of the Membranes (P.R.M.)

Patients whose membranes rupture prematurely in many instances involve the jeopardy of the foetus, since it is often difficult to decide whether to initiate labour and effect delivery of the foetus prematurely or to seek additional intrauterine maturation at the risk of amnionitis and foetal septicemia. The exact etiology is not clear, but it has been suggested that premature rupture of the membranes is not caused by membrane weakness, since membranes are capable of withstanding pressures that exceed those resulting from uterine contractions during labour[101].

In the 9 cases of premature rupture of the membranes studied, the mean maternal serum copper concentration at

delivery did not deviate to a statistically significant degree from the mean of normal pregnancy (figure 8). As seen from Table 7, the majority (7 cases out of 9) of these patients had serum copper concentrations in the range of 200-300 µg %, analogous to the control subjects. A larger percentage of cases fell into the 200-249 µg % bracket. Thus the frequency distribution of maternal serum copper levels in this group of patients exhibit a similar pattern to that of normal subjects.

H) Factors Affecting Copper Levels in Pregnancy

The onset of pregnancy is characterized by dramatic changes in maternal metabolic processes which are required for the growth and development of the foetus. Copper, being a critically important trace element necessary for the growth and development of many organ systems, plays a vital role in the maintenance and development of the foetus. Thus, as expected, marked changes in copper metabolism occur during pregnancy to meet the additional nutritional and physiological requirements imposed by both the foetus and mother.

a) Chemical Structure of Estrogens

The estrogens are one of the main hormones secreted by the placenta during pregnancy and are responsible for the development and maintenance of pregnancy. They exhibit a wide range of metabolic effects on the maternal and foetal organisms. Structurally, estrogens are four ringed, eighteen carbon compounds, which differ from other steroids by

lacking the C-19 methyl group which is normally attached to C-10 on the steroid nucleus. In addition, double bonds in the A ring of the steroid nucleus unsaturate the ring, and endow the eighteen carbon compounds with "estrogenic" activity. Estradiol, estrone, and estriol are the three major physiologic estrogens that account for the majority of estrogens in the human body[51]. Their chemical structures and interconversions are schematically represented in figure 12.

b) Biologic Effects of Estrogens

Estradiol - 17β is the most potent of the estrogens and as such is the major physiologic functional estrogen secreted by the syncytial cells of the placental trophoblasts. It is freely reversible with its less potent reduced form (at the C-18 position), estrone. Estriol is the physiologically weakest estrogen possessing only one-hundreth of the biologic activity of estrone and only one-five hundreth that of estradiol[147]. It is however the major excretory product of estrogen metabolism and estriol gluconate accounts for approximately ninety percent of all urinary estrogens excreted in pregnancy. Women in their menstrual cycle excrete an average 60 to 100 μg daily while pregnant females normally excrete more than 0.5 mg daily in the urine[51].

Although there has been a considerable amount of research on estrogen metabolism, the full biologic effects of the large quantity of estrogens secreted by the placenta

are not fully understood. The growth of uterine musculature is partly attributable to the effects of estrone and estradiol while estriol is known to antagonize this growth by suppressing the effects of both estrone and estradiol[114]; since the various estrogens possess differing expression of the sum total of their activities. Estrogens may also increase the strength of myometrial contractions by promoting increases in actinomyosin and adenosine triphosphate (ATP) concentrations in uterine muscle cells. This was demonstrated by Csapo[31] who observed an activation and stimulation of a pyridine nucleotide transhydrogenase system in the placenta and other tissues by estradiol and estrone. Inevitably, energy requiring biosynthetic processes could occur. Changes in the protein content as well as an increase in the thyroxine and cortisol binding capacity in the peripheral blood in pregnancy has been also attributed to placental estrogens[31].

c) Estriol in Pregnancy

The increasing amounts of excreted estrogens in pregnancy occur mainly as glucosiduronates or sulfates in the urine. Although the ovary also secretes the pregnant estrogens, it is the placenta that dominates their production. This was demonstrated by the finding that bilateral oophorectomy performed as early as the sixth week of gestation did not significantly alter the quantity of urinary estrogen excretion[51]. Estriol, being the most abundant estrogen secreted by the placenta, is also the fraction which demonstrates

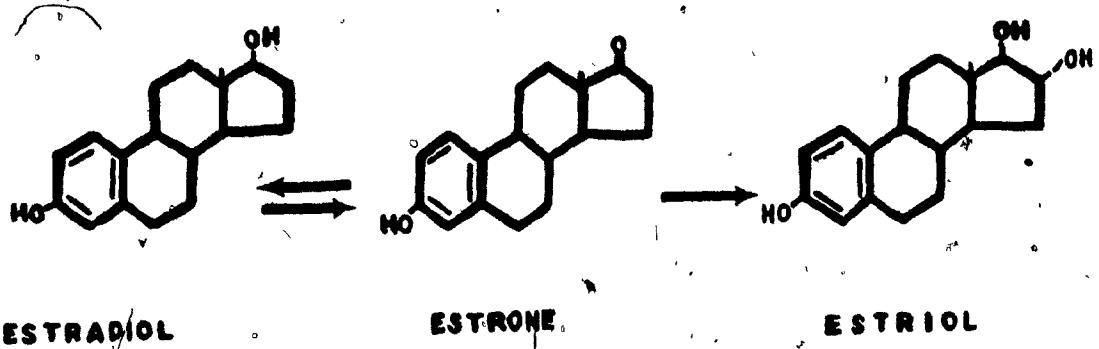

Figure 12 Chemical structures and metabolic interconversions of estrogens. Estradiol and Estrone are freely interconvertible, while conversion of Estrone into Estriol is not reversible. Estradial and estrone values (as measured in urinary excretions) increase only slightly, starting with day 168 with final values at term around 2 - 3 µg per 24 hours. A rapid rise in estriol values begins at day 140 with final urinary excretion values of 20-40 µg per 24 hours being observed at term (details in Fig. 13).

the most rapid increase in pregnancy as compared to estradiol and estrone. The ratio of estriol to estrone and estradiol in early pregnancy is of the order 2:1 while in the latter part of pregnancy this ratio increases five-fold in favour of estriol. At term, the urinary estriol concentrations have been shown to range from 10 to 50 mg daily[51]. This is graphically depicted in figure 13. In recent years, there has grown accumulating amount of evidence suggesting that the de novo synthesis of estrogens does not occur in the placenta. As is probably the case, substances synthesized outside the placenta, in both foetal and maternal endocrine glands, serve as precursors for the placental estrogens[51,94,114]. The principal estrogen biosynthetic pathways and their interelationship between the mother, placenta, and foetus are depicted in figure 14. Maternal and foetal dehydroepiandrosterone sulfate (DHAS) has been proposed as the precursor for placental estrone and estradiol. Active placental hydrolysis of maternal and foetal DHAS results in the formation of dehydroepiandrosterone (DHA) in the placenta which is then converted to androstenedione, androstenedione being freely reversible and interconvertible with testosterone. Aromatization of androstenedione, the process of which is not fully known, results in the formation of estrone which freely interconverts with estradiol[114]. The observation that both urinary and blood estriol levels fall immediately following foetal intrauterine death gives a further impetus to the possibility that the foetus provides

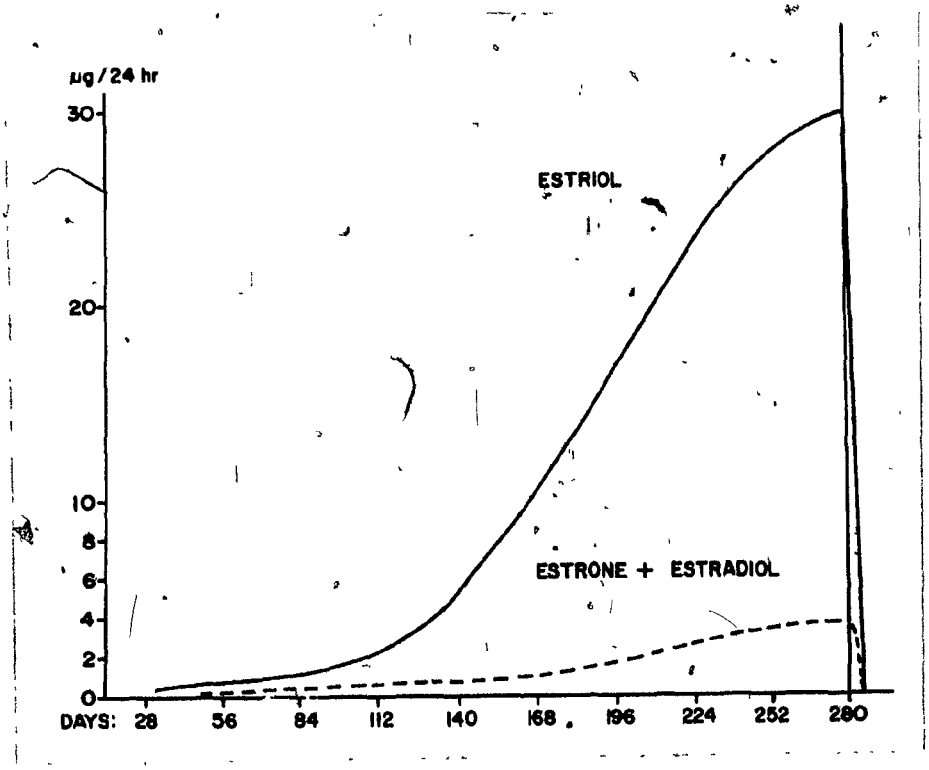

Figure 13 Urinary excretion of estriol and estrone - plus estradiol throughout pregnancy. (after Gold[51])

