TRACE ELEMENTS

IN

HUMAN PREGNANCY

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by

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ABSTRACT

Recently there has been much interest in the effects of trace elements on the fetal growth and development and congenital defects. Baseline data on these trace elements are few and new more accurate methods for their analysis are being introduced. This study was designed to determine such baseline data.

Methods were developed to determine zinc, copper, iron, manganese and chromium in serum and urine. A series of experiments were carried out to optimize the drying, charring, and atomizing parameters for each element in three different matrices: water, serum and urine. The method for chromium was hampered by the loss of the highly volatile chromium fraction and the analytical interference due to smoke and background absorption. The efficiency of the method was determined using three different methods: 1) a calibrated serum standard - Cation-cal; 2) serum samples analysed by method of additions; 3) serum analysed by flame atomic absorption.

Serum zinc, copper, iron, manganese and chromium levels were measured in pregnant women at 6-10, 11-16, 24-26 weeks of gestation, at labor, and at 3 days and 6-30 weeks postpartum and in nonpregnant women. Trace elements in the venous, umbilical cord serum were also measured.

The serum trace element levels in nonpregnant women were not influenced by the regular hormonal changes of the menstrual cycle. However, use of oral contraceptive agents resulted in a significant decline in serum chromium and increase in serum copper.

In normal pregnancy, maternal serum zinc and chromium levels declined, serum copper rose and no changes were observed in serum iron and manganese

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levels. Iron supplementation eliminated any observable decline which would be expected in pregnancy. No statistically significant differences were observed between pathological and normal pregnancies, and due to lack of adequate numbers in each category, statistical analysis could not be done. However, the data seems to suggest that serum zinc and copper levels were decreased in women with threatened abortions. In some cases decreased copper levels may be observed in women who miscarry; or with premature rupture of membranes; or women who undergo induction of labor. A decline in serum iron levels, at term, was observed in prolonged gestation.

Zinc, iron, manganese and chromium levels in the venous, umbilical cord serum were higher than the levels seen in maternal serum at term. Cord serum copper levels were only one-fourth of the maternal level at term. The fetus accumulates these trace elements from the mother for postnatal growth' and development. Serum copper in the fetus is low because the fetus does not synthesize the major cuproenzyme, ceruloplasmin, until onset of postnatal life; however, it is known that fetal liver copper content is much higher than adult liver copper content, suggesting prenatal storage of the metal. Slightly lower serum zinc and iron levels were observed in low birthweight neonates and low serum iron was seen in postmature neonates.

ACKNOWLEDGEMENTS

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Acrodermatitis enteropathica	AE
Alpha-aminoisobutyric acid	AIB
Chromium	Cr
Copper	Cu
Creatinine	Cre
Daltons	D
Deoxyribonucleic acid	DNA
Example	e.g.
Flameless atomic absorption spectrophotometer	FLAAS
Gastrointestinal tract	G.I. tract
Glucose removal rate	GRR
High molecular weight zinc binding ligand	HMW-ZnBL
Hour	h
Iron	Fe
Leukocyte endogenous mediator	LEM
Low molecular weight zinc binding ligand	LMW-ZnBL
Manganese	Mn
Oral contraceptive agents	OCA
Oral glucose tolerance test	OGTT
Protein-calorie malnutrition	PCM
Ribonucleic acid	RNA
Ribonuclease	RNase
Seconds	sec
Small-for-gestational age	SGA
Low birthweight	LBW
Thymidine kinase	TK
Total iron binding capacity	TIBC
Total parenteral nutrition	TPN
Zinc	Zn

1.   Literature review

1.1. Fetal nutrition

Pregnancy is associated with many complex and interrelated physiological and biochemical changes in the mother and her conceptus. The nutritional supply to the fetus, the endocrine milieu and the expression of the fetal genome determines the fetal ontogeny (Page *et al.*, 1976; Miller & Merritt, 1978). Upon implantation, the mother and fetus commence a partnership in which they must share the available energy supply and nutrients for growth and sustenance of life. The development of the fetus which is nurtured through the maternoplacental complex, is directly dependent on the mother's health and nutritional status. Impaired fetal or maternal blood flow, placental dysfunction and maternal malnutrition results in impaired metabolic and nutritive interaction between the mother and fetus (Winick, 1970; Winick *et al.*, 1973; Hahn, 1978).

The fetal diet has three main components: 1) calories (carbohydrates), 2) proteins, 3) vitamins and minerals. Protein-calorie malnutrition (PCM) in humans has been studied since World War 11 (Smith, C., 1947) and it is generally accepted that PCM in pregnancy decreases the birthweight of the newborn. It is suspected that infants born to malnourished mothers also suffer from varying degrees of mental retardation, depending on the timing and duration of malnutrition (Chase, 1975; Iyengar, 1975; WHO, 1974; Tizard, 1975; Guzman & Guthrie, 1976; Mora *et al.*, 1979; Vuori *et al.*, 1979).

Vitamins are micronutrients, essential to human metabolism (Pike & Brown, 1975). The knowledge regarding the effects of vitamin deficiencies in human pregnancies is not extensive; however, the fact that they are essential has been recognised and these micronutrients are generally supplemented in pregnancy (Kellog Nutrition, 1978).

About 99% of the total body weight is attributed to eleven major elements: carbon, hydrogen, oxygen, nitrogen, sodium, potassium, calcium, magnesium, phosphorous, sulfur and chloride. The other elements contribute less than 0.01% of the total body weight and are arbitrarily designated as 'trace elements' (Ulmer, 1977). The nutritional importance of three such trace elements, iodine in thyroid physiology (Chatin, 1852 in Prasad 1978), cobalt as a part of Vitamin B_{12} (Rickes *et al.*, 1948), and iron as a component of hemoglobin (Heath *et al.*, 1932), has been known for many years. Several other trace elements have been recently identified and added to the list of essential trace elements in man: zinc (Prasad *et al.*, 1963 b); manganese (Doisy, 1972); copper (Josephs, 1931); chromium (Glinsman & Mertz, 1966); and fluorine (WHO, 1970).

These essential trace elements participate in a large variety of metabolic processes because of their structural and functional roles in many key enzyme systems: carbonic anhydrase (zinc), lysyl oxidase (copper), and kinases (manganese). Therefore, despite their minute concentrations in the tissues, deficiencies of these new trace elements can produce a variety of structural and physiological abnormalities (Burch *et al.*, 1975; Reinhold, 1975). Until very recently the importance of these nutrients in man had not been seriously considered. Since animal studies have shown that specific nutritional deficiencies do occur and they can be detrimental to health, growth and development, it is worth considering their occurrence in man.

The problem of trace element deficiencies during human pregnancies presents a special concern, since nutritional deficiencies can severely affect the prenatal development and the postnatal maturation of the fetus. In animals, impaired development of the fetal skeletal and nervous systems and related structures are dominant features of copper, zinc and manganese

deficiencies during pregnancy (Williams, R. 1977). The role of these

newly recognized trace elements in human reproductive processes is still

largely unexplored. Some of the reported effects of zinc and copper

deficiencies are abnormal duration of pregnancy, poor efficiency of labor

and impaired fetal development (Jameson, 1976; Friedman *et al.*, 1969).

Trace element deficiencies have been reported in all income

families in industrialized countries (Hambidge *et al.*, 1972), and are

believed to be due to increased refinement of food which removes much of

these nutrients, (Klevay *et al.*, 1979; Solomons *et al.*, 1979). The 1975

Nutrition Canada survey in Saskatchewan reported that 12% of the pregnant

white population had inadequate intake of proteins and calories and 28%

of the white population had inadequate intake of calcium, iron and vitamins.

Overall nutritional deficiency was higher in the Eskimos (60%) and North

American Indians (58%). The survey did not assess the trace element

nutritional status of the Saskatchewan population. My project was a pilot

study designed to monitor trace elements: zinc, copper, manganese, chromium,

and iron, in a discrete population, i.e. pregnant women. An attempt was

also made to determine the effects of these deficiencies on the fetus and

mother.

1.2. Iron*

1.2.1. Distribution

Iron is the most abundant trace element in the body. The iron content

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*Tabulated summaries of the effects of trace element deficiencies are

presented at the end of the literature review.

of a 70 kg man ranges from 4-5 gms and is distributed as follows: 70.5% in hemoglobin, 3.2% in myoglobin, 26% in storage compounds (ferritin and hemosiderin), 0.1% in transport form (transferrin), 0.1% in the cytochromes and 0.1% in the catalases (O'Dell & Campbell, 1971).

Iron occurs in the blood as hemoglobin in the erythrocytes and as transferrin in the plasma, in an approximate ratio of 1000:1. Small quantities of iron as ferritin are also present in the erythrocytes and serum and represent only 0.2-0.4% of the total serum iron (Summers et al., 1974).

Hemoglobin (M.Wt. 65,000 D) is a complex of globin and four ferroprotoporphyrin or "heme" moities. The iron molecule is in the reduced (Fe^{2+}) form and reversibly binds to oxygen, thus permitting hemoglobin to function as an oxygen carrier. The iron content of hemoglobin is approximately 0.35% (g/dL hemoglobin). The hemoglobin level varies with age (Linder & Munro, 1973), sex, nutrition, pregnancy, altitude (Ganong, 1977), and disease states (Prasad, 1978). The hemoglobin level is high at birth (18-19 g/dL) and declines to about 12 g/dL at 3-4 months of age (Linder & Munro, 1973). At puberty the males have a more dramatic increase in hemoglobin content than the females and this difference is maintained throughout the adult life (Ganong, 1977). During pregnancy, a decline in hemoglobin is observed and this is primarily due to increased plasma volume and a concomitant iron deficiency, resulting in an anemic state (Page et al., 1976).

Serum iron is bound to a B_1-globulin called transferrin. Normally only 30-40% of transferrin carries iron and the remaining binding capacity is known as latent iron-binding capacity. The normal serum iron is higher in males (127 ±65 ug*/dL) than in females (113 ± 60 ug/dL). The serum

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* ug represents the term microgram

iron declines in anemia (0-78 ug/dL), infections (30-72 ug/dL) and in late pregnancy (22-185 ug/dL). The total iron-binding capacity (TIBC) of transferrin is increased in pregnancy and anemia (Cartwright & Wintrobe, 1949).

Excess iron is stored primarily in the liver as ferritin and hemosiderin compounds. During high demand for erythropoiesis and placental iron transfer to the fetus, the iron from these storage complexes is mobilized.

1.2.2. Metabolism

Iron absorption occurs primarily in the duodenum (Brown & Rother, 1963) in the ferrous (Fe^{2+}) form (Moore & Dubach, 1956). Most dietary iron is in the ferric (Fe^{3+}) form. The acid gastric milieu favors the reduction of ferric to ferrous for absorption (O'Dell & Campbell, 1971).

Iron is absorbed into the intestinal mucosal cell and then passes directly into the blood where it binds to transferrin. The existence of an iron-transport system has been suggested. It involves binding of iron to iron-receptor sites in the plasma-membrane of the intestinal epithelial cells. Interation of plasma transferrin with the binding sites releases the iron into the circulation (Evans & Grace, 1974).

The homeostatic mechanism for iron metabolism is not known. McCance & Widdowson (Pike & Brown, 1975) demonstrated that there is a unidirectional movement of iron across the intestinal mucosa and the body has no satisfactory means of excretion via either the gut or the kidney. The iron homeostatic control, therefore, depends on its absorptive mechanism (Granick, 1954). The present hypothesis, based on animal studies, is that the transfer of the absorbed iron from the mucosal cells into the body is dependent on its iron status. Therefore, there is increased transfer of

iron into the blood stream during increased erythropoeisis and deficient states and little or no transfer in sufficient states. This proposed mechanism is known as "Mucosal Block theory" (Granick, 1954; Munro & Drysdale, 1970). The mechanism of iron homeostatic control in man is not known and the application of the Mucosal Block theory in iron homeostasis is still being investigated (Prasad, 1978).

Dietary factors can promote or hinder inorganic iron absorption. Organic acids, such as ascorbic, lactic and pyruvic acid, promote absorption by reducing and chelating inorganic iron in the gut (Brown & Rother, 1963; Hallberg & Solvell, 1967). Amino acids also promote uptake of inorganic iron by forming amino acid-iron chelates (VanCampen & Gross, 1969). Absorption of iron is decreased in presence of phytates (Hallberg & Solvell, 1967), clay (Minnich et $al.$, 1968) and phosphates (Brock & Dramond, 1934) because they bind the inorganic iron and decrease its availability for absorption. High intakes of cobalt, zinc. (Gregor et $al.$, 1978 a), cadmium, copper and manganese. (Thomson & Valberg, 1972), also decrease iron absorption because they compete for the intestinal binding sites. Organic iron, i.e. hemoglobin, is readily absorbed from the gut and is not very sensitive to the presence of phytates or the organic acids in the intestine. (Hallberg & Solvell, 1967).

A normal subject retains 5-10% of the ingested iron and this is increased to 10-20% in iron deficient states such as anemia, in pregnancy, and in conditions that produce increased erythropoiesis (Schwarz & Kirchgessner, 1974). The absorbed iron is bound to transferrin and transported primarily to erythroid bone marrow for incorporation into hemoglobin, and to the liver and spleen for storage as ferritin and hemosiderin.

Since the work of McCance & Widdowson (1943) it has been known
that iron is not excreted in significant amounts in the urine, and fecal
iron represents unabsorbed iron. This is further supported by studies
in hemolytic anemia patients since they only excrete 0.5% of the
liberated iron. Small amounts of iron are lost with the bile (0.20mg),
urine (0.08mg), skin sweat (0.02mg) and the gastrointestinal tract (0.45mg).
The gastrointestinal iron represents the red blood cells and the mucosal
cells lost in the feces (McCance & Widdowson, 1943; O'Dell & Campbell, 1971).

1.2.3. Iron deficiency in humans

1.2.3.1. Infants and adults

Iron deficiency in human adults and infants is characterised by
listlessness and fatigue, palpitation on exertion and sometimes by a sore
tongue, angular stomatitis, dysphagia and koilonchyia. (Prasad, 1978). In
some cases of iron deficiency anemia, abnormalities of the G.I. tract, i.e.
achlorhydria, superficial and atrophic gastritis, have been observed
(Lees & Rosenthal, 1958).

Iron deficiency in infants is also manifested as anorexia, decreased
growth and poor resistance to infection (McFee, 1979). In many parts of
the world, zinc and iron deficiencies occur simultaneously. Zinc deficiency
adversely effects appetite, growth and resistance to infection; therefore,
it has been postulated that the above symptoms are manifestations of zinc
deficiency, rather than iron deficiency (Prasad, 1978).

Iron deficiency results in the development of hypochromic, microcytic
anemia, accompanied by a normoblastic, hyperplastic bone marrow that
contains little or no hemosiderin. The serum iron is decreased with a
concomitant increase in total iron-binding capacity. The plasma transferrin
saturation is decreased to 10% or less, implying an inadequate iron supply
to the erythroid tissues (Bainton & Finch, 1964).