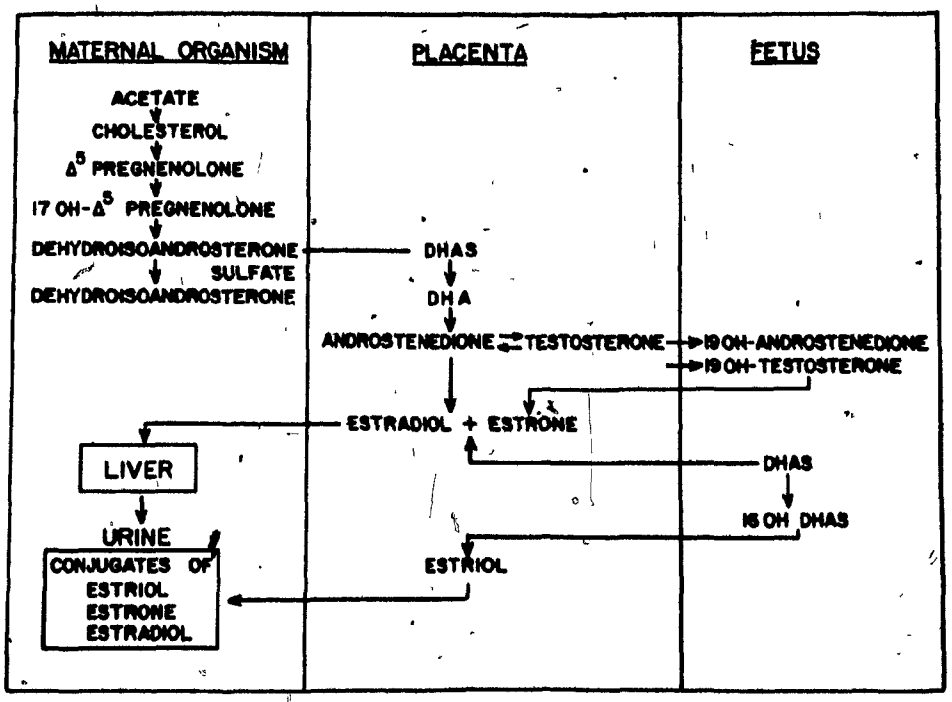

Figure 14 Biosynthetic pathways of estrogens in pregnancy (after Gold[51]).

precursors for estriol synthesis in the placenta. Magendantz and Ryan[94] have isolated a possible estriol precursor, 16∝-hydroxydehydroepiandrosterone from umbilical cord blood and this steroid was shown in vitro to be converted to estriol in the placenta by the sequence: DHAS ⟶ 16∝- OH - DHA ⟶ 16∝ - OH - androstenedione⟶ estriol. Thus both the mother and foetus must be taken into consideration in estrogen biosynthesis since an intricate interelationship exists between them and the placenta.

Urinary estriol excretion in pregnancy is now an accepted evaluative method for monitoring the progress and well being of the foetus. Studies estimating the urinary estriol concentrations in cases of complicated pregnancies are found in the literature[6,63,147]. The results of these studies strongly suggest that abnormal gestations are accompanied with low urinary estriol levels. Since a normal foetal-placental-uterine circulation and foetal-maternal function are a prerequisite for normal urinary excretion rates during pregnancy, any interruption in these interelationships will ultimately result in varying urinary estriol levels. Work done by Greene[53,54] has shown that when the urinary estriol excretion reaches the value of 12 mg/24 hours (or higher) within 48 hours of delivery, one can expect a healthy foetus except for cases of eclampsia or erythroblastosis fetalis. When the excretion falls into the range between 11.9 and 4.0 mg/24 hours during the last four to six weeks of pregnancy,

this indicates either a small infant or one in jeopardy. With values in this range, neonatal morbidity and mortality may be encountered. Estriol excretions of less than 4.0 mg/24 hours after the thirty third week of gestation are almost uniformly associated with foetal death. When estriol falls below 4.0 mg/24 hours, termination of pregnancy is considered, provided the foetus is of such size that it can tolerate an extrauterine environment[53,54]. Bernhard and Pfeifer[6] claim that a consistent urinary estriol decline to more than 30% indicates placental insufficiency and dysfunction. An estriol titre of less than 1 mg/24 hours would appear prognostically unfavourable and indicates an irreversible placental dysfunction and foetal death. Lundwall and Stakemann[92] have also quoted diagnostically unfavourable urinary estriol absolute values in cases of postmaturity: urinary estriol concentrations below 16 mg/24 hours indicate foetal stress and call for immediate delivery, either by a Caesarean section or by labour induction. A study by Bengtsson and Forsgren[4] demonstrated that in all cases of spontaneous abortion with foetal death studies, a sharp drop in estriol excretion occurred twenty four hours after intrauterine death which thereafter decreased slowly to very low values.

d) <u>The Effects of Estrogens on the Concentration of Serum Copper</u>

The significant rise of maternal serum copper in normal pregnancy was first reported by Krebs in 1928[80]

and has since been well documented[76,82,109]. Neither the significance nor the exact mechanisms accounting for the elaborate increases in serum copper concentrations in pregnancy are completely understood, and the maternal pregnancy hypercupremia still remains a subject of controversy. It has been postulated however, that it is the physiological increase in the amount of hormones synthesized by the placenta during pregnancy, namely the estrogens, that are in part responsible for the elevated serum copper concentrations. In certain diseases estrogen therapy has been demonstrated to increase serum copper levels[121]. Administration of estrogens and androgens in the form of estradiol benzoate and testosterone propionate to healthy geriatric patients significantly increased their serum copper content, which returned to normal preadministrative levels only eight weeks after discontinuation of estrogen administration[77]. Previous studies[26,60,107,129] have also clearly demonstrated a significant rise in serum copper levels in women taking oral contraceptives containing an estrogen, with a high correlation being established between the serum copper and ceruloplasmin levels in these females (figure 15). A high correlation was also found to exist between the ceruloplasmin and serum copper concentrations in the different stages of gestation during pregnancy[129]. It thus appears that the placenta, through the endogenous secretion of high amounts of physiologic estrogens, is of vital necessity for the marked hypercupremia

observed in pregnancy. The estrogens found in oral contraceptives and those naturally secreted by the placenta somehow appear to actively stimulate the rate of ceruloplasmin synthesis in the maternal liver. The increase in ceruloplasmin synthesis by estrogens occurs independently of the hepatic copper content and the estrogen action sites appear to be not only limited to uterine cells, but may be found on other tissues and cells, as has been demonstrated in the liver of fowl[132]. Although radioactive copper experiments clearly indicate that increased ceruloplasmin synthesis accompany elevated estrogen levels, the converse situation was shown not to be true. Decreased tissue estrogen levels accomplished by oophorectomy did not alter serum ceruloplasmin concentrations, indicating that the synthesis of ceruloplasmin is not dependent on estrogen production.[44] The mechanism of estrogenic stimulation of ceruloplasmin synthesis has been proposed by Evans[44] and is based upon the operon concept model of Jacob and Monod. Estrogen may be considered to act as an "inducer" for the synthesis of ceruloplasmin RNA templates which subsequently elevate the concentration of the ceruloplasmin protein. However, this proposition remains still to be elaborated upon since Evans did not specify for which protein, either the copper free ceruloplasmin precursor apoceruloplasmin or the actual ceruloplasmin molecule was there an increased synthesis of RNA templates. Nor did he mention whether the increased synthesis of RNA templates was

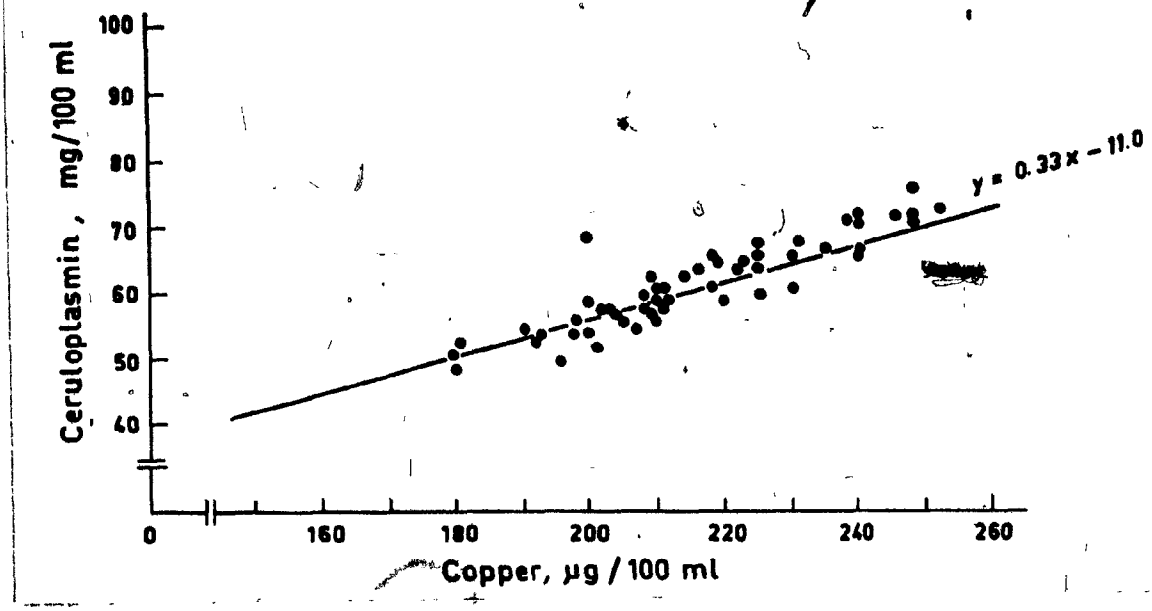

Figure 15 Corre-lation between serum copper and ceruloplasmin levels in women receiving estrogen containing oral contraceptives. This figure is shown to demonstrate that estrogens increase plasma ceruloplasmin concentrations. (after Schenker[129])

also accompanied by or independent of the rate of "charging" the apoceruloplasmin molecule with copper ions in the hepatic microsomes.

2. Umbilical Cord Serum Copper Concentrations at Delivery

A) Normal Pregnancy

The mean umbilical cord serum copper concentration of 49.42 µg% (S.D. ± 18.66) obtained in our study of 100 normal pregnancies agrees with the values of 55 µg %[131] and 50 µg %[159] reported by other authors. Henkin[65] observed a lower mean value of 29 µg %.

The mean umbilical cord serum copper concentration at delivery in our second study (Study II) was 63.51 µg % (S.D. ± 27.35), (figure 16). The frequency distribution of the normal umbilical cord copper levels are illustrated in Table 8. It can be seen that 71% of all the cases fell in the range of 20 - 79 µg %. Twenty nine percent of the total observed cases had serum copper levels ranging between 80 - 130 µg %, with only 6% of the values exceeding 110 µg %. Thus the copper content in the blood of the umbilical cord in normal pregnancy, whether from the umbilical artery or vein, is approximately four to five times lower than in the maternal blood[105].

B) Premature Labour

In the 7 studied cases of prematurity, the observed mean cord serum copper content of 42.83 µg % (S.D. ± 26.05) was lower than control values (figure 16). Schenker[130], on

GROUP	NO. OF CASES	MEAN UMBILICAL CORD SERUM COPPER LEVEL(μgCu/100 ml SERUM)
NORMAL PREGNANCY	106	63.51±27.35
PREMATURE LABOUR	7	42.83±26.05
POSTMATURITY	13	69.30±15.03
MATERNAL IRON DEFICIENCY ANEMIA	9	65.37±30.74
TOXAEMIA	6	62.17±31.02
PREMATURE RUPTURE OF THE MEMBRANES	9	60.33±24.98

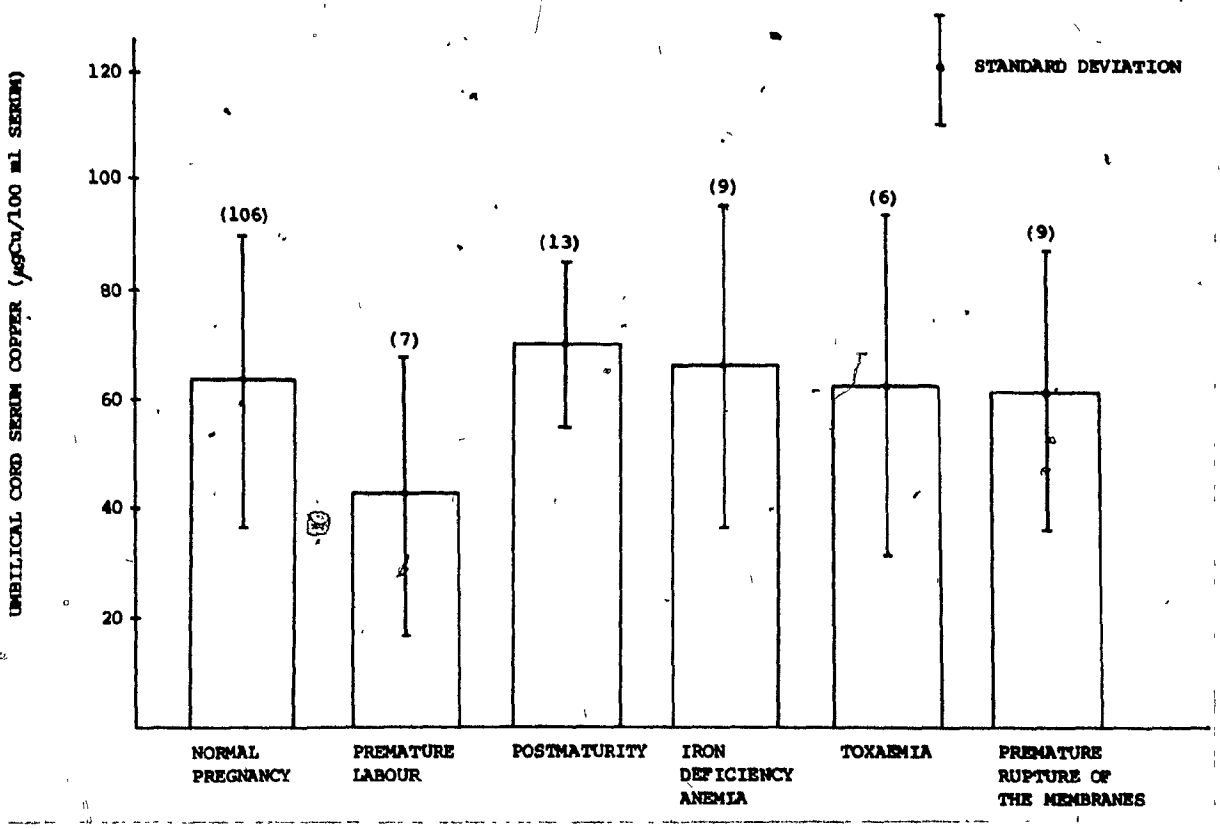

Figure 16 Mean Umbilical Cord serum copper concentrations (μg Cu/100 ml serum) at delivery in normal pregnancy and in various conditions.

CONDITION / SERUM COPPER LEVELS	NORMAL PREGNANCY NO. OF CASES	PREMATURE LABOUR NO. OF CASES	POSTMATURITY NO. OF CASES	ANEMIA NO. OF CASES	TOXAEMIA NO. OF CASES	P.R.M. NO. OF CASES
20-49 µg%	35(33%)	3(43%)	2(15%)	3(33%)	2(34%)	3(33%)
50-79 µg%	40(38%)	3(43%)	8(62%)	3(33%)	2(34%)	4(45%)
80-109 µg%	24(23%)	1(14%)	2(15%)	2(22%)	1(16%)	2(22%)
110-130 µg%	7(6%)	0(0%)	1(8%)	1(11%)	1(16%)	0(0%)
TOTAL	106	7	13	9	6	9

Table 8 Frequency distribution of Umbilical Cord serum copper levels at delivery (µg Cu/100 ml serum).

the other hand, did not observe any significant difference between the umbilical cord copper levels in normal and premature births. In our present study, six out of the total seven cases observed fell within the 20 - 79 µg % bracket and only one case had a copper concentration exceeding 79 µg%. An explanation as to the slight hypocupremia observed in the umbilical cord serum copper content of premature infants may be postulated in part from a study of Linder and Munro[91]. They advance that "considerable quantities" of copper are transferred from the mother to the foetus towards the "end" of pregnancy. However, they did not quantitatively determine the "end" of pregnancy, whether it be at the commencement of the third trimester or whether they meant the final gestational week that the extensive transfer of copper occurred. A decreased umbilical cord serum copper content in premature infants could mean that this transfer of maternal copper to the foetus had not yet occurred at the time of the premature delivery. It is logical to assume that a large transplacental copper transfer would result in increasing levels of copper in umbilical cord serum. As premature births in this study occurred between the thirty third to thirty sixth week of gestation, it may be postulated that the "extensive" transfer of maternal copper to the foetus via the placenta occurs only after the thirty sixth gestational week.

C) Postmaturity

In the 13 cases of postmaturity studied, the mean

umbilical cord serum copper concentration of 69.30 µg % (S.D. ± 15.03) was recorded. This is slightly higher (9%) than in the control subjects. Schenker[130] found a 10% increase of cord serum copper (65 µg %, S.D. ± 15.60) in his cases of postmaturity, although he did not attempt to explain this increase. It is probable that the already mature foetus in utero has an increased ability to synthesize and release ceruloplasmin into the blood stream. As yet, nobody has determined the ceruloplasmin vs "direct-reacting" copper concentration ratio in the cord serum of postmature infants. This would give an insight into the validity of our suggestion. Examination of Table 8 reveals that more than half (62%) of the cord serum copper values fall within the 50 - 79 µg % bracket while only 38% of the normal values fell within this range. Thus, unlike normal pregnancy, there appears to be a more specific concentration range (50 - 79 µg %) where the cord serum copper values fall in postmature cases. Comparitive examination of the standard deviations (± 27.35 in normal pregnancies vs ± 15.03 in postmature cases) further upholds this statement.