Formerly, it was believed that the primary effect of iron deficiency was decreased hemoglobin synthesis and therefore, decreased oxygenation of tissues. Beutler (1961), investigated the effect of iron deficiency on other heme enzymes and found a variable but significant decrease in activity of cytochrome C, succinic dehydrogenase and cytochrome oxidase. The variation in the degree of depletion is not fully understood but is believed to be related to the function, growth rate and cell turnover of the particular tissue. Iron deficiency is also believed to impair the bacteriocidal function of leukocytes by decreasing the activity of another heme enzyme, myeloperoxidase. This then results in decreased resistance to infection (Prasad, 1979).

Human infants between 4 and 24 months of age are at high risk for iron deficiency because of the rapid growth of the infants and the negative iron balance in the body (Cavell & Widdowson, 1964). Children born to frankly anemic mothers and premature babies have subnormal hemoglobin levels and iron stores (Sanchez-Madrid, 1975; McFee, 1973). If not supplemented at birth, the inadequacy of iron stores at birth and increased need for rapid postnatal growth accelerates the onset of anemia (Linder, 1978).

Adults at risk for iron deficiency are those suffering from chronic blood loss, malignancy or infection, and those on poor nutritional intake, (Prasad, 1978). Women of fertile age frequently (20-25%) have hypoferremia due to loss via menstruation (Rybo, 1966); pregnancy, and lactation. (Page *et al.*, 1976). Women on oral contraceptive agents (OCA) have a significant increase in serum transferrin (244 ug/dL in nonusers vs. 290 ug/dL in women on OCA) and TIBC (268.7 ug/dL in nonusers vs. 338.9 ug/dL

in women on OCA), with no effect on serum iron and other hematological indices; hemoglobin, hematocrit, red blood cell count, mean corpuscular hemoglobin content, and mean corpuscular volume, (Margen & King, 1975; Prasad *et al.*, 1975; Deeming & Weber,1978). The increase in transferrin and TIBC is believed to be due to estrogen rather than progesterone. (Powell *et al.*, 1970). Metabolic balance study in women on OCA showed a slight but significant (P<0.05) decrease in iron absorption (Margen & King, 1975). The investigators have cautioned against drawing a conclusive result because of the analytical problems and incompleteness of human balance studies. King *et al.*, (1978) investigated absorption of stable isotopes of iron, zinc and copper and found no significant difference between the OCA users and nonusers.

1.2.4. Iron in pregnancy

Iron requirement in pregnancy is increased. Nonpregnant women have a daily intake of 18mg of iron while the pregnant women need 36mg /day of iron. This increase is basically due to: 1) an increase in red blood cell production to maintain normal hematocrit concentration with 40-50% increase in plasma volume in pregnancy, 2) and for fetal hemoglobin synthesis. This need is further accentuated with multiple pregnancy and abnormal antepartum bleeding. The increased need for iron is met by a compensatory increase in absorption from the gut. At delivery, 300-400mg of iron are removed with the placenta and normal puerperal blood loss. This loss of iron is partially overcome by a gradual breakdown of excess erythrocytes produced in pregnancy. Lactation results in a further loss of 0.5-1.0mg/day of iron (McFee, 1979; Ashe *et al.*, 1979; Sanchez-Madrid, 1975).

The major effect of mild and moderate iron deficient anemia, in pregnant women, involves alterations in circulation. Anemia results in

decreased hemoglobin level accompanied with an increase in plasma volume, cardiac output and velocity of blood flow to maintain adequate peripheral tissue oxygenation. Other changes are decreased peripheral vascular resistance and shift in the hemoglobin-oxygen dissociation curve to the right, i.e. reduced affinity of oxygen for hemoglobin (McFee, 1973). Anemia usually occurs in late pregnancy coinciding with high fetal demand (Scott & Pritchard, 1967), and often results in increased incidence of complications. The incidence of toxemia is 2-5 fold greater in anemic pregnancies than in normal pregnancies (Chaudhuri, 1970; MacGregor, 1963). In severly anemic pregnancy inadequate placental perfusion and oxygenation results in intrauterine hypoxia. It is believed that the fetus tries to escape the hypoxic environment and precipitates premature termination of an anemic pregnancy (McFee, 1973). Another factor that affects anemic parturients is their inability to tolerate any appreciable blood loss without entering into cardiac failure, shock and death (Beischer et al., 1970).

Severe anemia is not a characteristic of a prosperous nation. However, moderate anemia is fairly common. The Nutrition Canada Survey (1975), on iron intake in pregnant women found 29% had inadequate iron intake and 5% were in the high risk category.

The bulk of fetal iron is obtained during the last trimester of pregnancy and the iron level increases as the pregnancy progresses to term. The placenta actively procures maternal iron and makes it available to the fetus. Approximately two-thirds of the fetal iron is incorporated into hemoglobin and the remaining one-third is stored in the liver as ferritin (Linder & Munro, 1973; Singla et al., 1979).

The effect of mild to moderate iron deficient anemia on the fetal development is not clear. Significant correlation (P$<$0.05) has been found between low birth weight (LBW) infants, and mothers deficient in iron at term (Bogden *et al.*, 1978 a, b; Whiteside *et al.*, 1968). However, fetal hair and cord serum iron level is normal in the LBW infants (Bogden *et al.*, 1978 a; Rios *et al.*, 1975; Hambidge & Baum, 1971; McFee, 1979). Low hemoglobin and serum ferritin levels were observed in other studies on LBW neonates born to anemic mothers. These children exhibit high TIBC and increased iron absorption (Sanchez-Madrid, 1975; Fenton *et al.*, 1977; Kelly *et al.*, 1978; Singla *et al.*, 1979).

Normal neonates are highly susceptible to anemia because of low postnatal intake, negative iron metabolic balance and high demand for growth. Precipitation of anemia in premature infants is greatly enhanced for they have low ferritin content and are in great need of iron supplementation (Saarinen, 1978).

An adequate fetal liver iron storage is extremely important for maintenance of postnatal growth, increasing number of red blood cells for the increasing blood volume, and for synthesis of heme enzymes. Heme enzymes, tryptophan oxygenase of liver (Linder, 1978), and leukocyte myeloperoxidase (Baggs & Miller, 1973), are both present in low concentrations at birth and iron deficiency results in decreased synthesis of myeloperoxidase (Weinberg, 1974). Decreased myeloperoxidase increases the neonate's susceptibility to infection (Linder, 1978).

Oski & Honig, (1978; Pollitt & Leibel, 1976) studied the effect of iron supplementation on behavior in anemic infants. Significant improvements in motor coordination, responsiveness, alertness and Bayley Mental Developmental Index were observed when compared to matched

unsupplemented anemic controls. Further research is being conducted to investigate the role of iron in these neurological disorders.

1.3.   Zinc

1.3.1. Distribution

An adult man is estimated to have 1.2-2.3 grams of zinc (Mikac-Devic, 1970).

Zinc is present in the erythrocytes, leukocytes, platelets and plasma. Almost all the zinc in the erythrocytes occurs in carbonic anhydrase (CA) and a small fraction is associated with other zinc-containing enzymes. Erythrocyte zinc accounts for 75-85% of total whole blood zinc content. Leukocytes account for only 3% of total blood zinc, but zinc concentration per cell is greater than in erythrocytes (Prasad, 1978). 30-40% of the plasma zinc is firmly bound to an α-macroglobulin, 60-70% is loosely bound to the albumin and 2-8% is bound to β-globulin and free amino acids (Parisi & Vallee, 1969; Prasad & Oberleas, 1970 b). The reported value for normal plasma is in the range of 1.12 ± 0.12 ug/mL and in serum 1.20 ± 0.20 ug/mL (Parisi & Vallee, 1969; Vallee, 1959). Serum zinc levels are 16% higher than plasma levels; 44% of it is contributed by disintegration of platelets during clotting, 39% from a slightly greater dilution of plasma and 4.0% from hemolysis (Foley, 1968). Plasma zinc has slight but definite circadian periodicity. Normal circadian rhythm does not change the values by more than 5-10% from the base value (Henkin, 1972; Guillard et al., 1979).

The plasma zinc levels decrease during pregnancy (0.60 ± 0.11 ug/mL at third trimester vs. 0.96 ± 0.13 ug/mL in nonpregnant women) and also with use of oral contraceptive agents (OCA); (0.65 ± 0.08 ug/mL in OCA

users vs. 0.96 ± 0.13 ug/ml in nonusers) (Halstead *et al.*, 1968).

Similar data have been presented by Henkin *et al.*, (1971 a);

Hess *et al.*, (1977 a, b), and Deeming & Weber, (1978). Hambidge &

Dreogmuller (1974) found decreases in serum zinc during pregnancy

but not in OCA users. A 30-50% decrease in plasma zinc is reported in

patients with trauma, inflammation (Beisel, 1976; Solomons, 1979)

and in patients with poor rates of healing (Pories *et al.*, 1976).

Decrease in blood zinc level is also observed in animals and humans

on zinc deficient diets (Mills, C. *et al.*, 1967; Wilkins *et al.*, 1972).

Table 1 lists most of the factors and mechanisms resulting in

hypozincemia.

The umbilical cord plasma zinc is twice that of the plasma zinc in

the mother at the third term of pregnancy, 0.83 ± 0.03 ug/mL in the cord

vs. 0.48 ± 0.03 ug/mL in the mother (Henkin *et al.*, 1971 a). The neonates

have approximately the same plasma zinc levels as the normal adult

(0.83 ± 0.03 ug/mL in cord vs. 0.90 ± 0.03 ug/mL in adults (Henkin *et al.*,

1973).

Zinc is also found in other body fluids: in sweat 1.15 ± 0.3 ug/mL

(Prasad *et al.*, 1963 a), and in saliva 0.08 ug/mL (Gregor & Sickles, 1979).

Urinary zinc excretion is very low, 0.5 to 0.8mg/day (Aamodt *et al.*, 1979;

Spencer *et al.*, 1979, 1976). The urinary excretion is increased 2-3

fold during protein catabolism such as starvation (Spencer *et al.*, 1976;

Fell *et al.*, 1973). In the neonates, the normal zinc excretion via the

kidney is 5 times that of the adult (Cavell & Widdowson, 1964).

The zinc content of human milk ranges from 1.3 to 3.9 ug/mL

(Cavell & Widdowson, 1964; Picciano & Guthrie, 1976; Johnson & Evans, 1978;

Kirksey *et al.*, 1979). The zinc content in multiparous and older women's

TABLE I Possible Causes of Conditioned Zinc Deficiency

Conditioning factors	Mechanisms
Dietary phytate, fiber, EDTA, polyphosphates, clay, and laundry starch	Chelation of zinc
Alcohol	Increased urinary excretion of zinc
Pancreatic insufficiency	Steatorrhea, lack of binding factor
Gastrectomy (partial or total)	Steatorrhea, achlorohydria
Diverticuli or blind loop	Steatorrhea, bacterial utilization
Intestinal mucosal disease	Steatorrhea, exudation, increased secretions, mucosal block
Cirrhosis of the liver	Increased urinary loss
Other liver diseases	Steatorrhea
Renal failure	Anorexia
Malabsorption Syndrome	Intestinal loss of zinc
Nephrotic Syndrome	Proteinuria
Renal tubular disease	Failure of tubular reabsorption of zinc
Dialysis	Removal of zinc in dialyzate
Hemolytic anemias	Urinary excretion of RBC zinc
Neoplastic disease	Anorexia, catabolism
Psoriasis	Loss of skin cells
Burns	Exudation, catabolism
Parasitic infection	Chronic blood loss, steatorrhea
Iatrogenic	
Antimetabolite drugs	Catabolism
Antianabolic drugs	Nonanabolism
Chelating drugs	Increased urinary excretion of zinc
Parenteral alimentation	Failure to add zinc to I.V. fluids
Surgical trauma	Catabolism
Genetic and congenital defects	
Hemolytic anemias	Urinary excretion of RBC zinc
Pancreatic defect	Lack of zinc binding factor (?)
Intestinal defect	Mucosal block
Renal tubular defect	Failure of tubular reabsorption of zinc
Diabetes mellitus	Increased urinary excretion of zinc
Congenital absence of the thymus gland	Unknown
Mongolism	Unknown
Acrodermatitis enteropathica	Possibly impaired intestinal absorption of zinc
Inflammation	
Infectious disease	Mobilization of zinc by LEM and subsequent urinary excretion
Collagen disease	Catabolism
Tissue necrosis	Catabolism
Pregnancy	Fetal uptake of zinc

Sandstead et al., 1976. (Reproduced with permission of Academic Press, N.Y.)

milk is greater than in the milk of the younger and primiparous women.
Diurnal variations are also observed in milk zinc concentration with
the highest concentration in the morning (Picciano & Guthrie, 1976).
No difference in milk zinc concentration is observed between women
who were on oral contraceptive agents (OCA) and those who were not
on OCA prior to pregnancy (Kirksey et al., 1979).

Body zinc has been assessed using urine, hair, plasma, serum,
whole blood and various other body tissues. The most practical,
convenient means of monitoring body zinc is by taking urine, hair or
blood samples. Normal urinary zinc excretion is very small and it
increases with hepatic and nephrotic disorders (Sandstead et al., 1976).
The hair zinc does not always reflect body zinc status and is most useful
in determining prolonged deficiency (Erten et al., 1978; Aggett & Harris,
1979; Solomons, 1979). Plasma or serum has been considered a good and
convenient index to monitor body zinc status (Walker & Kelleher, 1978;
Butrimovitz & Purdy, 1978). Since 60-70% of plasma zinc is bound to
albumin, plasma zinc alone is not an accurate index in patients with
hypoalbuminemia or protein-calorie malnutrition (Solomons, 1979).

1.3.2. Metabolism

Dietary zinc absorption in animals occurs throughout the small
intestine, but most rapidly in the duodenum and proximal jejunum
(Evans, 1976 b; Methfessel & Spencer, 1973). Zinc absorption in humans
must occur in the upper intestine since [67]zinc appears in the plasma
within 20 minutes of oral administration (Aamodt et al., 1979). Impaired
zinc absorption in patients with Crohn's disease (McClain et al., 1980;
Sturniolo et al., 1978), ileojejunal bypass (Anderssen et al., 1976;
Atkinson et al., 1978) and celiac sprue (Solomons et al., 1976) also

suggest that zinc absorption takes place in the upper G.I. tract.

The mechanism of absorption from the gut is not clear, but a hypothesis, based on the *in vitro* studies has been presented by Cousins, (1979). A portion of the dietary zinc is actively transported across the mucosal brush border into the intestinal cells (Kowarski *et al.*, 1974; Atherton *et al.*, 1979). It is not known if the transport carrier is common to all divalent cations or unique to zinc. The amount of zinc absorbed is dependent on the intestinal cell zinc content. The newly formed zinc "pool" within the intestinal cell, is either shunted into high molecular weight zinc-binding ligand (HMW-ZnBL) and metallothionein or is transferred to the plasma. Zinc is taken up from the basolateral membrane by transferrin and transported to the liver (Evans, 1976 b).