D) Toxaemia, Premature Rupture of the Membranes (P.R.M.), and Maternal Iron Deficiency Anemia

In cases of pregnancy complicated by toxaemia, premature rupture of the membranes, and maternal iron deficiency anemia, the mean umbilical cord serum copper concentrations of 62.17 µg % (S.D. ± 31.02), 60.33 µg %

(S.D. ± 24.98), and 65.37 µg % (S.D. ± 30.74) respectively were not significantly different in comparison to normal pregnancy (figure 16). The frequency distribution of umbilical cord serum copper concentrations in these conditions with respect to the controls were similiar and no significant variation was observed to occur in any bracket range. These findings agree with those of Schenker[130], who found no significant differences in the umbilical cord copper levels in toxaemic patients and those with premature rupture of the membranes with respect to normal values.

E) Foetal Copper Levels

a) Blood Copper

According to our studies, no significant correlation (Pearson product moment correlation coefficient, $r = -0.02$) was found to exist between the maternal serum copper levels and the umbilical cord serum copper concentrations in normal pregnancy. The increases of maternal serum copper are due exclusively to an increase in the synthesis of ceruloplasmin, with the direct-reacting pool of serum copper remaining fairly constant throughout pregnancy. Since no correlation was established between the elevated maternal serum copper, or mainly ceruloplasmin concentrations, and the foetal serum copper concentrations, it appears reasonable to assume that the constitution of foetal serum copper is largely composed of the albumin complexed direct-reacting copper. Increases or decreases of ceruloplasmin concentrations do not appear

to change the serum copper constitution of the foetus. This finding lends support to the finding of Henkin[65] that at term, the percentage of direct-reacting copper in maternal serum was 9% of the total serum copper whilst 91% was accounted for by ceruloplasmin. In the case of the foetus, 21% of the total serum copper was in the form of free "ultrafiltrable" copper while the rest of the serum copper remained in the bound form. Scheinberg[124] estimated a slightly higher percentage of direct-reacting copper in foetal serum. Out of a total of 30.5 µg % copper in the foetal serum, 22 µg % was bound to ceruloplasmin whereas 8.5 ug % (28%) was free. Quantitative enzymatic determinations of ceruloplasmin in the bound fraction of foetal serum indicated that in the foetus, as in the mother, almost all bound copper is carried by ceruloplasmin[66]. These results indicate that quantitatively, foetal serum binding of copper is not significantly different from that of normal adults. However, the presence of high copper concentrations in many foetal tissues in comparison to normal adult values would seem to indicate that specific differences in foetal tissue metal binding (i.e., mitochondrocuprein) do exist.

In the case of the foetus, the situation is converse to the hypercupremia observed in the mother. As Table 9 demonstrates, the concentration of maternal serum copper at term (250 µg%) is approximately five times higher than that of the newborn. It is puzzling and yet unknown why the total

copper concentration in the serum of the immature foetus (200 µg%) should be so high in comparison to the values encountered in the full term mature foetus. The marked difference in maternal and foetal serum copper concentrations is due to the relative concentration of ceruloplasmin on either side of the placenta. Henkin[65,66] has demonstrated that although at term the total serum copper concentration in mothers was approximately twice the normal nonpregnant level, the mean concentration of unbound direct-reacting copper was not significantly different from the levels in nonpregnant women (Table 10). Other studies[135] have demonstrated that it is the albumin complexed direct-reacting copper that accounts for the greater proportion of total plasma copper at birth in comparison to 8% or less in the plasma of the adult. Since the concentration of total "free" plasma copper is not significantly different on either side of the maternal-placental-foetal gradient, the observed foetal hypocupremia is not due to the reduction in the levels of direct reacting copper but is due to a substantial decrease in the concentration of ceruloplasmin. The low serum ceruloplasmin concentration in the foetus may be explained by the relative incapacity and immaturity of the foetal liver to synthesize the copper-bound ceruloplasmin molecule[91]. It was originally believed that the foetal liver was nonfunctional in the de novo biosynthesis of ceruloplasmin, lacking both the ability to synthesize the apoceruloplasmin precursor for ceruloplasmin and to subsequently

"charge" the apoenzyme with copper ions in the hepatic microsomes. Recent evidence however, indicates that in the case of the human infant the concentration of the precursor apoceruloplasmin in plasma is similar to the concentration present in normal adult plasma[135]. This indicates that the hepatic capacity to synthesize the apoprotein is not hindered in the foetus, but that only the ability to charge apoceruloplasmin with ionic copper is "underdeveloped" at birth. The low ceruloplasmin concentration of foetal serum may also be attributed to the existing placental barrier which does not allow the passage of the large ceruloplasmin molecule; only the direct-reacting copper is transferred to the foetus[45,65]. During the first week after birth, the concentration of copper in neonatal serum increases from the newborn level of 50 to 150 µg/100 ml serum; the ceruloplasmin level, measured by its enzymic activity, demonstrates a sixfold increase. After the first week, the concentration of copper falls again to approximately 100 µg/100 ml serum and remains at that level throughout childhood and adult life[59].

B) Total Body and Hepatic Copper

The foetal liver is the main depot site for the copper transferred from the mother. It contains more than half of the total foetal body copper (average 7 mg% as compared to 0.75 mg % in adults). This foetal hepatic copper accumulation occurs in association with mitochondrocuprein, which is unique to foetal and neonatal life and

	Foetus		
	Immature	Full Term	Mother
Fe	40	I60	60
Cu	200	50	250
Zn	300	I25	70

Table 9 Iron, copper and zinc in maternal and foetal serum (µg/100 ml serum). (after Widdowson[159])

	Nonpregnant	Pregnant
	(µg/100 ml)	
Serum copper		
Bound	94	203
Free	13 ± 2	18 ± 2
Total	107 ± 3[a/]	222 ± 14[a/]

Table 10 Comparision of serum copper in nonpregnant and pregnant women. (after Henkin[65])

accounts for a large percentage of the total copper present in the liver. Because of its specific high affinity towards copper, it binds and incorporates the transferred maternal copper and thus prevents the copper in the foetal liver from reaching toxic levels. Neonatal mitochondrocuprein is a highly insoluble protein containing a large amount of cysteine groups, probably in the form of disulfide bonds and contains about four percent copper. It is probably made up of peptides of a molecular weight similiar to the copper containing enzyme, cytocuprein[115]. Twenty percent of the hepatic copper is localized in the nuclear fraction of parenchymal cells, which declines to 9% in the adult. The remainder of the hepatic copper is distributed throughout the soluble cytosol and other cellular organelles. Postnatally, at 60 days of age, the infant attains the adult levels of 20% of total liver copper in each of these fractions, with the now most abundant cytosol fraction accounting for an approximate 50% of the total hepatic copper[116].

The total body copper of infants is significantly higher than in mature adults. It can be seen from Table II that the copper concentration in the full term human foetus (0.47 mg Cu/100 grams fat-free tissue) is about three times as high as in the normal adult (0.17 mg Cu/100 grams fat-free tissue)[159]. The extensive maternal-foetal copper transfer, especially in the third trimester of pregnancy, accounts for the high concentration of copper in the newborn. An

upper limit for copper accumulation appears to exist, at least in rats; this is not exceeded when large amounts of copper are added to the diet[42].

Widdowson[159] has studied the intakes and excretions of copper in normal human infants and found that on the average, infants were in copper balance. A relationship was established between the dietary intakes of copper in milk and the amount of copper that was excreted in the faeces; the babies with the highest copper intakes excreted the most copper in the faeces. Nonsignificant negative balances were occasionally observed and were always due to high faecal excretions of copper and not to urinary copper excretions, urine containing negligible amounts of copper. It is suggested that constant homeostatic mechanisms are already functioning early in life which maintain the copper in the body in a constant equilibrium. Thus, copper losses and gains may be easily replenished and excreted from the normal diet.

It would appear that nature has provided a mechanism to prevent the development of copper deficiency during the period of life when little copper is provided in the diet. Milk, being the almost exclusive dietary supply of the neonate, contains a low copper concentration (0.08 - 0.18 mg Cu/litre undiluted milk)[83] and as such a copper deficiency could develop in infants on a milk diet low in copper. Considerable quantities of maternal copper are transferred to the foetus during the terminal stages of pregnancy and substantial

	Foetus 20 Weeks Gestation	Full Term Newborn	Adult
Fe	5.80	9.40	7.40
Cu	0.32	0.47	0.17
Zn	2.00	1.90	2.80

Table II Relationship between the concentration of iron, copper, and zinc in foetal and adult tissues (mg/100 grams fat-free tissue). (after Widdowson[159])

portions are retained in the liver in association with mitochondrocuprein. During suckling, the low dietary intakes of copper result in the removal of the stored hepatic copper which is then increasingly transferred and incorporated into plasma ceruloplasmin and other essential copper containing enzymes. During the early postnatal period, the hepatic mitochondrocuprein is still present in the parenchymal mitochondria. With increasing age of the infant, induction of adult proteins by "basic mechanisms which are common to many adaptive processes within the complex mammaliam organism" occur[91]. An overall schematic representation of copper metabolism in pregnancy is depicted in figure 17.

3. Placental Copper Concentrations at Delivery

The full term human placenta measures from 15 to 20 centimeters in diameter, is 2 to 3 centimeters thick, and weighs between 500-600 grams. The placenta can be divided into both foetal and maternal portions. The foetal portion, consisting of the chorion frondosum with villi, is covered with amnion underneath which run the branching vessels that enter and leave the umbilical cord. The external surface attached to the uterus and divided into irregularly shaped cotyledons is the maternal portion of the mature placenta. The function of the placenta is to serve as an organ for the synthesis, degradation, and transfer of substances between mother and foetus. It is thus a basic foetal support system responsible for maintaining excretory pathways. At the same

time the placenta provides the foetus with a physical and chemical barrier against certain toxic agents and microorganisms that would be lethal to the developing foetus[56,130].

A) Normal Pregnancy

The mean placental copper content at delivery in our series was recorded as 8.45 µg/gram dry weight (S.D. ± 2.52). On a wet weight basis, because of dilution of copper, the mean was calculated as 1.52 µg/gram (S.D. ± 0.45), (figure 18). It appears therefore that the placenta has a higher copper content than most organs. According to Tipton and Cook[150], who have not measured placental copper, the only organs which contain higher copper concentrations are the brain (21.9 µg/gram dry weight), liver (18.8 µg/gram dry weight), kidney (11.9 µg/gram dry weight), heart (14.0 µg/gram dry weight), duodenum (10.5 µg/gram dry weight) and jejunum (8.97 µg/gram dry weight). It may be seen from Table 12 that 83% of the cases had placental copper concentrations in the range of 4.0 - 9.9 µg % on a dry weight basis. Seventeen percent of the values ranged between 10.0 - 16.0 µg%.

B) Premature Labour

In 7 cases of prematurity with delivery occuring between the thirty third and thirty sixth gestational week, the mean placental copper concentration was recorded as 10.15 µg/gram dry weight (S.D. ± 1.48), (figure 18). This mean is significantly (20%) higher than that of normal

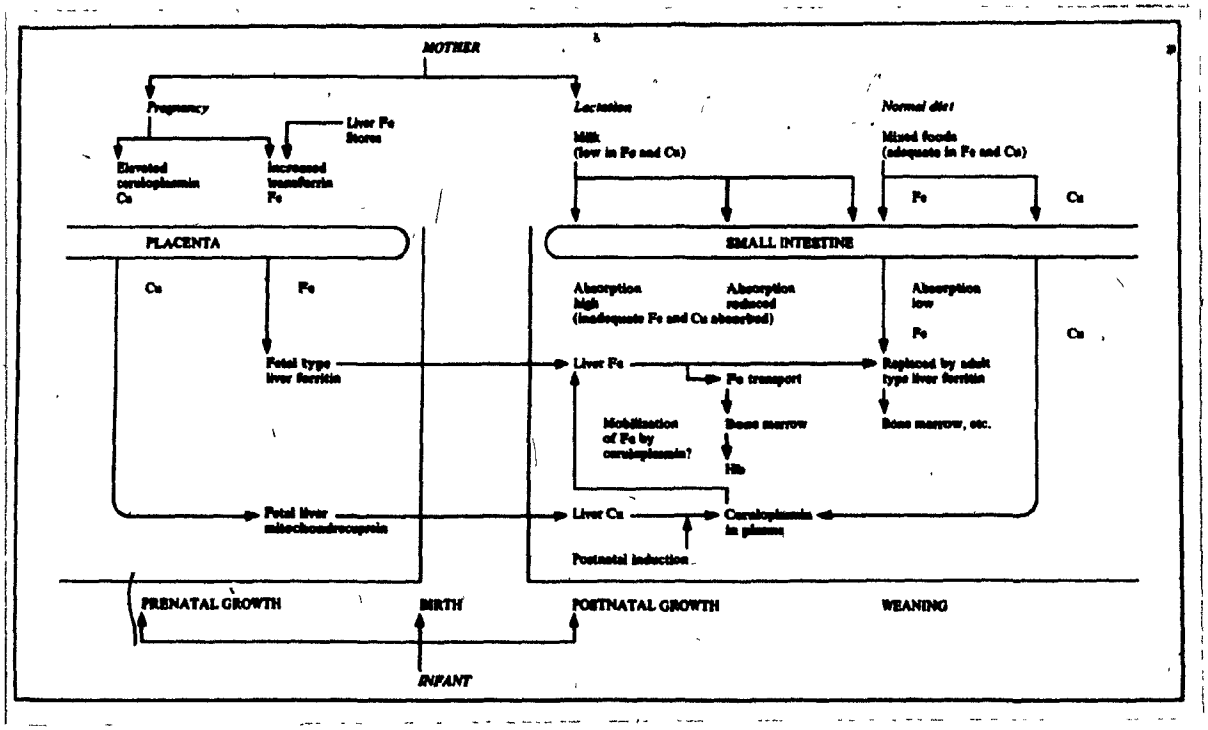

Figure 17 Schematic representation of copper metabolic pathways in pregnancy. (after Linder and Munro[91])

pregnancy. The values in all cases fell in the range of 7.0 - 12.9 µg % and in 4 of these cases the values were over 10 µg%. Thus the frequency distribution of placental copper concentrations in premature births was more specific (Table 12).

C) Other Conditions (Postmaturity, Toxaemia, Premature Rupture of the Membranes)

Placental copper concentrations were measured in an additional 28 cases, including postmaturity, toxaemia, and premature rupture of the membranes. The mean values obtained did not differ significantly from those in normal pregnancy (figure 18). These included 13 cases of postmaturity (8.72 µg%, S.D. $\pm$ 1.63), 6 cases of toxaemia (9.110 µg %, S.D. $\pm$ 2.80), and 9 cases of premature rupture of the membranes (8.29 µg %, S.D. $\pm$ 1.88). If these 28 cases were added to the 106 cases of normal pregnancy, the frequency distribution of placental copper concentrations would not be significantly changed (Table 12).

D) Source of Placental Copper and Mechanisms for Maternal-Foetal Transfer

The above data permit some evaluation of the possible role of the increase of copper in pregnancy. It appears certain that the placental copper concentration does not reflect the maternal serum copper values. Although the maternal serum copper values are increased in toxaemia and decreased in cases of premature rupture of the membranes

GROUP	NO. OF CASES	MEAN PLACENTAL LEVEL (μgCu/gm DRY WEIGHT)	MEAN PLACENTAL LEVEL (μgCu/gm WET WEIGHT)
NORMAL PREGNANCY	106	8.45±2.52	1.52±0.45
PREMATURE LABOUR	7	10.15±1.48	1.73±0.39
TOXAEMIA	6	9.11±2.80	1.69±0.68
POSTMATURITY	13	8.72±1.63	1.60±0.36
PREMATURE RUPTURE OF THE MEMBRANES	9	8.29±1.88	1.48±0.42

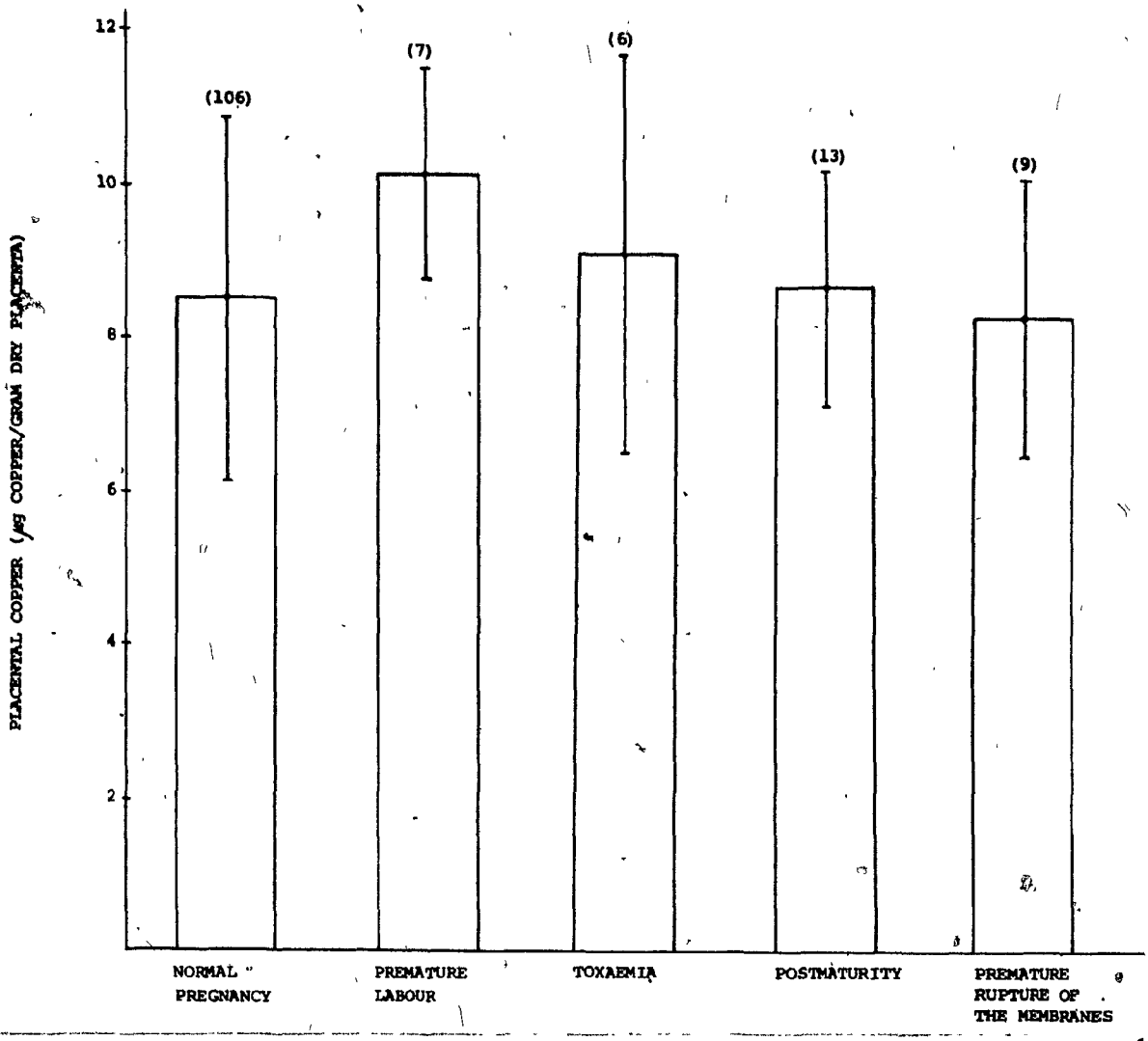

Figure 18 Mean Placental copper concentrations at term (μg Cu/gram dry weight) in normal pregnancy and in various conditions.