The absorption mechanism is closely linked to the homeostatic control of zinc metabolism (Cousins, 1979). In the zinc deficient animals the dietary zinc is absorbed at a high rate and is mostly bound to HMW-ZnBL with a significant portion being transferred to the plasma. In sufficient states, the rate of absorption is decreased and most of the intestinal cell zinc is bound to metallothionein and is rendered unavailable for absorption (Richards & Cousins, 1976, 1977). Metallo-thionein has been found to be an inducible, intracellular ligand and it has been proposed that body zinc status programs the rate and extent of zinc absorption, in part via changes in the concentration of intestinal metallothionein (Cousins, 1979; Aggett & Harris, 1979). Low molecular weight zinc-binding ligand (LMW-ZnBL), similar to HMW-ZnBL, has been isolated from rat intestinal cells and is believed to be involved with zinc translocation in the intestinal cells (Evans *et al.*, 1975). Similar LMW-ZnBL has been isolated from human and rat milk and is believed

to be essential for zinc absorption in the neonates (Hurley *et al.*, 1977).

Average zinc absorption in man ranges from 31-51% of the intake (Spencer *et al.*, 1976, 1979). The absorption of this metal is dramatically affected by the composition of the diet. The zinc content of plant and animal foods are similar (Aggett & Harris, 1979) but the bio-availability of zinc is greater with animal foods (O'Dell, 1969; Matseshe *et al.*, 1978; Solomons, 1979). The plant foods have a high percentage of phytates which bind to zinc and prevent its absorption (Becker & Hoekstra, 1971). Complexation of zinc also occurs with fiber, hemicellulose and cellulose resulting in 30% increase in fecal excretion (i.e. decreased absorption) of zinc (Reinhold *et al.*, 1976; Drews *et al.*, 1979). Divalent cations, cadmium and copper, decrease zinc absorption by competing for the binding sites in the gut. Calcium and phosphates also decrease zinc absorption by potentiating the complexing of the metal to the phytates present in the gut (Reinhold *et al.*, 1976; Spencer *et al.*, 1979; Ahokas *et al.*, 1980). Zinc absorption is also decreased in disease states affecting the G.I. tract, e.g. Crohn's disease (McClain *et al.*, 1980), and impaired protein synthesis (Aggett & Harris, 1979). In the neonates, the absorption of zinc is highly dependent on the availability of LMW-ZnBL in the diet. This ligand is present in the human breast milk but not in the cow's milk (Eckhert *et al.*, 1977), therefore, the infant's diet substantially determines its zinc status (Shaw, 1979).

The absorbed zinc is transported from the gut to the tissues bound to transferrin. The metal is incorporated at varying rates into different tissues. The bones, hair and nail have slow uptake and zinc in these tissues is not available for metabolic use (Hurley &

Swenerton, 1971). The spleen, pancreas and kidney have rapid uptake
and high turnover. The major organ in zinc metabolism is the liver.
The liver cytosol has an inducible zinc-binding protein, hepatic
metallothionein, which functions as temporary storage prior to its
utilization in essential functions (Cousins, 1979).

The main excretory route of zinc is the G.I. tract. The fecal
zinc represents unabsorbed zinc from the diet and intestinal cells
and the secretions into the G.I. tract (Aggett & Harris, 1979). The
urinary excretion of zinc is very low, 0.5mg/day to 0.8mg/day (Aamodt
et al., 1979; Spencer et al., 1976, 1979). Urinary excretion increases
2-3 fold during a period of high protein catabolism, such as starvation
and muscle loss (Spencer et al., 1976; Fell et al., 1973). Cirrhotic
and burn patients also exhibit increased urinary excretion of zinc
because of tissue destruction (Sullivan & Burch, 1976).

Use of oral contraceptive agents decreases plasma and serum zinc
concentrations (Prasad et al., 1975; Halstead et al., 1968). Hess et al.,
(1977 a) observed that serum zinc in OCA users on marginal zinc intake
declined by 47% when compared to 21% in nonusers on marginal zinc intake.
The urinary excretion declined 83% in OCA users and 62% in nonusers.
The fecal excretion is decreased, i.e. increased absorption by 40% in both
groups. The use of OCA influenced the response to the low-zinc diet and
the dramatic drop could be due to increased tissue uptake of zinc. However,
the endogenous zinc loss in OCA users and nonusers is the same, about
1.6mg/day (Hess et al., 1977 b). King et al., (1978) found no effect
of OCA on zinc absorption in the body and metabolic balance studies
(Margen & King, 1975) revealed no difference between OCA users and non-
users.

1.3.3.   Zinc Deficiency in Man

1.3.3.1. Growth, sexual maturation, appetite and taste

The first documented cases of zinc deficiency were described in late 1950's when Vallee *et al.*, (1957) reported decreased serum and hepatic zinc in cirrhotic patients. However, nutritional zinc deficiency was not documented until 1963 (Prasad *et al.*, 1963 a, b). Eleven young men, average ages of 21 years, were brought to Saadi Hospital, Shiraz, Iran (1960) with severe anemia. Other clinical symptoms were dwarfism, retarded skeletal maturation, hypogonadism, hepatosplenomegaly, rough and dry skin, mental lethargy and geophagia. Hookworm and schistosomiasis infections were not present. The nutritional history revealed that these patients ate only unleavened wheat bread and had negligible intake of animal protein. Laboratory results showed severe iron deficiency, normal liver function, and serum proteins, except for decreased alkaline phosphatase activity. No hypothyroidism or hypoadrenalism was found. Following institution of a well balanced diet for 12 months, anemia was alleviated, linear growth and sexual maturation resumed. The explanation for arrest of linear growth and gonadal maturation could not be explained by iron deficiency alone (Prasad *et al.*, 1961). The clinical picture in zinc deficient animals is growth failure, testicular atrophy, parakeratosis and decreased serum alkaline phosphatase activity (Tucker & Salmon, 1955; Vallee, 1959). Prasad *et al.*, (1963 a) suspected these patients to be zinc deficient because these patients had a high phytate intake which was known to decrease iron and zinc absorption (Vallee, 1959), and the clinical picture was similar to those observed in zinc deficient animals. Subsequently in 1963 b, Prasad *et al.*, described a group of young

Egyptian men with the same clinical profile. Their plasma, sweat, hair and red blood cell zinc levels were decreased, they had enhanced turnover rate of plasma [65]zinc and decreased excretion of [65]zinc in stool and urine in comparison to the controls. Their magnesium, copper and vitamin profiles were within the normal range. The patients on adequate protein and zinc diet responded better than those on adequate protein diet without zinc (Prasad *et al.*, 1963 a; Sandstead *et al.*, 1967).

Coble *et al.*, (1966) studied the same group of patients. The plasma zinc in normals, Prasad's patients and a new group of "hypogonadal dwarfs" were within the same range and therefore, Coble concluded that these patients had delayed sexual maturation rather than zinc deficiency. Prasad *et al.*, (1976) compared Coble's "normals" to a matched group from Cairo and North America and found that Coble's normals exhibited poor growth and sexual maturation and therefore, concluded that Coble's "normals" were not normal. Since 1966, many investigators have carried out controlled blind studies in various parts of the world: in the Middle East (Ronaghy *et al.*, 1969, 1974; Halstead *et al.*, 1972, 1974); in Australia (Holt *et al.*, 1980), and in the United States of America (Erten *et al.*, 1978; Butrimotvitz & Purdy, 1978; Hambidge *et al.*, 1972, 1976; Walravens & Hambidge, 1976) and have observed that zinc is a limiting, essential nutrient for human growth and development.

Growth retardation and impaired learning ability have been observed in very young zinc deficient rats (Halas *et al.*, 1977; Peters, 1978), and Rhesus monkeys (Sandstead *et al.*, 1978). Recent investigations into infant's and young children's nutrition indicate that hypozincemia is quite common in low-income families and to a certain extent in middle and high income groups. Hambidge *et al.*, (1976) evaluated hair and

plasma zinc in 74 low income pre-schoolers in Denver, Colorado.

These children were selected on the basis of their low height

percentile. Two-thirds of the children had a 26% decrease in plasma

and hair zinc, suggesting inadequate zinc nutrition. Another

study (Hambidge *et al.*, 1972) evaluated 338 high and middle income

normal subjects between ages 0-40 years. Ten out of 132 children

between the ages four and seventeen years had hair zinc concentrations

below the mean ± 2 standard error suggesting a zinc deficient status.

Eight of these children had a history of poor appetite, had below

average growth and also exhibited impaired taste acuity. Improved taste

acuity and increased hair zinc was observed in zinc deficient children

supplemented with zinc. Hair zinc in children with growth hormone

deficiency, panhypopituitarism, familial and primordial short stature

were within normal range, therefore, the above children displayed

growth retardation due to nutritional zinc deficiency.

The probable mechanism of action of zinc in growth retardation

is impaired nucleic acid and protein synthesis resulting in impaired

cell division and growth (Prasad & Oberleas, 1974). Prasad and his

colleague monitored thymidine kinase (TK) activity in rapidly regenerating

tissue of rat liver, at 6, 13, and 17 days after onset of zinc

deficient diet. The TK activity was 70% of pair-fed controls by the

6th day and 13th day and it was not measurable by the 17th day. Thymidine

kinase is essential for DNA synthesis, a prerequisite of cell division.

In long-term deficiency, the utilization of amino acids in protein

synthesis is also impaired. This could be due to decreased synthesis

of ribonucleic acid (RNA) by the RNA polymerase or increased degradation

by the ribonuclease (RNA ase) (Hsu *et al.*, 1969). The RNAse activity,

and the enzyme in RNA degradation is increased in zinc deficient rat testes while the DNA-dependent - RNA polymerase activity is decreased (Somers & Underwood, 1969 a). In experimental mild zinc deficiency in humans (Prasad *et al.*, 1978), the plasma ribonuclease (RNA ase) activity is increased by 200%, plasma ammonia is increased and weight loss is observed, suggesting increased degradation of RNA.

Impaired skeletal growth observed in zinc deficiency is probably due to decreased bone alkaline phosphatase activity (50-60% decrease in rats) and decreased zinc concentration in the endochondral growth site. The exact mechanism involved in retarding skeletal maturation is not known but zinc supplementation has been found to have a dramatic beneficial response (Kirchgessner *et al.*, 1977; Haumont & McLean, 1966; Vincent, 1963; Hurley & Everson, 1963). In a double-blind study, with 12-14 year old boys Ronaghy *et al.*, (1969) observed increased thickness of the metacarpal bones in response to zinc supplementation. Analysis of sponge connective tissues in zinc deficient humans has revealed a decreased content in total protein, collagen and ribonucleic acid and decreased activity of TK (Prasad *et al.*, 1978).

In a recent study, zinc deficiency has been associated with low serum growth hormone. In hypophysectomized zinc deficient rats, both zinc and growth hormone were needed for optimum growth and, therefore, zinc and growth hormone may be complementary, but independent growth factors (Prasad *et al.*, 1969; Root *et al.*, 1979).

Growth inhibition also results partly from impaired appetite, i.e. anorexia (Prasad *et al.*, 1963 b; Sandstead *et al.*, 1978; Chesters & Quaterman, 1970). In zinc deficiency, both animals and humans exhibit inanition but the biochemical cause is not known. Hypogeusia, i.e.

decreased taste acuity, has also been observed in zinc deficient children and adults (Henkin *et al.*, 1971 b; Ronaghy *et al.*, 1974) and zinc supplementation enhances their taste responsiveness. The biochemical lesion is believed to be in the synthesis of a zinc metalloenzyme, gustin. Gustin is postulated to be essential for taste (Henkin *et al.*, 1975), and hypogeusia may play a role in precipitating anorexia.

Zinc deficient male animals have retarded sexual maturation and testicular atrophy (Millar *et al.*, 1958). Injection of testosterone increases the growth rate and maturation of secondary sex characteristics in the rats, but has no effect on spermatogenesis and testicular atrophy. However, zinc supplementation improves spermatogenesis and reverses the testicular atrophy. The availability of sufficient zinc for incorporation of high concentrations into sperm seems to be essential for the maintenance of spermatogenesis and the survival of the germinal epithelium (Millar *et al.*, 1960). In sexually mature zinc deficient rats, the testosterone levels are low (77% of pair-fed control) and serum leutinizing hormone (LH) is increased. In sexually immature zinc deficient rats, follicle stimulating hormone (FSH) is also increased, reflecting disruption of the Sertoli cells. The FSH and LH secretory responsiveness to hypothalamic LH-RH is greatly enhanced (Root *et al.*, 1979). The exact action of zinc on the gonadal hypothalamic-pituitary axis is not known, but there is no doubt that zinc does have a profound effect on testicular function (Root *et al.*, 1979). In the females, zinc deficiency disrupts all phases of reproduction and is reversible with zinc supplementation. Early zinc deficiency in rats and Rhesus monkeys impairs the estrous cycle and conception. Zinc deficiency during pregnancy results in a high incidence of fetal abnormalities and maternal deaths (Sandstead *et al.*, 1978;

Hurley, 1976).

1.3.3.2.   Keratogenesis

Parakeratotic lesions are characterised by hyperkeratinization
with failure of complete nuclear degeneration of the epithelial cells
and are found in most zinc deficient animals   (Todd *et al.*, 1934;
Tucker & Salmon, 1955; Barney *et al.*, 1967).   Parakeratotic lesions
in man have not been widely reported.   Prasad *et al.*, (1963 b)
observed dry and brittle skin in the zinc deficient Egyptian boys.
Facial eczema and thin, glazed and fragile skin have been observed in
severely zinc deficient infants and elderly people   (MacMahon *et al.*,
1968; Arakawa *et al.*, 1976).   Acrodermatitis enteropathica (AE), an
autosomal recessive inherited disease, is also characterized by skin
lesions.   These lesions occur mainly on the extremities and around
the body orifices.   The AE patients respond well to zinc supplementation.
Klingberg *et al.*, (1976) reported an occurrence of true parakeratosis
in a young man on penicillamine treatment for Wilson's disease.   The
patient had developed zinc deficiency during his treatment with the
chelating agent and it had manifested itself as parakeratosis, alopecia
and bilateral centrocorneal scotoma.   Cortisone and various vitamin
therapy did not improve these symptoms.   Due to similarities between the
patient's symptoms and swine parakeratosis, the patient was supplemented
with zinc and all his symptoms were alleviated.   The patient had 50% of
normal plasma zinc before onset of treatment.   The levels were within
normal limits within one year of supplementation.