CONDITION / COPPER CONCENTRATIONS	NORMAL PREGNANCY	PREMATURE LABOUR	TOXAEMIA	POSTMATURITY	P.R.M.
	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES	NO. OF CASES
4-6.99µg%	28(26%)	0(0%)	2(33%)	3(23%)	3(33%)
7-9.99µg%	60(57%)	3(43%)	2(33%)	6(46%)	5(55%)
10-12.99µg%	12(11%)	4(57%)	1(17%)	4(31%)	1(11%)
13-16.00µg%	6(6%)	0(0%)	1(17%)	0(0%)	0(0%)
TOTAL	160	7	6	13	9

Table 12 Frequency distribution of Placental copper concentrations at delivery (µg Cu/gram dry weight).

and postmaturity, the placental copper concentrations in these conditions remain within normal limits. In premature labour, the maternal serum copper values are decreased and the placental copper is increased. It appears logical to assume that the placenta concentrates copper for the purpose of transferring the metal to the foetus at several stages of pregnancy. Since plasma ceruloplasmin does not wholly account for the placental copper constitution, other forms of copper are probably also associated with and stored in the placenta. These forms of copper may possibly consist of the maternal direct-reacting copper retrieved from the plasma or of copper containing enzymes.

The first source of copper which possibly contributes to the copper content of the placenta is the maternal direct-reacting copper pool. Controversy exists concerning the mechanism of maternal copper transfer to the foetus. Henkin[65,66] has postulated a "copper transfer" model in which only the direct-reacting copper is available for the unidirectional maternal-foetal transfer of copper. Copper transferred to the foetus appears to move across the placenta by passive transfer with an existant maternal-foetal gradient of 13 µg/100 ml plasma. We believe that it is important to determine whether the copper in transfer was still loosely bound to albumin in its preexistant maternal state, whether the copper became associated with some other unidentified substance, or whether the copper became freely ionizable

in course of the transfer. Taradaiko[144] has carried out copper determinations in the umbilical artery and vein and in retroplacental blood. He agrees with Henkin[65] that only the copper not bound to ceruloplasmin is transferred from the mother and is found in the umbilical cord blood. However, Taradaiko concludes that maternal copper does not appear to cross the placenta by passive diffusion but that copper accumulates in the placenta and is transported to the foetus by an active transport mechanism according to foetal needs. In our study of premature labour, the higher placental copper levels on the maternal side concomitant with lower foetal serum copper concentrations would appear to indicate that the placenta is "holding" the copper stores for the foetus. This "holding" of copper stores would seem to contradict Henkins[65,66] proposal of passive placental diffusion of copper and indicate that direct-reacting copper is stored in the placenta, which would support the proposal of Taradaiko[144] that copper is actively transported to the foetus.

A second, though small, source of placental copper may be the copper containing enzymes. Southren[137] has shown that one such enzyme, diamine oxidase (DAO), is markedly increased during gestation. It has also been suggested by Southren[138] that the increase of DAO levels is due to an increased production of a specific amine by the foetus, possibly a histamine - like compound which acts both as

an inducer and natural substrate for the enzyme. DAO actively deaminates histamine and other diamines, which in their undegraded form could act as potentially harmful agents to the developing foetus; therefore a self-defensive mechanism on the part of the foetus can be assumed. Southren[137] has demonstrated that the placenta is the site of DAO synthesis, more specifically the retroplacental decidua, which synthesizes the enzyme and secretes it into the plasma. Thus at least part of the placental copper content may be attributed to the presence of diamine oxidase.

Our studies do not answer the question of whether the concentration of "free" copper in the placenta occurs gradually throughout pregnancy or whether it only occurs in the terminal stages of pregnancy to supply the mature foetus on the way to begin a separate existence. It appears to be more logical to assume that some regulartory mechanisms exist in the placenta, which permit a moderate supply of copper to the foetus during gestation with the massive transfer occuring after the thirty sixth gestational week.

4. Maternal Iron Deficiency Anemia

Approximately 95% of the anemias encountered in pregnancy are due to iron deficiency. The increased demand for iron imposed by the foetus combined with a frequently inadequate diet and initially insufficient maternal iron stores at the onset of pregnancy may lead to a microcytic, hypochromic iron deficiency anemia. The total iron requirement during pregnancy was established to be about 800 mg, including

500 mg of iron necessary for the mother for expansion of erythrocyte volume plus 300 mg required for the mature foetus and placenta; an additional 50 mg of iron lost in blood during the parturition should be replaced (figure 19). Out of the total 3500 - 4000 mg of iron normally present in the adult, circulating hemoglobin accounts for approximately 70% of this total while 25% is stored as ferritin in the bone marrow, spleen, and liver. To maintain an adequate supply of iron for the increased metabolic needs of pregnancy, a daily dietary intake of eighteen milligrams of iron with an additional daily oral or parenteral medicinal administration of 30 - 60 mg elemental iron should be followed[56,130]. Foods high in iron content include liver, egg yolks, oysters, and nuts; it should be remembered that these ingredients are also of high caloric content and therefore are not always recommended.

In the 9 cases of maternal iron deficiency anemia included in our study, the mean maternal serum copper concentration of 280.37 µg % (S.D. $\pm$ 47.07) was higher than the normal mean of 257.91 µg %. The mean umbilical cord serum copper concentration of 65.37 µg % (S.D. $\pm$ 30.74) was not significantly different from the normal mean of 63.51 µg % observed in other cases. All cases of anemia had hemoglobin concentrations of less than 10 gm/100 ml serum (mean 9.2 G %) and a hematocrit of 30 percent or less, these criteria generally being accepted as indicative of maternal iron deficiency

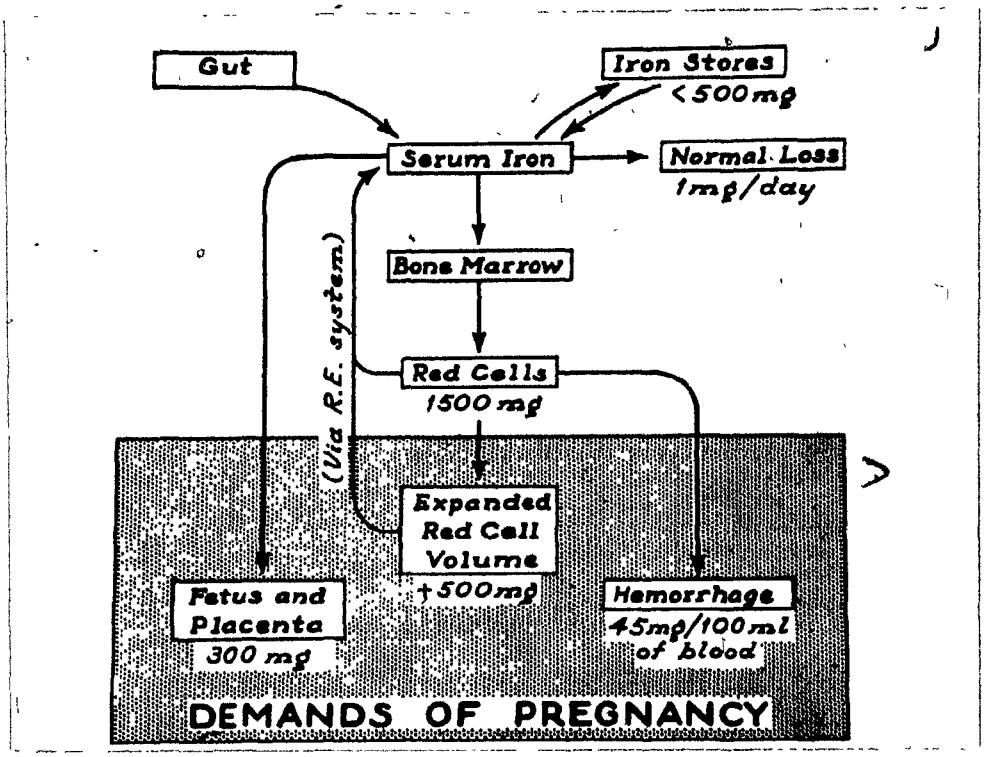

Figure 19 The iron requirements of normal pregnancy. (after Greenhill[56])

anemia. Wajcicka[157] observed an elevation of maternal serum copper in a two-fold increase when compared to that observed in normal pregnancy. It appears that higher maternal serum copper concentrations concomitant with decreased maternal hemoglobin concentrations represent a possible relationship between these two values. We found that a statistically significant inverse correlation (correlation coefficient, $r = -0.82$) exists at term between the maternal serum copper concentration and the maternal hemoglobin level.