The parakeratotic skin disorder observed in the zinc deficient
animals is believed to be due to abnormal nucleic acid and keratin
metabolism.   In zinc deficiency, there is increased urinary excretion

of sulfur (Somers & Underwood, 1969 b), and 30% decrease in

incorporation of $1-^{14}C$ cysteine in the liver and the kidney proteins

(Hsu, 1976). The major portion of the epidermal layer of skin is made

up of albuminoid proteins called keratins which have a high content

of cysteine. In the skin of zinc deficient rats there is a 65% decrease

in incorporation of cysteine into the skin protein, therefore, zinc

deficiency may be the cause of impaired keratin synthesis resulting

in parakeratotic lesions.

1.3.3.3. Vitamin Metabolism

The bilateral centrocorneal scotoma in Klineberg's patient (1976)

was thought to be due to Vitamin A deficiency since the patient did have

chemical hypovitaminosis A. Supplementation with 500 U/day of Vitamin A

did not increase the plasma Vitamin A level or clear the corneal lesion.

However, shortly after zinc supplementation, normal plasma Vitamin A

level was measured and marked corneal improvement was observed. Animal

studies have shown that in zinc deficiency hepatic synthesis of

retinal binding protein, transport protein for Vitamin A, is decreased

(Smith,, *et al.*, 1973). Huber & Gershoff (1973 a) investigated

and found decreased activity of retinal reductase and retinal

dehydrogenase in the retina of zinc deficient rats. These studies

suggest that mobilization of Vitamin A from the liver and retinol

metabolism of the vitamin is impaired by zinc deficiency. A similar

decrease in Vitamin A mobilization has been observed in zinc

deficient pregnant dams (Duncan & Hurley, 1978). Morrison, *et al.*,

(1978) studied Vitamin A and zinc interaction in cirrhotics with

abnormal dark adaptation. He found that zinc supplementation was

essential to attain the normal threshold of dark adaptation.

Carbonic anhydrase, a zinc metalloenzyme, is present in high concentrations in the retina (O'Rourke *et al.*, 1972). The significance of zinc deficiency on retinal carbonic anhydrase activity is not yet apparent, but it has been postulated that zinc deficiency may result in eye lesions due to impaired enzyme activity (Klingberg *et al.*, 1976). Patients with acrodermatitis enteropathica, an inherited disease of zinc deficiency, also have optic atrophy and subacute myelo-optic neuropathy and malabsorption resulting in zinc deficiency which is believed to be the causative factor (Sturtevant, 1980).

1.3.3.4 Wound healing

Strain *et al.*, (1960) observed that burned and wounded rats on a zinc contaminated diet healed faster than the controls. The healing rate was improved by as much as 40% of the control and the beneficial effect was only evident 12-13 days after supplementation. Similar response has been observed in zinc supplemented rats with ulcers, burns, and surgical incisions (Sandstead & Shephard, 1968; Oberleas *et al.*, 1971; Elias & Chvapil, 1973).

Zinc deficiency in the hospital population occurs at a high frequency caused by a combination of nutritional and pathological conditions (Pories *et al.*, 1976). Based on animal studies, Pories *et al.*, (1967 a, b) investigated the effectiveness of zinc in healing in humans operated for pilonidal sinuses. The medicated patients healed 34-36 days faster than the controls, and like the animals, it took 12-13 days for the beneficial effect to become evident. The beneficial effect of zinc in patients with cutaneous ulcers (Pories *et al.*, 1971; Serjeant *et al.*, 1970) and burns

(Larson, 1970; Pories *et al.*, 1976) has also been reported. Increased zinc in diets of zinc sufficient patients does not improve the healing rate. Flynn *et al.*, (1973) demonstrated a correlation between decreased healing rates and prolonged and chronic corticosteroid therapy. Increased corticosteroids decreases the mobilization of zinc from the liver (Lewis *et al.*, 1973) and oral zinc supplementation in such patients improved the healing rate.

Wound healing is an active, complex biological process that requires an intact protein sythesis machinery. Zinc deficiency has been associated with abnormal nucleic and protein synthesis (Wacker, 1976; Sandstead & Rinaldi, 1969). Savlov *et al.*, (1962) found 50% increase in ^{65}zinc uptake at the site of tissue injury. Collagen, the main fibrous protein, is largely responsible for the tensile strength in healing wounds. McClain *et al.*, (1973); Elias & Chvapil, (1973) found no definitive role of zinc in collagen synthesis, but increased nucleic acid synthesis precedes the deposition of collagen. McClain *et al.*, (1973); Fernandez-Madrid *et al.*, (1973, 1976) have found decreased uptake of ^{14}C-thymidine in zinc deficient wounded tissue and they postulate that the metal plays a role in the elementary step necessary for cellular proliferation and protein synthesis.

1.3.3.5. Other effects of zinc deficiency in animals and man

Zinc deficient animals have an irregular and delayed response to intravenous glucose load but they respond normally to an oral glucose load test (Quaterman *et al.*, 1966; Hendricks & Mahoney, 1972). Oral glucose administration is believed to be more efficient in releasing insulin from the pancreas (Hendricks & Mahoney, 1972) possibly by the

release of a "factor" by the duodenal mucosa which aids insulin

release (Fasel *et al.*, 1970). A poor intravenous glucose tolerance

test has been hypothesized to be due to either poor secretion or

increased degradation of insulin (Hendricks & Mahoney, 1972). *In vitro*

studies with zinc sufficient and deficient pancreas have shown that

the release of a protein with insulin-like activity by the deficient

pancreas is decreased by 55% and supplementation increases the release

to near normal levels (Huber & Gershoff, 1973 b). Zinc has been

established as an integral component of insulin (Maske, 1957).

Autoradiographic studies have shown that loss of ^{65}zinc from the rat

pancreas is different for acinar and islet tissues (McIssac, 1955).

The acinar cells lose radioactivity very rapidly while the islet cells

have a high and fairly constant zinc concentration for 92 hours. These

studies indicate that zinc accumulates in the insulin-storing granules

of the islets and the intracellular zinc concentration appears related

to the functional state of the islet. The mechanism of zinc uptake by

the cells is passive, facilitated diffusion (Ludvigsen *et al.*, 1979).

Koo & Turk (1977) studied the ultrastructure of pancreatic acinar

cells and found that zinc deficiency resulted in marked cellular

alterations: a reduction in zymogen granules, basal accumulation of

lipid droplets, prominent lysosome bodies, abnormal endoplasmic

reticulum and Golgi apparatus. The knowledge regarding zinc and

insulin metabolism is not clearly defined but a 50% decrease in zinc

and a 25% decrease in insulin has been observed in the diabetic

pancreas (Scott & Fischer, 1938). Martin-Mateo *et al.*, (1978) found

increased (30%) serum zinc in diabetic patients and suggested that

there is decreased storage or a chronic hypersecretion of insulin in

hyperglycemic patients. Pidduck & Wren (1970) also found increased

urinary loss of zinc in the diabetics by some unknown mechanism.

Zinc deficiency does not result in an overall decrease in activity

of all zinc metalloenzymes. The sensitivity of the enzyme is

dependent on both the zinc-ligand affinity and their turnover rates in

the affected tissues (Prasad & Oberleas, 1970 a). Zinc deficiency

results in a dramatic decrease in activity of serum alkaline phosphatase (48%),

bone alkaline phosphatase (21%), intestinal alkaline phosphatase (20%),

pancreatic carboxypeptidase A (24%) and B (50%) and alcohol dehydrogenase

(28%). Carbonic anhydrase, glutamic, lactic and malic dehydrogenases;

aldolases and phosphokinases are also zinc-metalloenzymes but no definite

decrease in activity due to zinc deficiencies have been demonstrated

(Kirchgessner et al., 1976). Despite the decrease in activity of a

variety of enzymes no clear cut relationship has been demonstrated

between the varied anomalies of zinc deficiency and enzyme activity

(Prasad et al., 1969). However, the rapidity with which biochemical

changes arise in response to zinc depletion and its disappearance upon

repletion helps to identify some of the primary sites of metabolic

function of zinc (Mills et al., 1969).

Another interesting aspect of zinc metabolism in the body is the

marked depression of plasma zinc in response to acute stress. The

initial depression in plasma zinc is due to an abrupt redistribution

from blood to liver in response to a hormone-like substance termed

leukocyte endogenous mediator (LEM). LEM stimulates the liver to

synthesize a large number of acute phase reactant proteins and zinc

may function as a component or co-factor in synthesis of acute phase

proteins (Beisel, 1976). True hypozincemia, due to negative zinc

balance in the body, develops later in the disease process. These factors include a lessening of dietary intake due to anorexia and loss of zinc from excessive sweating, diarrhea and excretion via the kidney.

Zinc has also been found to be associated with cell membranes and is believed to regulate the function of macrophages, platelets and lymphocytes (Chvapil *et al.*, 1976). It has also been hypothesized to be a factor in the etiology of coronary heart disease (Klevay, 1974, 1975). Klevay's hypothesis is that an increased ratio of zinc and copper results in hypercholesterolemia and increased mortality due to coronary heart disease. Increased zinc and copper have been observed in diabetics and they do have increased incidence of arteriosclerosis (Martin-Mateo *et al.*, 1978).

1.3.4. Pregnancy and early postnatal development

1.3.4.1. Animals

The effects of zinc deficiency in female rats are decreased food intake, weight loss, hemoconcentration and difficult parturition, i.e. prolonged labour and excessive blood loss. Morbidity at or immediately after parturition is exhibited by 38% of the dams (Apgar, 1973, 1977; Hurley *et al.*, 1971). To control the effect of inanition on fetal development, all zinc deficiency experiments are carried out with pair-fed and ad-libitum fed controls.

A high incidence of congenital malformations results from maternal zinc deficiency during pregnancy. Severe zinc deficiency during gestation (day 0 to 21) in normal healthy female rats results in resorption of 54% of the implantation sites, decrease in birth weight (by 50%) of full term young, and 98% of the live births exhibit gross congenital abnormalities of skeletal and all soft tissues (Hurley *et al.*, 1971; Mills *et al.*, 1969;

Warkany & Petering, 1972). Transitional zinc deficiencies in pregnant rats are also tertogenic. Zinc deficiency during days 6 to 14 of the rats' gestational period results in an array of anomalies in 46% of the live births, decrease in their birth weight and also failure to thrive. Deficiency for the first 10 days of gestation results in malformation in 22% of the full term fetuses (Hurley et al., 1971). Low zinc intake between day 10 and 12 of gestation also results in a small but significant percentage of malformed young (Warkany & Petering, 1972).

In mild zinc deficiencies, the fetal rats display intrauterine growth retardation, poor postnatal survival, decreased brain and liver size and weight and marginally affected placenta (McKenzie et al., 1975). In another study with mild zinc deficiency in rats, the fetuses displayed severe abnormalities of long bones (in 23% of the fetuses), ribs (in 59%) and vertebrae (in 50% of live births). The litter size was the same as the zinc sufficient dams (Hickory et al., 1979). Fosmire et al., (1977) studied the maternal and fetal response to various suboptimal levels (1, 2, 3, 5, 11, 25mg/L) of zinc intakes. The food consumption and weight gain of the dams were related to the level of zinc supplementation and this effect was most evident after day 18, which coincided with increased fetal zinc accumulation. An increase in fetal demand for zinc appears to precipitate a more severe state of deficiency. The fetal weight gains were also related to the level of zinc supplementation but their tissue zinc concentrations were independent of maternal zinc intake. The uniformity of tissue zinc concentration in pups of dams on 2, 3, 5, 11mg/L zinc suggest that the fetus has priority for the available zinc. Zinc repletion in late pregnancy (day 18) results in an increase in fetal and maternal weight gain and an increase in maternal spleen and mammary gland weight. The dams also

exhibit increased tolerance for stress of parturition (Apgar, 1973, 1977).

Zinc deficiency during the suckling period also has a very deleterious effect on development of the young. Suckling pups on zinc deficient dams have poor weight gain, decreased DNA content in the brain (50% of pair-fed control) and liver (33% of pair-fed); decreased (25%) proteins in both of these organs and decreased total brain lipids (Sandstead et al., 1972; Fosmire et al., 1976). Postnatal zinc deficiency does not result in an equally generalized reduction of DNA synthesis. The brain is more vulnerable to such deficiency than any other organ. Zinc deficiency results in 30-50% decrease in cytoplasmic, nucleoplasmic and chromosomal nonhistone proteins in liver and in brain. The synthesis of histone proteins is decreased by 50-60% in the brain of deficient animals indicating impaired cell division and impaired microneuronal proliferation (Duerre et al., 1977; Mckenzie et al., 1975; Eckhert & Hurley, 1977). The importance of zinc can also be observed in pregnant rats with high cadmium intake. Since zinc and cadmium compete for absorption sites in the gut (Spencer et al., 1976), the presence of high zinc in the diet decreases the uptake of cadmium and alleviates the growth retarding effects of the toxic levels of cadmium (Ahokas et al., 1980).

An interesting aspect of zinc deficiency is the rapidity with which it occurs (Swenerton & Hurley, 1968; Hurley et al., 1971). The rapid effect of zinc deficiency arises from the need for a constant source of zinc to maintain the plasma levels. The plasma zinc levels in dams on a 24 hour regime of zinc deficient diet are 40% of the controls (Dreosti et al., 1968). This fast change is brought about by the lack of mobilization of zinc from maternal stores. Bone zinc in deficient postpartum rats is the same as the nonpregnant rats, indicating that the pregnant rats do not mobilize bone zinc

to meet the need for normal fetal development. Pregnant rats on calcium
and zinc deficient diets do not diplay any of the fetal teratogenic effects
seen in zinc deficient animals. The bone resorption to meet calcium need
increased the availability of the bone zinc which normally is unavailable
for mobilization (Hurley & Swenerton, 1971; Hurley & Tao, 1972).

The present evidence suggests that impaired nucleic acid synthesis
causes abnormal embryonic development. DNA synthesis was studied by
measuring the uptake of tritiated thymidine (^{3}H-thymidine). The uptake
of ^{3}H-thymidine was 70-75% of normal in the zinc deficient embryos at
12 days of gestation, suggesting that DNA synthesis is depressed (Swenerton
et al., 1969). These results are consistent with work of other investigators
with other systems (Eckhert & Hurley, 1977; Mckenzie et al., 1975; Vojnik
& Hurley, 1977; Duerre et al., 1977; Fosmire et al., 1976) indicating that
zinc is essential for DNA synthesis. Enzyme studies have shown that thymidine
kinase activity in all tissues is greatly depressed (Dreosti & Hurley, 1975).
Other effects noted are decreased synthesis of lung lecithin and phospha-
tidylethanolamine in zinc deficient fetuses (Vojnik & Hurley, 1977).

Behavioral studies on mildly zinc deficient rats (Caldwell et al.,
1976) have shown that the animals are lethargic, have impaired learning
ability and increased level of emotionality. The dams have impaired
maternal behavior, i.e. failure to sever and consume the umbilicus, to
clean and nurse the pups and also inability to retrieve the stray pups
and build nests. The pups also displayed impaired behavior (Caldwell et al.,
1976). Halas et al., (1976, 1977) found that prenatal zinc malnutrition
in rats had different effects on male and female progeny. The male
progeny had impaired ability to avoid shock and were less aggressive than
the male pair-fed pups at 75 days of age. The female offspring display

enhanced aggression at all ages but had no impairment in their learning ability or avoiding shock (Halas *et al.*, 1976, 1977). Peters (1977) reported increased aggression and impaired affiliation behavior in zinc deficient male adult rats. Sandstead *et al.*, (1978) studied the maternal and offspring behavior in zinc deprived Rhesus monkeys. The dams refused to care for their infants and the infants of zinc deficient dams played and explored less than the control infants. They also associated with their mothers for a greater percentage of the time and were less active. The behavioral abnormalities found in the infants of the zinc deprived dams may have been the result of injury to the fetal brain during rapid intrauterine growth and differentiation. Sandstead postulates that human infants, born to zinc deficient mothers may suffer from nutritional injury during the critical developmental period and have associated behavioral problems.

1.3.4.2. Human

The serum and plasma zinc concentration decreases during pregnancy (Halstead *et al.*, 1968; Hambidge & Droegmuller, 1974; Jameson, 1976). It is not known whether the decrease is purely physiological or an expression of a deficiency state implying a risk to mother or child (Prasad & Oberleas, 1970 a; Hambidge & Droegmuller, 1974). The administration of contraceptive steroids has been reported to decrease the plasma and hair zinc levels in some studies (Halstead *et al.*, 1968; Deemings & Weber, 1978) but not in other studies (Hambidge & Droegmuller, 1974), therefore, it is not certain whether the estrogen or the progesterone decreases body zinc.

An indepth study by Jameson (1976) has revealed that women with low serum zinc, 0.903 ± 0.13 ug/mL vs. 1.04 ± 0.12 ug/mL (P 0.01) in normal

pregnancies, at 14 weeks of gestation have pre- and postmature infants, inefficient labor and atonic bleeding. Women with threatened abortions also have low zinc levels. Six out of the ten malformed infants were born to mothers with hypozincemia. Coeliac patients are known to have malabsorption and low serum zinc (Solomons *et al.*, 1976) and seven coeliac women in Jameson's study showed long-standing infertility and two of them showed secondary infertility after giving birth to malformed infants by abnormal delivery. Improved taste acuity was observed in zinc deficient pregnant women supplemented with zinc and only one of seven treated patients gave birth to immature twins while four infants of 13 untreated mothers were dysmature. The treated group had shorter labor and very small blood loss while six of the untreated group had severe hemorrhage (blood loss >1000 mL) with signs of uterine atony. The importance of zinc in human reproduction is further supported by the very high incidence of low birth weight (<2500 g) and congenital malformations -anencephaly (Neldner, 1974), achondroplastic dwarfism (Epstein & Velder, 1960), spontaneous abortion (Verberg *et al.*, 1974) in patients partially treated for the disease, acrodermatitis enteropathica. Human achondroplasia is a rare inherited disorder and 80% of the cases are results of new mutation. Normal parents of achondroplastic children have significantly reduced zinc in their hair (130 ± 50 ug/g vs. 186 ± 40 ug/g in normals) suggesting that zinc deficiency may be a contributing factor in the pathogenesis of this mutation (Collipp *et al.*, 1979).

Amniotic fluid bacterial infection in late pregnancy results in congenital pneumonia. Amniotic fluid analysis of such patients revealed decreased bacteriocidal activity and low zinc concentrations (Schlievert *et al.*, Tafari *et al.*, 1977; Applebaum *et al.*, 1979). *In vitro* zinc

supplementation to amniotic fluid medium improved the bacteriocidal activity of the fluid. Tafari *et al.*, (1977) supplemented the women with zinc but observed no change in their amniotic fluid bacteriocidal activity. Failure to observe changes in the bacteriocidal activity could be due to inadequate zinc supplementation, since the zinc level in the amniotic fluid was lower than amniotic fluid of well nourished pregnancies and it was also lower than inhibitory levels required in *in vitro* reversion studies (Applebaum *et al.*, 1979). Kynast *et al.*, (1978, 1979 ; Chez *et al.*, 1978) found that amniotic fluid zinc level increased with increasing fetal gestational age in late pregnancy and distinctly low zinc levels were associated with intrauterine growth retardation. Shearer *et al.*, (1979) found no correlation between gestational age, high risk score, and amniotic zinc levels at 13-19 weeks of pregnancy.

The mechanism by which zinc moves across the placenta to the fetus is not known. Total and protein bound zinc concentrations in umbilical sera at delivery are greater than in the maternal sera, but the "free" zinc is greater in maternal sera than in the cord sera (Henkin *et al.*, 1971 a). Henkin and his colleagues (1971 a) suggest that a passive transfer mechanism is involved in transplacental zinc flux. Human placental zinc concentration, approximately 1.0 ug/100 g wet weight, is also greater than the maternal serum concentration at term, approximately 0.56-1.00 ug/mL (Alexiou *et al.*, 1977).

Between 28 and 36 weeks of gestation the human fetus accumulates zinc at approximately 30 ug/kg/day (Dauncey *et al.*, 1977). At term, the fetal body has about 60 mg of zinc, about one-quarter of which is in the liver and one-third is in the bones (Widdowson, 1974). The total plasma Zn

level 0.83 ug/mL, is within the adult normal range, 0.90 ± 0.03 ug/mL

(Henkin *et al.*, 1971 a). The plasma zinc decreases to less than normal,

0.65 ± 0.05 ug/mL by five months, before returning to normal adult levels

within 12 months (Henkin *et al.*, 1973). The mechanism for decreased

plasma zinc is an interaction between zinc intake, gestational maturation

and metabolism (Sann *et al.*, 1980). The human milk zinc level ranges from

1.40-3.95 ug/mL and its content is greatest in the morning. Zinc is also

higher in the milk of multiparous women than in the milk of primigravid

women (Picciano & Guthrie, 1976). The zinc level of human breast milk is

greater than the zinc levels in either the processed cow's milk or in the

formulas. (Johnson & Evans, 1978; Hambidge *et al.*, 1979). The zinc content

of breast milk starts to decline after the 5th day of lactation and by the

3rd month it is decreased by 40% (Vaughan *et al.*, 1977). The absorption

of zinc by the neonate is dependent not only on the availability of zinc

but also on the presence of low-molecular weight binding (LMW-BL) protein.

LMW-BL is present in large quantities in human milk but not in cow's milk

(Eckhert *et al.*,1977). Bogden *et al.*, (1978 a, b ; Hambidge & Baum, 1971 ;

Sann *et al.*, 1980 ; Gibson & deWolfe, 1979) found no difference in hair

and plasma zinc concentration between normal neonates and small-for-

gestational age (SGA) neonates. The SGA infants maintained a positive zinc

balance and seemed to be attempting catch-up growth (Dauncey *et al.*, 1977).

The preterm infants have been found to have low zinc concentration in their

femur bone (McIntosh *et al.*, 1974) and also have a negative zinc balance for

up to 60 days postnatally (Sann *et al.*, 1980; Dauncey *et al.*, 1977). The

importance of zinc nutrition is most evident in premature infants on

parenteral nutrition (Michie & Wirth, 1978; Arakawa *et al.*, 1976;

Sivasubramanian & Henkin, 1978), which is known to be deficient in zinc

(Hauer & Kaminski, 1978). These infants display retarded growth rates, behavioral and dermatological changes typical of zinc deficiency.

The importance of zinc in postnatal development was demonstrated in another study by Walravens & Hambidge, (1976). They monitored growth in 34 normal neonates on zinc supplemented and unsupplemented cow's milk. Anthropometric measurements at 6 months, were significantly different (P 0.05) between the two groups. Zinc supplementation is correcting an underlying deficiency state.

Low maternal zinc intake has been reported in pregnant American women of Mexican descent (Hunt et al., 1979), Egyptian and Iranian women (Sever & Emmanuel, 1973). Sever also noted a high frequency of central nervous disorders and malformations but no relationship has been established between reduced zinc intake and high frequency of these disorders.

Acrodermatitis enteropathica (AE), a rare and usually fatal disorder of zinc absorption offers a good model to study the effect of zinc deficiency, both in utero and postnatally. The clinical features are hypozincemia, eczematous skin lesion, alopecia, diarrhea, impaired growth and mental disturbances (Moynahan, 1974; Lombeck & Bremer, 1977). These symptoms first appear shortly after weaning, and subside when human milk, not bovine milk, is fed. It can also be alleviated by oral zinc therapy. The low molecular weight zinc-binding ligands in human milk, which enhance zinc absorption are thought to be responsible for the curative effect of human milk. Pregnancies in AE females, if not adequately treated with diidohydroxyquin, results in abnormal fetal development, achondroplastic dwarfism. Diiodohydroxyquin, an 8-hydroxy-quinolone derivative, alleviates the symptoms of AE, possibly by increased intestinal absorption of zinc (Verberg et al., 1974). Human milk is also beneficial in alleviation of the symptoms and

recent studies have shown that it contains LMW-ZnBL which has been characterized as picolinic acid (Evans & Johnson, 1980).

1.4.   Copper

1.4.1. Distribution

Total body copper for a 70 kg man is 70-80 mg (Cartwright & Wintrobe, 1964; Sass-Korstak, 1965). One-third of the body copper is in the liver and brain combined, one-third in the musculature and the remaining third is distributed in other tissues. The liver content is related to its function as a storage organ and the only site for synthesis and release of the most abundant cupro-enzyme, ceruloplasmin. The liver Cu content is sensitive to diet, age and disease. The human newborn has 6 to 10 times higher liver copper than the adult, i.e. 230 ug/mg in neonates vs. 35 ug/mg in adults (Bruckmann & Zondek, 1939) and it decreases in transition from infancy to early years of life (Mason, 1979). Subnormal liver copper is found in animals suffering from copper deficiency and the level increases upon supplementation: 153 ug/mg in normal, 5.7 ug/mg in deficient and 136 ug/mg after supplementation (Allcroft et al., 1959). Abnormally high levels of liver copper are found in biliary tract disease and hepatitis and very low levels in Menke's kinky hair syndrome (Gubler et al., 1957).

The brain has an uneven distribution of copper. It is highest in the grey matter (33.0 ug/g vs. 23.3 ug/g in the white matter). There are also regional differences with cerebellum (29.2 ug/g) and basal ganglia (24.6 ug/g) having a higher concentration than the brain stem (8.0 ug/g) and cerebral cortex (14.9 ug/g) (Cartwright & Wintrobe, 1964).

In adults 50% of the blood copper is in the red blood cells (RBC). Erythrocuprein contains 60% of the RBC copper and the remainder is loosely bound to unidentified proteins (Wintrobe et al., 1953; Cartwright &

Wintrobe, 1964; Prasad, 1978). Normal RBC copper levels (1.1 ug/mL)
have been reported in Menke's disease (Williams, D. *et al.*, 1977) and in
pregnancy (Scheinberg *et al.*, 1954).

The plasma copper is found in two forms - one firmly bound to
ceruloplasmin (80-90%) and the other reversibly bound to albumin and
amino acids (Evans, 1973). The albumin and amino acid bound copper is
believed to be the labile pool (Wintrobe *et al.*, 1953). Plasma cupro-
enzymes, cytochrome oxidases and monoamine oxidase, contribute about
1-2% to the nonlabile plasma copper (Sass-Kortsak, 1965). Ceruloplasmin,
an a-globulin with molecular weight of 151,000 D, is only synthesized in
the liver. It is believed to function as ferroxidase, i.e. oxidizes the
ferrous ion for transport via transferrin and is sensitive to hormonal
stimuli. Estrogen, adenocorticotrophic hormone, corticosteriods and
adrenal medullary hormones increase ceruloplasmin synthesis
(Evans, 1973). Plasma copper has a diurnal variation, 5-10% above baseline,
with peak levels in the morning (Guillard *et al.*, 1979; Lifschitz & Henkin,
1971). Significant changes in serum copper are observed in pregnancy, with
age, oral contraceptive agents, hormones and diet (Table 2).

Normal urinary copper excretion is less than 30 ug/day (Solomons,
1979). An increase in urinary copper excretion is observed in Menke's
infants (Williams, D. *et al.*, 1977), in patients on parenteral nutrition
(Solomons, 1979) and in women on OCA (Margen & King, 1975).

1.4.2.   Metabolism

The site of maximal absorption of copper varies among different
species (Evans, 1973). The primary absorption site for copper in man is
the stomach and duodenum (Bearn & Kunkel, 1955). Orally administered
[64]copper in rats increases rapidly and reaches a peak within 0.5 hours

Table 2a <u>Copper and ceruloplasmin levels in human plasma</u>

	References	Copper μg/mL ± SD	Ceruloplasmin mg/dL ± SD
<u>Plasma</u>			
Male	Hambidge, 1974	0.92 ± .12	31 ± 4
Females	Hambidge, 1974	1.07 ± .23	31 ± 5
	Prasad, 1975	1.38 ± 1.02	
	Prasad, 1975	1.43 ± 0.35	
	Margen, 1975.		38 ± 13
Females on OCA			
	Hambidge, 1974	2.21 ± 0.62	57 ± 21
	Prasad, 1975	2.41 ± 0.56	
	Prasad, 1975	2.27 ± 0.65	
	Margen, 1975		46 ± 12
Pregnant 16 weeks			
	Hambidge, 1974	1.62 ± .27	
Pregnant 36 weeks			
	Hambidge, 1974	1.92 ± .24	71 ± 24
Pregnant, term			
	Bogden, 1978	1.99 ± .06	71 ± 24

Table 2b. <u>Copper and ceruloplasmin in human serum</u>

References	Copper μg/mL ± SD	Ceruloplasmin mg/dL ± SD
Serum		
Females Friedman, 1968	1.21	
Gregor, 1978	1.06 ± .21	
Females without menarche		
Gregor, 1978	1.03 ± .21	
Average (no sex differentiation)		
Martin, 1978	1.57 ± .11	13.5 ± 5
Martin, 1978	1.25 ± .29	
Zidar, 1978	0.85 ± 1.55	20-60
Pregnant, term		
Shaw, 1980	2.21 ± .54	91 ± 13
Cord blood		
Bogden, 1978	0.51 ± .06	
Hambidge, 1974	0.29 ± .11	
Shaw, 1980	0.29 ± .11	10 ± 6
Neonate		
5 days Shaw, 1980	0.47 ± .9	
3 months Shaw, 1980	0.81 ± .17	
6-12 months		
Shaw, 1980	1.11 ± .19	
6-12 years		
Shaw, 1980	1.09 ± .17	

after administration and then the absorption rate decreases to a constant level for several hours (Marceau *et al.*, 1970). Copper is dissociated from the ingested foodstuff as ionic or copper-amino acid complex. The copper-amino acid complex is then actively transported across the intestinal mucosa. The copper-amino acid complex represents the rapidly absorbed copper observed in the plasma after [64]copper administration (Crampton *et al.*, 1965; Kirchgessner & Grassman, 1970). The ionic fraction traverses the mucosal membrane and binds to metallothionein, a copper-binding protein, by forming a mercaptide with the sulfhydryl groups. As the copper dissociates from the metallothionein, it either diffuses directly into the plasma or becomes complexed for transport into the lumen for excretion (VanCampen & Mitchell, 1965). Diagrammatic representation of copper metabolism is presented in Figure 1.