A) Relationship between Ceruloplasmin and Iron Metabolism

Recent evidence indicates that ceruloplasmin, more recently referred to as ferroxidase, is involved both in vivo and in vitro in iron mobilization and in the rate of ferric-transferrin formation, which ultimately results in biosynthesis of hemoglobin[47,110]. Storage iron in tissues, in the form of a ferric-ferritin complex, is released from within the ferritin protein "shell" by a reduction of the attached iron from the ferric to the ferrous state. Divalent iron is then released into the plasma[47]. The known preferential binding of iron free apotransferrin to ferric iron is achieved by a reoxidation of Fe II to Fe III in the plasma. The proposal by Frieden[47] and Osaki[110] states that ceruloplasmin is the enzyme responsible for the oxidation of ferrous to ferric iron in the plasma. The ferric form may bind with apotransferrin to form the iron transfer molecule, transferrin,

which distributes iron to all tissues. Some evidence exists supporting this hypothesis. Ascorbate in the plasma is a sufficiently potent reducing agent that can spontaneously convert any ferric iron to the ferrous state.[47] Helbock[64] claims that in the absence of ceruloplasmin, the rate of ferrous iron oxidation in the plasma would not be adequate to meet the ferric - transferrin demands of the erythroid bone marrow cells for the biosynthesis of hemoglobin and other iron containing proteins. Further experiments in vitro demonstrated that the ceruloplasmin - catalyzed oxidation of ferrous iron is ten to one hundred times faster than the nonenzymic oxidation under conditions that may be expected in vivo. The presence of 40 μ M ascorbate was sufficient to retard markedly the net rate of the nonenzymic oxidation of ferrous iron, while an excess of plasma ascorbate was found not to affect the ceruloplasmin - catalyzed oxidation of divalent iron. It was furthermore postulated that ceruloplasmin, by enhancing the creation of significant concentration gradients between intracellular iron in the storage organs and the iron concentration of the capillary system, may be partly responsible for the movement of storage iron to plasma.[47,110] It was suggested that this is accomplished by inducing a rapid efflux of iron from the reticuloendothelial system[64]. A summary of the overall working hypothesis is schematically represented in figure 20.

In no other physiological conditions such a great demand and extensive utilisation of iron exists as in pregnancy. A 15% increase in the maternal hemoglobin mass and a 6% increase in the red blood cell volume occur in the demands of both foetal and maternal tissue oxygenation[89]. Increased absorbability of ferrous iron in the mucosal cells of the upper gastrointestinal tract results in a greater storage of iron in maternal tissues, particularly in the liver[46]. A higher percentage of maternal transferrin saturated with iron may possibly be related to the elevated ceruloplasmin levels of pregnancy. The increased ceruloplasmin levels may partly provide an increase in tissue iron mobilization as well as a faster plasma ferrous iron oxidation rate, ferrous plasma iron also being substantially increased from increased absorption and from transport from storage sources. It appears logical to assume that the greater saturation of maternal transferrin during pregnancy imposes a greater demand for the oxidation of ferrous to ferric iron in the plasma. Since this oxidation is presumably catalyzed by ceruloplasmin, greater plasma concentrations of this copper enzyme, biosynthetically induced by the estrogens in pregnancy, may be needed to efficiently handle the increased amounts of plasma ferrous iron which must be catalytically oxidized. As previously mentioned, the absence of ceruloplasmin under normal conditions may result in an inadequate transfer of ferric-transferrin to the bone marrow for hemoglobin synthesis[110].

In the case of pregnancy, where iron demands for hemoglobin synthesis greatly exceed those of normal conditions, the increased transfer of ferric-transferrin to the erythroid bone marrow results from an increased coupling of ferric ions to apotransferrin, the latter presumably being achieved from increased ferroxidase oxidation of plasma ferrous iron. Not only is there a greater demand for transferrin by the maternal bone marrow cells, but increased saturation levels of maternal transferrin is of prime importance for the transfer of iron to the foetus, since the mother's plasma appears to be the only important source of iron for the foetus[46]. Fletcher[46] observed that the maternal transfer of iron to the foetus via transferrin occurs extremely rapidly. Within twelve minutes of introducing labelled iron into the mothers plasma, it was detected in the foetus. It appears that a high and constant amount of "saturated" transferrin must be readily available in the maternal plasma, not only to supply the greater demand of maternal hematopoietic organs, but to make iron rapidly available to the placenta for foetal transfer. This rapid and constant availability of "saturated" transferrin can presumably be maintained by increased ceruloplasmin concentrations which accelerate the rate of ferrous to ferric iron oxidation in the plasma and thus generate ferric ion availability for transferrin formation.

Our observations of a maternal hypercupremia and

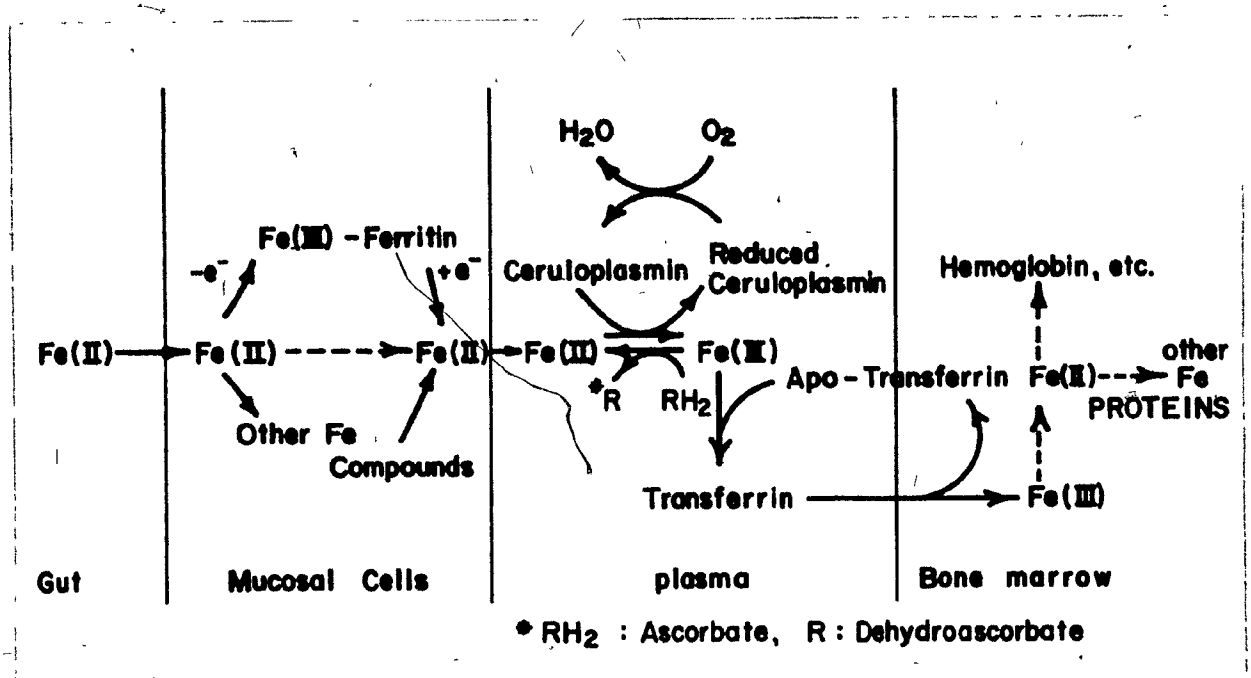

Figure 20 Schematic presentation of ceruloplasmin involvement in iron metabolism. (after Osaki[111])

a statistically significant inverse relationship (correlation coefficient, $r = -0.82$) between maternal serum copper concentrations and maternal hemoglobin concentrations in cases of maternal iron deficiency anemia may be explained on the basis of the "ceruloplasmin - iron oxidation theory". Decreased concentrations of iron in maternal storage tissues in anemia may result in a smaller than normal amount of ferrous iron that is made available to the plasma. Subsequently less ceruloplasmin may be needed for the oxidation of ferrous iron to the ferric state. Since there is a deficiency of iron and not of copper, it would be expected that initially normal levels of ceruloplasmin should be found in the plasma. A decreased oxidation rate concomitant with lower plasma ferrous iron concentrations may result in an equilibrium shift which leads to a higher percentage of ceruloplasmin not being reduced and degraded in oxidizing ferrous iron. Thus, possibly more free "unutilised" ceruloplasmin may be found in the plasma, which may partly account for the maternal hypercupremia in cases of iron deficiency anemia.

On the basis of these facts, it may be tentatively assumed that one of the main functions of the elevated maternal plasma ceruloplasmin concentrations in pregnancy is to increase the rate of ferrous iron oxidation to saturate plasma transferrin. A greater saturation of transferrin with iron occurs not only to meet the demands of increased maternal hemoglobin synthesis but to also maintain a constant and readily available supply of plasma iron which is

available for the foetus by constant passage through the placenta.