The intestinal metallothionein has a two-fold function. It passively binds copper within the intestinal mucosa and insures that an adequate supply of the metal is removed from the dietary source and temporarily stored. The copper-binding protein may also represent a mucosal block to protect against absorption of copper to toxic levels. The copper that has not been absorbed into plasma is excreted as a metallothionein complex with the sloughing off of the epithelial cells (Evans, 1973).

Dynamic studies with radioactive copper have been carried out in humans. The isotope is found within one to two hours after administration and it is bound to plasma albumin and amino acids. This is followed by a sharp decline in the plasma with a concomitant increase in liver radioactivity. The liver then synthesizes and releases new ceruloplasmin into the plasma within 2 to 3 days after the isotope administration (Earl *et al.*, 1954; Bush *et al.*, 1955; Bearn & Kunkel, 1954).

GUT	EPITHELIAL CELL	PORTAL BLOOD	HEPATIC CELLS	BILE

Cu-MM

Cu-BP ←

BP

$MM + Cu^{+2}$ → Cu^{+2} ----→ Cu^{+2}

amino acid (AA)

Albumin

Cu-AP — Cu-AA → Cu-AA

Albumin-Cu-AA

**

Cu-BP

BP

Apo-enzyme

Cu-AA → AA + BA → Bile

Cu-enzyme (Cp)

Cu-AA

Apo-enzyme

Cu-enzyme

Albumin

EXTRA-HEPATIC CELLS
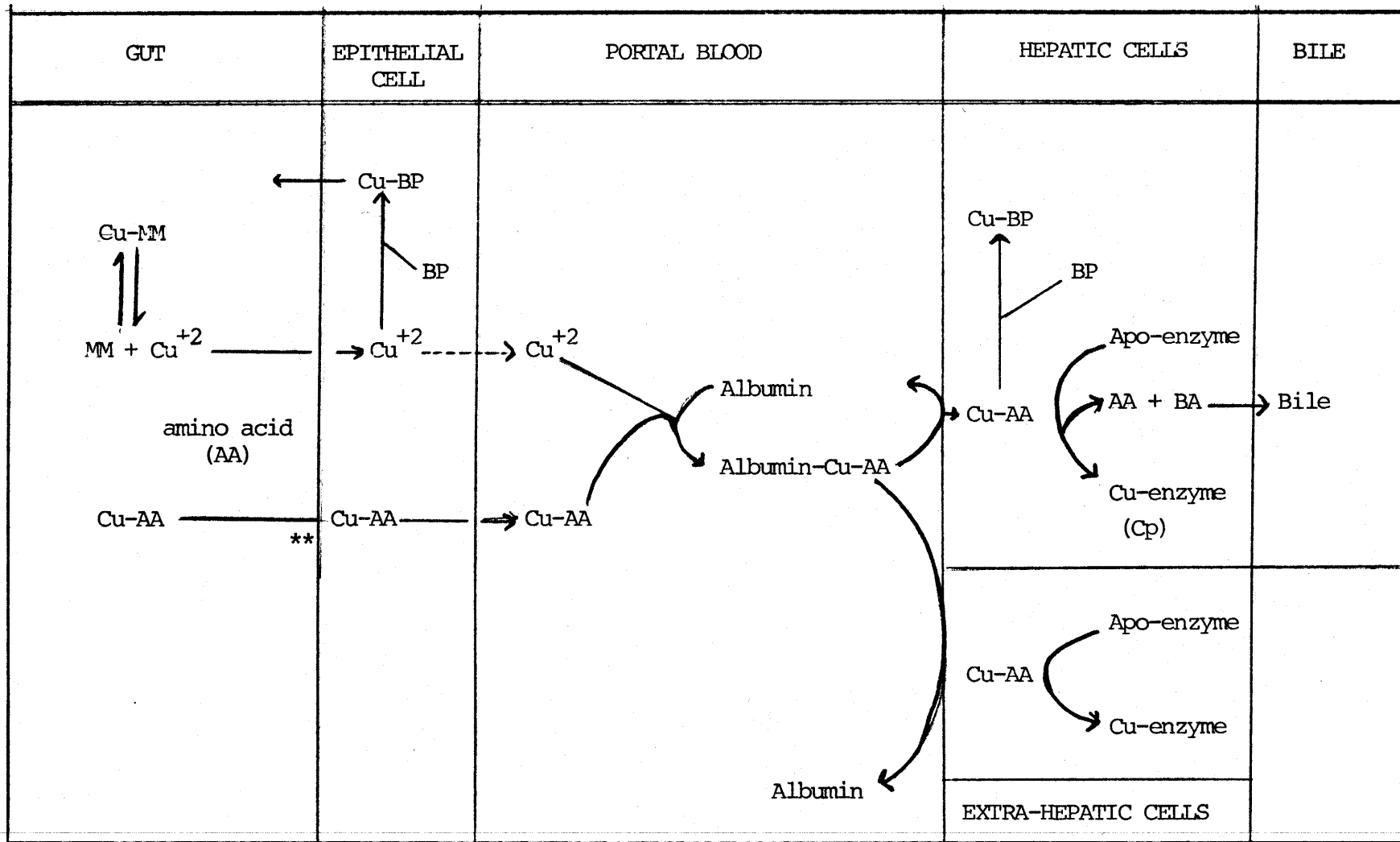
FIGURE 1: Copper metabolism in mammalian system. Abbreviations: MM - macromolecules; Cu - copper BP - binding proteins; AA - amino acids; BA - bile acids; Cp - ceruloplasmin. The (**) indicates an energy dependent process.

The proportion of dietary copper that is actually absorbed in humans has not been well studied; however, present data suggest that 40-60% of ingested copper is absorbed with wide individual variations (Deeming & Weber, 1978). Copper absorption is significantly impaired in cases of severe and diffuse diseases of the small bowel as in sprue (Sternlieb & Janowitz, 1964) and in patients with intestinal bypass (Zidar et $al.$, 1978) due to a decrease in absorptive sites and in protein-calorie malnutrition due to a generalized decrease in amino acids and protein content (MacDonald & Warren, 1961). The small-for-gestational age babies have increased absorption of copper from the from the gut (Dauncey et $al.$, 1977) in response to increased need for growth and poor hepatic copper stores.

In animals and man, several chemically similar elements (zinc, cadmium, calcium and molybedenum) are known to precipitate copper deficiency (Evans, 1973; Gregor et $al.$, 1978 b; Spencer et $al.$, 1979). Van Campen, 1968 demonstrated that zinc interacts with copper at a site either in or on the intestinal mucosa. Evans et $al.$, 1975 identified that cadmium and zinc displaced copper from sulfhydryl binding sites on the metallothionein; therefore, the alteration in copper homeostasis is produced by competitive inhibition for the binding sites. Other antagonists to copper absorption are ascorbic acid and dietary copper complexes. Ascorbic acid decreases the binding of of copper by metallothionein from both the intestine and liver. The vitamin interacts with the protein and inhibits the formation of mercaptides (Evans et $al.$, 1970). A diet of raw meat, unlike cooked meat, results in copper deficiency because the metal may be complexed to organic molecules that release copper only after denaturation

(Moore *et al.*, 1964). Vegetable proteins are a poor source of copper because all vegetables have phytic acid which binds copper and decreases its availability for absorption (Davis *et al.*, 1962; Moore *et al.*, 1964; Evans, 1973; Drews *et al.*, 1979). Copper can also be complexed with anions present in the gut. Sulfides are well known inhibitors of copper absorption because copper sulfide salts are insoluble and unavailable for formation of complexes for mucosal transport (Bowland *et al.*, 1961).

After absorption from the intestine, copper is transported to the liver primarily as an albumin and to some degree as amino acid complexes (Bearn & Kunkel, 1954). The preferential binding sites for copper in the albumin molecule are the alpha-amino nitrogen from histidine and two nitrogens from the peptide backbone. Species variation in binding of copper in plasma is determined by the presence or absence of the above binding sites in the albumin molecule (Bradshaw *et al.*, 1968; Appleton & Sarker, 1971).

Albumin releases its loosely bound copper at the hepatocyte membrane receptors. The metal is then transported to the cytosol where it binds to the liver metallothionein. Most of the hepatic copper is utilized in the synthesis of ceruloplasmin, superoxide mutase, cytochrome C and other cupro-proteins. These are then released into the plasma and they constitute 93% of the plasma copper. The liver also releases uncomplexed copper into the plasma to maintain the labile and exchangeable pool of the metal. The labile pool is mostly bound to albumin and to a somewhat lesser extent to amino acids (Evans, 1973).

The main route for copper excretion is the G.I. tract. Fecal copper represents the unabsorbed dietary copper. Endogenous Cu is excreted via the biliary tract (major fraction), gastric and intestinal mucosa (Evans & Cornatzer, 1971, Spencer *et al.*, 1979). The excreted copper is bound to a high molecular weight biliary protein and is unavailable for reabsorption. The removal of copper is severely retarded in the presence of protein synthesis inhibitors (Gregoriades & Sourkes, 1968). Urinary excretion of copper is negligible, 0.5-3.0% of the daily intake (Cartwright & Wintrobe, 1964). Cupuria will generally occur in advanced stages of pathological conditions affecting primarily homeostatic mechanisms such as biliary excretion, copper storage, ceruloplasmin synthesis (Evans, 1973). Other minor routes of excretion are sweat, saliva and menstrual blood.

1.4.3. Copper Deficiency in Animals

1.4.3.1. Anemia and iron metabolism

Iron insensitive progressive anemia was reported in 1928 in rats (Hart *et al.*, 1928). These animals were cured of anemia when given ash of beef liver. The curative acid extract of the beef liver was rich in copper and the addition of copper salts to the normal chow of the anemic rats alleviated their disorder. Similar anemic conditions have been diagnosed in sheep (Beck, 1941), pigs (Smith, S. *et al.*, 1944), ewes and lambs (Bennetts & Chapman, 1937) and man (Josephs, 1931). The hematological findings are microcytic, hypochromic anemia, decreased erythrocyte half-life and decreased plasma and tissue iron (Lee *et al.*, 1968; Lahey *et al.*, 1952; Gubler *et al.*, 1952; Bush *et al.*, 1956). The morphological and biochemical similarities between iron and copper deficient states suggests that copper deficient anemia is a result of

impaired iron metabolism (Gubler *et al.*, 1952).

Copper deficient swine on oral iron supplements had lower than normal total body iron (Gubler *et al.*, 1952 ; Lee *et al.*, 1968, 1976) and a 66% decrease in absorption of radioactive iron (Lee *et al.*, 1976). Detailed study on iron absorption revealed that orally supplemented copper deficient animals had iron granule deposits with the columnar epithelial cells and macrophages of the lamina propria. No such deposits were seen in intramuscularly iron supplemented or control animals. These observations suggested that the iron absorption was impaired and the defect lay in transport of iron from the mucosa to the plasma. Decrease in plasma iron, in the presence (Evans, 1973) or absence (Lee *et al.*, 1976) of normal total body iron, is accompanied with hypoceruloplasmenia (Roeser *et al.*, 1970). Administration of ceruloplasmin induces prompt increase in plasma iron by mobilization of stored iron from the liver and gut (Ragan *et al.*, 1969; Roeser *et al.*, 1970; Lee *et al.*, 1976) and an equivalent dose of inorganic copper is ineffective. However, a larger dose of copper salt increases plasma iron after a 30 minute lag and this coincides with an increase in ceruloplasmin (Lee *et al.*, 1976). These studies indicate that ceruloplasmin plays an important role in mobilization of iron from the cells to the plasma. Ceruloplasmin oxidizes cellular ferrous to ferric iron which binds to transferrin for transportation to heme biosynthetic sites. In an *in vitro* study, Osaki *et al.*, (1966) proposed that ceruloplasmin, by virtue of its "ferroxidase" activity, enhances the incorporation of absorbed iron into apotransferrin before its transport to the hemoglobin synthetic sites.

Certain facts, however, do not directly support the conclusions

of Osaki's *et al.*, study. In Wilson's disease, an inborn error of
metabolism affecting copper homeostasis, no anemia is observed despite
very severe hypoceruloplasmania (Prasad, 1978). Another route for iron
metabolism has been proposed and two nonceruloplasmin ferroxidases
have been isolated from human serum. The full physiological significance
of the nonceruloplasmin ferroxidases is not fully understood (Lee
1976; Topham & Friedman, 1970).

Bone marrow failure with ineffective erythropoiesis is observed
in copper deficiency anemia. Kinetic studies indicate that plasma
iron turnover is normal but incorporation of iron into the heme molecule
is reduced. The hemebiosynthetic enzyme profile is normal (Lee
1976) but mitochondrial iron uptake is decreased (Goodman & Dallman,
1969). Mitochondrial iron uptake must precede hemebiosynthesis and
cytochrome oxidase catalyzes this uptake by reducing ferric (Fe^{3+}) to
ferrous (Fe^{2+}). This provides a steady supply of iron for heme
synthesis (Lee *et al.*, 1976). The observed decrease in mitochondrial
uptake during copper deficiency is due to deficiency of the cupro-enzyme,
cytochrome oxidase (Gallagher *et al.*, 1956).

1.4.3.2. Cross-linking Proteins

Skeletal abnormalities associated with copper deficiency have
been observed in many mammalian species, including man (Baxter *et al.*,
1953; Underwood, 1977; Yuen *et al.*, 1979). The histological changes
observed are thinned cortices, broadened epiphyseal cartilages, and a
low level of osteoblastic activity (Follis & Bush, 1955). The copper
deficient bones are very fragile but the bone ash, calcium and
phosphorous content of these bones are normal (Baxter *et al.*, 1953).

The primary biochemical lesions in the bones of copper deficient animals, are reduced activity of amine or lysyl oxidase (Harris *et al.*, 1974; Siegel *et al.*, 1970). Amine oxidase is a cupro-enzyme and is essential for oxidative deamination of epsilon-amino groups of lysine residues. These lysine residues are involved in cross-linking of the collagen polypeptide chains. A decrease in cross-linkages reduces the structural integrity of the collagen and increases bone fragility (Rucker & Murray, 1978).

Another amine oxidase activity related disorder is the "falling disease" (Bennetts, 1948). The disorder is characterized with degeneration of the myocardium and replacement fibrosis (Bennetts, 1948). In 1961, O'Dell demonstrated a derangement of aortic elastic tissue in copper deficient chicks. The deficient animals had an elevated content of lysine but low desmosine and isodesmosine, the cross-linking amino acids of elastin. The latter two amino acids are formed by condensing the deaminated epsilon-amino groups of lysine. The deaminating enzyme is called amine oxidase. Copper deficient animals have reduced activity of the deaminating enzyme in the aorta and liver. Since copper is the key regulator and may be the major determinant of the steady state tissue levels of the enzyme, deficiency of the metal will impair elastin formation (Hill *et al.*, 1968; Harris 1976).

1.4.3.3. Central nervous system

Congenital or delayed neonatal ataxia in animals is a nervous disorder, characterized by incoordination of movement (Underwood, 1977). The pathological and histological findings in ataxic animals suggests a demyelinating encephalopathy and the significant changes are necrosis and degeneration of the neurons and the nerve fibers in the brain stem

and the spinal cord (Howell *et al.*, 1964; Everson *et al.*, 1968).

Howell *et al.*, (1964) did not detect any by-products of the degenerating

myelin and suggested that the lesion in the white matter of an ataxic

spinal cord may be one of myelin aplasia. The biochemical findings

are compatible with myelin aplasia. The primary lesion is the marked

decrease in activity of cytochrome oxidase, the copper containing

terminal respiratory enzyme in the large motor neurons of the red nucleus

and ventral horn of the grey matter in the spinal cord (Fells *et al.*, 1965).

Gallaghar & Reeve (1971) found two major biochemical dysfunctions at

early stages of the disease: impaired cytochrome oxidase associated

with loss of the prosthetic group haem-*A* and impaired mitochondrial

phospholipid synthesis. It has been hypothesized that a copper deficiency

results in decreased cytochrome oxidase activity, therefore decreased

ATP synthesis results in inhibition of aerobic metabolism and phospholipid

synthesis. This inhibition leads to myelin aplasia, since it is composed

largely of phospholipid.

The ataxic signs in the copper deficient animals are similar to

Parkinson's disease. O'Dell *et al.*, (1976) found decreased catecholamines,

dopamine and serotonin, in the anterior brain stem and corpus striatum.

Supplementation with copper increased only the dopamine levels and not

the serotonin and there was no reversal of the locomotor disorder. A

60% decrease was seen in the dopamine levels in the corpus striatum of

copper depleted post-weanling rats and copper repletion did not improve

this low level (Feller & O'Dell, 1980). The investigators proposed that

copper deficiency depresses a catalytic function in the adrenergic pathways

and also affected a structural component of the dopaminergic system during

development.

1.4.3.4. Pigmentation and Keratinization of Hair and Wool

The pigmentation process of sheep and lamb is susceptible to copper status of the animal and is considered a very sensitive index of copper deficiency (Smith & Ellis, 1947). It has been suggested that the conversion of tyrosine to melanin is defective because it is catalyzed by copper containing polyphenyl oxidases (O'Dell, 1976). The copper deficient animals also exhibit impaired keratinization. The tensile strength of the wool or hair is reduced and the elastic properties are abnormal (Bennetts & Chapman, 1937). The characteristic physical properties of hair is dependent on the presence of disulfide groups that provide the cross-linkages of keratin and on the alignment and orientation of the keratin fibrillae. Copper deficient hair has more sulfhydryl groups and fewer disulfide groups and it seems that copper is required for the incorporation of disulfide groups in keratin synthesis (Burley & deKoch, 1957).

1.4.3.5. Pregnancy

The daily dietary copper requirement during pregnancy in rats is increased from 2-3 ug/mL to 6-8 ug/mL (Cerklewski, 1979). The hepatic copper store is not decreased but the biliary excretion is decreased to one-third the normal with a concomitant increase in dietary intake (Terao & Owens, 1977). However, the maternal hepatic copper store in cattle is markedly decreased in late pregnancy (Allcroft et al., 1959) and the hepatic vein has the highest concentration of copper during gestation (Russ & Raymunt, 1956).

The fetal rat accumulates copper in the latter third of pregnancy (Seeling et al., 1977; Terao & Owens, 1977) and the hepatic copper level is above the adult level (12.4 ug/g fetal hepatic copper vs. 3.31 ug/g

in the dam). The serum copper is approximately one-third to one-fourth the adult level and is primarily due to low ceruloplasmin levels. During the first postnatal week, the hepatic copper stores double (12.4 ug/g at term vs. 27.7 ug/g in first week) and by six weeks the stores decrease to adult level (3.1 ug/g at six weeks of age vs. 3.3 ug/g hepatic copper in adult). The serum ceruloplasmin level starts to increase (11.9-14.4 U/mL at birth vs. 43 U/mL at 24 h after birth) and by the fourth week the values are within adult range. The main source of copper in postnatal life is the maternal milk. In the rats the level of copper in milk drops at about the fourth day postpartum (Terao & Owens, 1977).

Gestational copper deficiency in ewes and guinea pigs (intake 6 ug/mL) results in neonatal ataxia, characterized by incoordination and paralysis of the hind legs. This deficiency affects brain development through impaired phospholipid synthesis, hypomyelination and catecholamine synthesis (Section 1.4.3.3.) (Bennetts & Chapman, 1948; Allcrofts 1959; Everson *et al.*, 1967, 1968). Other abnormalities in the offspring of copper deficient rats are abnormal separation of aortic elastic lamina and less elastin than normal. These offspring are also anemic, nonviable and are afflicted with edema and subcutaneous hemorrhages (O'Dell, 1971, 1968). In very severe copper deficiency, in rats, there is normal conception but the fetal development ceases by the thirteenth day and necrosis of the placenta is evident by the fifteenth day (Howell & Hall, 1969). Very mild copper deficiency during rat pregnancy results in decreased litter size and decreased fetal hepatic copper stores (1.32 ug/g in pups of slightly deficient dams 3 ug/mL copper vs. 1.53 ug/g in pups of dams on 9 ug/mL copper) (Cerklewski, 1979).

An autosomal recessive mutant gene - crinkled (cr), in mice, displays phenotypic similarities to copper deficient animals and is especially true for hair growth and pigmentation. A high copper diet (500 ug/mL) during pregnancy and lactation suppresses the expression of the gene in a homozygous mutant. The gene and trace element interaction during development also increases the survival days of the mutant mice over the nonsupplemented mutants (Hurley & Bell, 1975).

1.4.4.   Copper Deficiency in Man

1.4.4.1.   Infants and adults

A syndrome characterized by hypocupremia, hypoferremia, hypoproteinemia, edema and hypochromic anemia, responsive to copper rather than iron, has been observed in infants. Several investigators (Josephs, 1931; Sturgeon & Brubacker, 1956; Schubert & Lahey, 1959; Zipursky, 1958), treated a series of infants suffering from "secondary hypochromic" anemia with iron and iron/copper supplements. The rationale behind the treatment was based on the animal studies by Hart *et al.*, (1928). Josephs. (1931) found accelerated hemoglobin synthesis when copper was given in addition to iron. This acceleration was most evident when the hemoglobin was above the 50% level, for above this point the hemoglobin curve from cases on iron alone tended to plateau whereas the infants on iron/copper supplements continued to have increasing hemoglobin level up to about 70%. Copper accelerated hemoglobin synthesis but had no effect on the reticulocyte maturation.

In 1964, Cardano *et al.* reported on the development of anemia, neutropenia and severe demineralization of bone in some of the malnourished infants on modified cow's milk diet with an adequate intake of vitamins and iron. These children showed no signs of malabsorptions

and total serum proteins and albumin were normal. Oral copper
sulphate supplementation had a prompt and dramatic reversal of the
anemic symptoms. Cardano also observed that despite the persistent
neutropenia, the patients had normal neutrophil response to infection.
In severe and prolonged deficiency, maturation of granulocyte series in
the bone marrow was also arrested. Cardano and Graham, (1966) also
found that neutropenia responded better to free copper than ceruloplasmin.

Recently, a number of papers have documented copper deficiency
in infants and adults on prolonged total parenteral nutrition (TPN)
and in patients with intestinal bypass surgery (Heller *et al*., 1978;
Karpel & Pedan, 1972; Vilter *et al*., 1974; Dunlap *et al*., 1974; Fleming
et al., 1976; Zidar *et al*., 1978). All copper deficient patients exhibit
subnormal ceruloplasmin and erythropoietin levels, anemia and neutropenia.
Addition of copper to the TPN results in dramatic improvement in the
abnormal hematological picture.

Severe copper deficiency in infants is accompanied by skeletal
disorders. Osteoporosis, with enlargement of costochondral cartilages,
followed by cupping and flaring of metaphysis of long bones with spur
formation and submetaphyseal fracture, periosteal reactions and
spontaneous fractures, especially of the ribs, are manifestations of
copper deficiency in infants. These skeletal changes are usually
referred to as "scurvy-like" changes, and are suggestive of "the battered
child syndrome". Supplementation with copper causes withdrawal of these
abnormalities (Heller *et al*., 1978; Cardano & Graham, 1966). Deficiency
of the copper-containing oxidases, essential for the cross-linking of
bone collagen, is believed to explain these manifestations adequately
(Mason, 1979).

1.4.4.2. Menke's disease

This is a progressive brain disease inherited as a sex-linked recessive trait. It was first reported by Menkes and his colleagues in 1962. The disease is an inherited defect in copper absorption: a congenital copper deficiency. Therefore, Menke's disease and nutritional copper deficiency have certain features in common: 1) usual occurrence in infancy, 2) subnormal plasma levels of copper and ceruloplasmin, 3) 3) tortuosity and defects in elastin of the aorta due to lack of lysyl oxidase, 4) scorbutic-like changes in costochondral junctions and epiphyses of long bones, and 5) decreased pigmentation of skin or hair. Menke's disease differs from the state of dietary copper deficiency in the following respects: 1) alterations of hair structure, 2) highly variable and often extensive lesions involving both white and grey matter of the cerebrum and cerebellum, due to lack of cytochrome oxidase which may be responsible for hypothermia, convulsive seizures and mental retardation, 3) absence of anemia and neutropenia, and 4) unresponsiveness to orally administered copper other than significant increases in plasma levels of copper and ceruloplasmin (Mason, 1979; Menkes, 1972).

The manifestations of this metabolic disorder are believed to begin *in utero* and continue into postnatal life. However, the underlying principle of the metabolic disorder is not known and it is not clear if there is abnormality in placental transfer, binding of copper or in production of copper-containing enzymes. The characteristics of Menke's disease are very similar to that observed in "crinkled" mutant mouse. There may be genetic similarities between the two disorders (Hurley & Bell, 1975).

1.4.4.3. Pregnancy and early postnatal development

Plasma copper level in pregnancy increases 2 to 3 fold and is almost entirely due to increased ceruloplasmin synthesis. The erythrocyte copper content and the free copper concentration remains constant through the pregnancy (Scheinberg *et al.*, 1954; Henkin *et al.*, 1971 a; Mason, 1979). Beginning during the first trimester, the serum copper (ceruloplasmin) rises progressively with each lunar month (Friedman *et al.*, 1969; DeJorge *et al.*, 1965; Heijkenskjold & Hendenstedt, 1962). Since there is no evidence of alterations in absorption or excretion during pregnancy, the copper for increased ceruloplasmin synthesis and fetal need is believed to come from maternal hepatic stores. Autopsy studies have shown that hepatic copper is lower in pregnant women than in nonpregnant women and this observation tends to support the above hypothesis. The large increment of about 25% in plasma volume during gestation and maintenance of high plasma copper levels places a special demand upon maternal stores (Mason, 1979).

The stimulus for increased ceruloplasmin synthesis is believed to be estrogen. Women on OCA have much higher plasma copper and ceruloplasmin levels: 31 ± 5 ug/dL in nonusers vs. 57 ± 2 ug/dL ceruloplasmin in OCA users; 1.07 ± 0.23 ug/mL copper in nonusers and 2.21 ± 0.62 ug/mL in users (Shaw, 1980; Hambidge & Dreogmuller, 1974; Prasad *et al.*, 1975; Deeming & Weber, 1978; King & Margen, 1978; Margen & King, 1975). Margen & King, (1978) carried out metabolic balance studies with copper isotopes in OCA users and nonusers. The investigators found no significant changes in uptake or excretion of the metal due to the OCA. No change in plasma or serum copper is noted in adults on progesterone (Briggs *et al.*, 1970) and in those with intrauterine device (Daunter & Epstein 1973).

However, estrogen alone may not be responsible for increase in ceruloplasmin since estrogen level drops more rapidly after delivery or abortion than serum ceruloplasmin and it takes up to four weeks to attain nonpregnanat copper levels (Borglin & Heijkenskjold, 1967; Johnson, 1961).

Abnormally high serum copper and ceruloplasmin levels have been observed in pre-eclampsia and eclampsia (Fattah *et al.*,1976; O'Leary *et al.*, 1966) and hydatidiform mole (Heijkenskjold & Hedenstedt, 1962). Low copper levels in pre-eclampsia has been reported by another investigator (Friedman *et al.*, 1969) and is compatible with a decrease in placental cupro-enzyme mono- and diamine oxidase (DeMaria, 1964; Sandler & Coveny, 1962). Decreased serum copper levels are also observed in cases of missed abortion and very high levels in threatened abortion (Friedman *et al.*, 1969).

Nonceruloplasmin copper readily diffuses across the placenta by passive transfer (Henkin *et al.*,1971 a). Studies on umbilical vein and artery copper levels, indicate that 28-30% of the copper is removed from the circulation (Dokumov, 1968). The high level of copper in the placenta (13.5 ug/g) (Poczekaj *et al.*, 1963) and fetal liver (Widdowson *et al.*, 1972) reflects the efficiency of this transfer.

The time and magnitude of accumulation of copper by the fetus is not clear. The fetal liver copper rises exponentially from the 20th week to term and is higher than that of the adult liver, 64 mg/100 g of fetal liver to 0.5 mg/100 g fresh tissue of an adult liver (Widdowson *et al.*, 1972). The concentration of liver copper in premature and full term infants is highly controversial. Iyengar, (1972) and Butt *et al.*, (1958) reported higher copper levels in infants less than 36 weeks vs. those at

term, while Sultanova (1970) reported the opposite. Bogden *et al.*, (1978 a, b) and Gibson & deWolfe, (1979) found no difference in umbilical cord copper levels between premature and term infants. Krishnamachari & Rao, (1972) found decreased plasma copper and ceruloplasmin in infants born to undernourished mothers. Despite the accumulation of copper by the fetus, the circulating copper level at birth is only one-fourth to one-fifth of the adult and over 80% is bound to ceruloplasmin (Henkin *et al.*, 1971 a). Despite the presence of ceruloplasmin in the fetus, there is no evidence that the fetal liver can synthesize ceruloplasmin and, therefore, it has been suggested that the protein may cross the placenta (Sass-Korstak, 1965). Upon the onset of postnatal life the liver starts to synthesize ceruloplasmin and the plasma copper levels reach that of an adult by the 2nd to 3rd month of life (Henkin *et al.*, 1973). The unusually high concentrations of copper in the liver and other tissues at birth act as a reserve to ensure an adequate supply of copper to meet the demands of increasing ceruloplasmin and other cupro-protein synthesis and ensure normal hematopoietic and maturational changes during the postnatal life.