CHAPTER 5

CONCLUDING COMMENTS AND SUMMARY

1. It is well established that copper is an essential trace element in all living organisms, due to its participation in enzymatic catalysis, particularly with reference to oxidative enzymes such as the cytochrome oxidases in the respiratory chain. Environmental copper is present in the soil, water, and atmosphere and thus invariably in foodstuffs. Foods rich in phospholipids, particularly organ meats, contain high concentrations of copper and are responsible for meeting the daily nutritional requirements in man and animals.

2. Data on normal copper metabolism in man indicates that the average daily dietary intake is in the range of 2 to 5 mg. Ingested copper is absorbed mainly from the duodenum and upper jejunum. At least two absorptive mechanisms have been postulated, in association with specific proteins: the superoxide dismutase and a "metallothionein" protein. The presence of other transition elements, inorganic sulfate, and calcium carbonate in the intestinal lumen interfere with the absorption of copper, either by competitively binding to copper recepter sites or by chemically altering the copper complex.

3. Copper is found in two forms: a firmly and a loosely bound form, present in both the plasma and the erythrocytes. In plasma, the firmly bound copper is incorporated in the ceruloplasmin molecule (93%) while the loosely bound copper accounts for the direct-reacting copper (7%). In erythrocytes, erythrocuprein accounts for the firmly bound copper (60%) while 40% of the copper is localized in the more labile "protein" fraction. The mean serum copper concentrations reported are 109 µg % for men and 120 µg % for women. No sex differences exist in the copper concentration of erythrocytes. The copper content in 100 ml of packed erythrocytes has been reported as 89 µg. Hypocupremia (as in hepatolenticular degeneration and nephrosis) and hypercupremia (as in Hodgkin's disease, leukemia, and bacterial infections) have been described to occur in association with various clinical conditions in man.

4. Copper is metabolized in the liver with resulting incorporation into ceruloplasmin, the physiological role of which is not completely understood. The liver contains 10% of the body copper, with lesser amounts being present in the brain, kidneys, heart, and hair in that order. The main excretion route for copper is the biliary tract; renal excretion is responsible for a negligible percentage (less than 1%). Copper deficiency and

toxicity states in humans are rare, due to the presence of specific homeostatic mechanisms which maintain the body copper within constant limits. Because animals lack such mechanisms, they are more susceptible to copper deficiency and toxicity effects. Many naturally occuring diseases in animals have been ascribed to copper defiency, such as enzootic neonatal ataxia in sheep, as well as several conditions due to copper toxicity.

5. In the current studies, the maternal serum copper concentrations were determined using atomic absorption spectroscopy technique in 206 cases of normal pregnancy and 144 cases of pregnancy with various conditions, selected from 2032 pregnancies. The mean maternal serum copper concentration in normal pregnancy at term was recorded as 259.60 µg %. This is significantly higher than serum copper levels in nonpregnant women, which was found to be 117.38 µg %.

   In cases of intrauterine foetal growth retardation, the observed mean serum copper concentration of 183.44 µg % was significantly lower than the mean of control subjects.

   In both premature labour and postmaturity cases the trend in serum copper concentrations was lower. This is explainable by the fact that the peak of maternal serum copper concentration is reached at term, and therefore

sampling carried out before that date will yield a lower copper concentration. Similarly in postmaturity cases, the maternal serum copper levels begin to decline after term. These differences were not statistically significant when compared to those delivering at term.

In cases of toxaemia and gestational diabetes, the maternal serum copper levels tended to be higher than normal. In these cases, subliminal liver changes may be responsible for the increased release of copper into the circulation.

6. The copper concentrations of umbilical cord serum as well as placental copper levels were determined in 106 cases of normal pregnancy and 44 cases of pregnancy with various conditions. The mean umbilical cord serum concentration in normal pregnancy was recorded as 56.45 µg % which is approximately 5 times lower than the maternal serum copper concentration at term.

In cases of premature labour, the observed mean umbilical cord serum copper content of 42.83 µg % was lower than in the controls. This may be explainable by the fact that the extensive transplacental transfer of maternal copper to the foetus had not yet occurred at the time of premature delivery. In postmature cases, a 9% increase in the umbilical cord serum copper content as compared to normal pregnancy was observed: the already mature

foetus in utero may be commencing the biosynthesis of ceruloplasmin. The umbilical cord serum copper concentration in cases of toxaemia and maternal iron deficiency anemia did not deviate significantly from the mean of normal pregnancy.

7. The mean placental copper concentration in 106 cases of normal pregnancy was found to be 8.45 µg Cu/gram dry weight and 1.52 µg Cu/gram wet weight.

   In premature births, the mean placental copper concentration of 10.15 µg Cu/gram dry weight was higher (20%) than that of normal pregnancy. In cases of toxaemia and postmaturity, no significant differences in the placental copper levels were observed when compared to normal pregnancy.

8. Some postulates may be suggested as explanations of the data recorded in our studies. A significantly lower value of maternal serum copper was observed in cases of intrauterine foetal growth retardation; this may be related to placental dysfunction, alterations in the foetal-placental-maternal metabolic interelationship or to quantitative decreases of estrogenic secretion by a small placenta. The reduction in the amount of estrogens secreted by the placenta may result in a decrease of ceruloplasmin synthesis, since the induction rate for ceruloplasmin RNA

templates would be reduced by the lack of sufficient levels of estrogens. The maternal hypercupremia of toxaemic patients may be a result of hepatic damage, not recognizable clinically, with release of stored copper into the blood stream. In gestational diabetes, which is often associated with toxaemia, the slight maternal hypercupremia may also result from hepatic damage. In premature births, the maternal hypocupremia is probably explainable by the fact that serum copper concentrations reach their peak levels at term; therefore sample estimations before this date should yield lower values. Similiarly, the maternal hypocupremia of postmature cases is most likely due to the fact that serum copper levels decline after the 38th gestational week; therefore sample estimations after this date should yield lower values. A quantitative decrease in the amount of estrogens secreted by the placenta, due to placental dysfunction of prolonged pregnancy, may also be responsible for lower serum copper levels in these cases.

9. The finding that no statistically significant correlation (correlation coefficient, $r = -0.02$) exists between the umbilical cord serum copper levels and maternal serum copper concentrations in normal pregnancy indicates that ceruloplasmin is not the major copper constituent of foetal serum, as is the case in maternal serum. Maternal ceruloplasmin levels do not appear to be reflective of foetal serum

copper concentrations. Experimental studies support the view that the direct-reacting pool of copper represents the major constituent of foetal serum.

10. A decrease in the concentration of serum copper in the umbilical cord and increased placental copper levels observed in premature birth cases indicates that the placenta may be storing additional copper which is transported to the foetus by an active process in the terminal stages of pregnancy. This extensive transfer probably occurs after the 36th gestational week. The fact that there is statistically no significant correlation between the placental copper concentrations and the maternal serum copper bound to ceruloplasmin indicates that the copper content of the placenta is mainly of the direct-reacting fraction and copper incorporated into enzymes specific for gestation.

11. No data is available in the literature, to the best of our knowledge, to indicate the possible significance of the elevated ceruloplasmin levels in pregnancy. Several observations arising from our studies are presented; these seem to support the "ceruloplasmin - iron oxidation" theory and indicate a possible role of ceruloplasmin in the metabolism of iron in pregnancy.

The physiological increase of ceruloplasmin concentration

during pregnancy may be related to the increased maternal gestational demands for iron. The augmented iron mobilization from maternal iron stores as well as the maintenance of transferrin-saturated iron by plasma ferrous oxidations may be the proposed function of ceruloplasmin; this refers to usage by maternal hematopoietic organs and for transplacental transfer to the foetus. A highly significant inverse relationship between the maternal hemoglobin concentration and maternal serum copper concentration in cases of maternal iron deficiency anemia suggests that less ceruloplasmin is needed for oxidizing the ferrous ion in the plasma as well as for mobilization of maternal iron stores. A decreased ceruloplasmin utilisation would result in a decreased degradation of the protein, with resultant elevation of serum copper concentration.

12. Obstetrical research in the area of developing a dependable method for the assessment of the intrauterine environment should continue to reduce perinatal mortality and morbidity. Accurate measurements of gestational copper status may prove useful in the determination of foetal well-being. The current urinary estriol assays utilised for the detection and monitoring of "high" risk patients poses some problems. The estriol concentrations increase significantly only

in the last trimester of pregnancy; therefore this data is not indicative of complications which may occur earlier. The sample collections (twenty four hour urine specimens) involve a tedious procedure. In trying to apply estriol determinations to clinical practice, a rapid and accurate method of determining urinary estriol levels in pregnant women is still to be found. Estriol assays are done only in specially equipped laboratories and involve a large time factor (three days to do the Brown analysis).

Maternal serum copper concentration determinations may be used as diagnostic aids in conjunction with other presently known diagnostic methods for evaluating foetal status. The advantages of serum copper measurements over urinary estriol assays consist of the speed of sample procurement and analysis, economy, and sensitivity. The detection of complications as early as the tenth week of gestation may be aided by this method. Serial serum copper determinations throughout pregnancy, depicting a general trend of copper levels, could be employed in the assessment of foetal welfare in different cases. Single maternal serum copper determinations could also serve as valuable aids in the diagnosis of "high" risk patients. Mothers having markedly low serum copper concentrations than what one would normally expect in

the third trimester of pregnancy should be assessed further with respect to possible foetal growth retardation or pending to abortion.

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