The importance of the fetal hepatic copper stores is most evident in premature neonates on low copper milk diet. Dauncey *et al.*, (1977) carried out metabolic balance studies on preterm neonates on mother's milk and found them to be in negative balance for about 2 months postnatally. Al-Rashid & Spangler (1971) were presented with a premature infant on formula who had anemia, neutropenia, apnea, osteoporosis and failure to thrive. Copper intake was poor and upon copper supplementation the child made a remarkable recovery. Other investigators have also reported similar clinical cases (Griscom *et al.*, 1971; Seely *et al.*, 1972; Ashkenazi *et al.*,

1973; Sann *et al.*, 1978; Yuen *et al.*, 1979). Sivasubramanian & Henkin, 1978 observed reversible behavioral disorders in preterm infants on copper deficient TPN. Low birth weight term infants may also have decreased copper storage since they have increased copper retention capacity (Dauncey *et al.*, 1977).

1.5. Chromium

1.5.1. Distribution

Reliable data on the distribution of chromium in the tissues is very sparse because, until recently, chromium analysis has been hampered with the loss of the analyte during analysis and interference with nonspecific absorption (Shapcott *et al.*, 1977; Guthrie *et al.*, 1978 a, b; Routh, 1979).

Chromium is distributed throughout the human body in low concentrations (Schroeder, 1968). The tissue chromium levels vary with age and geographical location (Schroeder 1968, Schroeder *et al.*, 1961). The chromium level in most tissues is highest at birth and starts to decline by 45 days of age (27.0 ug/g in spleen at 0.45 days vs. 4.1 ug/g at 45 days to 10 years) and this decline continues to old age (16.6 ug/g in liver at 10 years to 1.3 ug/g at 80 years). The level in the liver and kidney does not decline until after 10 years of age (17.9 at birth vs. 16.6 at 10 years vs. 4.6 ug/g in liver after 10 years) and hair chromium level declines from 0.9 ug/g at birth to approximately 0.4 ug/g by 2-3 years of age (Hambidge & Baum, 1972; Gurson, 1977).

Significant variation in adult tissue chromium levels due to geographical locations have been observed: 0.8 ug/g chromium in liver in U.S. vs. 1.3 ug/g in Africa vs. 2.1 ug/g in Near East and Far East;

5.4 ug/g chromium in liver in Alaska vs. 2.3 ug/g in the U.S. Mainland.
These variations are believed to reflect differences in dietary chromium
intake.

The reported normal values for chromium in blood differ markedly
and reflect the lack of reliable analytical methodology. A comparison
of recent normal ranges is given in Table 3.

Plasma chromium levels decline in pregnant women: 2.97 ± 0.11 ug/L
in pregnancy vs. 4.70 ± 0.15 ug/L in nonpregnant women (Davidson & Burt,
1973). Levels are also decreased during stress and infection: 1.5 ug/L
in normals vs. 0.5 ug/L during sandfly fever (Perkerak et al., 1975) and
in coronary heart disease: 6.09 ug/L vs. 2.51 ug/L in diseased patients
(Newman et al., 1978; Schroeder et al., 1970).

Low hair chromium is observed in premature infants (0.32 ± 0.04 ug/g)
and in low birth weight (LBW) infants (0.15 ± 0.03 ug/g vs. 0.97 ± 0.16
ug/g in normals) (Hambidge, 1974). The levels are also decreased in hair
of diabetic children (0.56 ug/g in diabetics vs. 0.85 ug/g in normals)
(Hambidge et al., 1968); in adult diabetic females (0.27 ± 0.65 ug/g
in diabetics vs. 0.52 ± 0.91 ug/g in normal, Rosson et al., 1979) and
in parous women (0.75 ug/g in nulliparous vs. 0.22 ug/g in parous women)
(Hambidge & Rodgerson, 1969; Mahalko & Bennion, 1976).

Urinary chromium excretion in healthy adults is: 7.2 ± 0.4 ug/day
(Mitman et al., 1975); 8.4 ± 5.2 ug/day (Hambidge, 1971); 1.09 ug/day
(Routh, 1979); 0.8 ± 0.4 ug/day (Guthrie et al., 1978 a, b). These
variations in urinary chromium excretion again are due to the problems
associated with chromium analysis. The urinary chromium excretion increases
by three-fold in insulin-dependent diabetics (Doisy et al., 1976; Hambidge,
1974) and in newborn infants, up to 3 days of age (Gurson, 1977). Urinary

Table 3.   Chromium levels in human serum and plasma

	Levels μg/L	Method	References
Fasting serum			
	6.1 ± 3.3	Neutron Activation	Newman et al., 1978
	0.14	FLAAS*	Kayne et al., 1978
Fasting Plasma			
	0.3 - 6.0	FLAAS	Rabinowitz et al., 1980
	1.8 ± 2.3	Neutron Activation	Liu et al., 1978
	1.3	FLAAS	Doisy et al., 1976
	$1.5 \pm .14$	FLAAS	Pekarek et al., 1975
	1.58	FLAAS	Pekarek et al., 1974
	$4.7 \pm .15$	FLAAS	Davidson et al., 1973
	13	Emission Arc	Hambidge et al., 1971

* - FLAAS - Flameless atomic absorption spectrophotometer

excretion is also increased in glucose load challenge (Gurson & Saner, 1978 a).

1.5.2.   Assessment of chromium status

[51]Chromium studies have indicated that blood chromium disappears into the tissue rapidly after injection and the tissue chromium is not in equilibrium with the blood. The plasma chromium level in the fasting state, therefore, is not considered a meaningful indicator of the stores except in extreme cases of deficiency (Glinsman & Mertz, 1966; Mertz, 1969). It is believed that a much more reliable indicator is the plasma chromium response and urinary chromium excretion in presence of large glucose intake (Glinsman et $al.$, 1966; Mertz, 1969).

The normal plasma chromium response to glucose challenge is an increase in the circulating level of chromium (Glinsman et $al.$, 1966; Mertz, 1969). However, decrease in plasma chromium in response to glucose load in normals has been observed by other investigators (Pekerak et $al.$, 1975; Liu & Morris, 1978; Davidson & Burt, 1973) and no changes in plasma chromium has been reported by Hambidge & Droegmuller, 1974).

Despite the controversy over the type of plasma chromium response, no chromium "response" is consistently noted in diabetics with abnormal glucose tolerance (Glinsman & Mertz, 1966; Liu & Morris, 1978), in patients with sandfly fever (Pekerak et $al.$, 1975) and in pregnancy (Davidson & Burt, 1973).

Urinary chromium excretion and chromium creatinine ratio (Cr/Cre) is significantly higher ($P < 0.01$) in diabetic and malnourished children. The Cr/Cre ratio increases (4.30 ng before OGTT vs. 7.31 ng after OGTT) in response to oral glucose load test in normal individuals and no

significant changes are seen in diabetics (15.95 ng before OGTT vs. 16.57 ng after OGTT) and their families (6.58 ng before OGTT vs. 7.54 after OGTT) (Gurson & Saner, 1978 a, b). The positive response of urinary Cr/Cre ration to glucose load test is considered the best indicator of body chromium status (Gurson & Saner, 1978 b; Mertz, personal communication, 1978).

1.5.3.   Chromium metabolism

In vitro studies with rat intestine suggests that the primary site of chromium absorption is the small intestine (Chen *et al.*, 1973). Hahn & Evans, (1975) postulated that the absorption mechanism for this metal is similar to zinc. These investigators observed increased ^{51}Cr absorption in zinc deficient rats and this increase was prevented by oral zinc administration. Chromium and zinc are also found in the same mucosal supernatant fraction suggesting that the intestinal binding ligand may be the site of competition between these two metals.

Absorption of chromium is dependent upon its valency. Experimental work by Donaldson & Barreras (1966) in humans and rats has shown that only 0.5% of the inorganic chromium (Cr^{+6}) is absorbed. No significant absorption of inorganic Cr^{+3} is observed (Gurson, 1977). The presence of anions in the gut also affects chromium absorption and their mechanism of action is not understood (Chen *et al.*, 1973). Oxalates significantly increase absorption while phytates decrease absorption. Increased acidity of the gastric juices also decrease chromium absorption by reducing absorbable chromium to inaccessible chromates (Donaldson & Barreras, 1966). At neutral pH, amino acids e.g. glycine, serine and methionine, prevent the formation of large inaccessible polynucleate chromium complexes and enhance absorption. Absorption capacity in

mice declines with age (Underwood, 1977), while no such phenomenon was observed in humans (Doisy *et al.*, 1971).

Absorbed chromium from the gut is transported to the tissues as a siderophilin (transferrin) - chromium complex (Hopkins & Schwarz, 1964). All tissues have rapid uptake and the plasma is cleared of ^{51}Cr within a few days but the whole body radioactivity disappears more gradually. The fact that the tissues retain ^{51}Cr much longer than the plasma suggests that there is no equilibrium between the tissue stores and circulating chromium (Hopkins, 1965; Glinsman & Mertz, 1966). Chromium entering the tissues is distributed among the subcellular fractions: 49% in nuclear material; 23% in supernatant and remainder between microsomes and mitochondria (Edwards *et al.*, 1961).

Chromium is excreted mainly via the kidney and very small amounts are lost in the feces via the bile and small intestine (Hopkins, 1965; Collins *et al.*, 1961).

1.5.4.   Chromium deficiency in animals

1.5.4.1. Carbohydrate metabolism

Impaired glucose tolerance is observed in rats maintained, for several weeks, on a diet of 30% tortula yeast supplemented with selenium and Vitamin E. Addition of an equal amount of brewer's yeast or wheat casein improved the glucose intolerance. The curative factor is called the glucose tolerance factor or GTF (Mertz & Schwarz, 1955, 1959). An organic complex containing trivalent chromium was extracted from brewer's yeast. Addition of this extract or 20 ug of inorganic trivalent chromium improved the abnormal glucose tolerance in rats on tortula yeast diet (Schwarz & Mertz, 1959; Woolliscroft & Barbosa, 1977). Squirrel monkeys raised on standard commercial chow have a high frequency of impaired

carbohydrate metabolism and like rats, they respond favourably to Cr^{+3} supplementation (Davidson & Blackwell, 1968).

Chromium deficient rats have unusually low (50% of the control) liver glycogen after 18-hour fast and the rate of ^{14}C-glucose incorporation into cardiac and hepatic glycogen, in response to exogenous insulin is 60% of the control. This suggests that impaired carbohydrate metabolism is due to decreased insulin response of chromium deficient tissues (Roginski & Mertz, 1969; Schroeder, 1966).

In *in vitro* studies, the insulin response of chromium deficient rat epididymal fat pads is improved when chromium is added to the experimental media. The rate of glucose uptake into the fat pads is increased by 67% and its incorporation into fat by 76%. No effect of chromium supplementation is observed on insulin independent action, i.e. incorporation of acetate into fat (Mertz *et al.*, 1961).

Similar insulin/chromium responses have been observed in studies with glucose uptake by crystalline lens (Farkas & Roberson, 1965), galactose uptake by adipose tissue (Mertz 1969), swelling of the mitochondria in response to insulin (Campbell & Mertz, 1963). No positive response due to chromium supplementation is observed in poor insulin responsive tissues, e.g. the diaphragm (Mertz, 1969). The beneficial effect of chromium supplementation only occurs in the presence of insulin and it also decreases the dosage required to elicit a response (1.6 X 10^{-3}U in nonsupplemented mitochondrial preparation vs. 1.6 x 10^{-4}U of insulin upon supplementation) (Mertz, 1969).

The mode of interaction between these two agents, chromium and insulin is not clear (Mertz, 1969). There are five theoretical possibilities: 1) chromium maintains the optimal tertiary configuration

of insulin, 2) inhibitor of tissue insulinase, 3) increase initial
binding of insulin to the tissues, 4) a cofactor for insulin sensitive
glucose transport carrier. There is no evidence to support these first
four hypothesis. The fifth hypothesis: chromium acts as a catalyst in
the initial reaction between insulin and specific membrane sites.
Polargraphic studies have shown that chromium initiates the formation
of disulfide linkages between the intrachain disulfide of insulin and
sulfhydryl groups of the cell membrane by participating in a tertiary
complex (Christian *et al.*, 1963). This is believed to be the first step
through which insulin increases glucose flux into the tissues (Mertz, 1974).

The biological activity of chromium is dependent on its valency
and the organic complex. Of all the inorganic chromium compounds,
only chromium chloride ($CrCl_3$) has any biological activity. Chromium
fraction isolated from brewer's yeast or GTF is a trivalent chromium
complexed to nicotinic acid, glycine, glutamic acid and cystein.
Administration of GTF with insulin had a 10 fold higher response than
those produced by inorganic chromium (Mertz, 1969). The dose response
curve of insulin with and without GTF clearly shows that GTF potentiates
the action of insulin, in a manner quantitatively similar to that of
inorganic chromium. Doisy *et al.*, (1973) found that genetically
diabetic rats supplemented with GTF had improved glucose tolerance
response, unlike those on inorganic trivalent chromium chloride.

The composition of GTF is known but its exact structure has not
been elucidated. Toepfer *et al.*, (1977) synthesized GTF-like compounds
by mixing appropriate molar proportions of the trivalent chromium,
amino acids (serine, glycine and glutamic) and nicotinic acid. The
biological activity of the synthesized compound was similar to that of

the natural GTF, isolated from brewer's yeast (Toepfer *et al.*, 1977);
Tuman *et al.*, 1978).

In severe chromium deficiency, the animals (rats) exhibit glycosuria
and fasting hyperglycemia (Schroeder, 1966). The hyperglycemic
response is observed in 86% of the adult rats and 46% of the young
rats (3rd and 4th generation). The hyperglycemic response was
significantly more marked ($P < 0.001$) in the females than the males.
Glycosuria occurred in 55% of these rats as compared to only 10% in
the supplemented group. Schroeder (1966) also observed that chromium
supplementation did not improve the impaired carbohydrate metabolism in
Vitamin E and selenium deficiency liver necrosis but it did delay the
onset of the liver necrosis; this is believed to be due to increased
glycogen storage in chromium sufficiency (Schroeder, 1966; Mertz &
Schwarz, 1955).

Kraskeski *et al.*, (1979) have studied the effect of insulin on
radio-chromium distribution in normal and diabetic rats. Retention
of ^{51}Cr is decreased by 32% in diabetics but by only 17% in insulin-
treated diabetics. The tissue:serum ratio of ^{51}Cr for all tissues is
decreased in diabetes and administration of insulin shifts the tissue:
serum ratio, for all the tissues except the pancreas, towards the normal range
(bone: serum ^{51}Cr - 2.7 ± 0.2 in controls; 1.4 ± 0.07 in diabetics;
2.6 ± 0.2 in insulin treated). The pancreas of insulin treated diabetics
has higher retention of ^{51}Cr (0.22 ± 0.01% of ^{51}Cr dose in treated vs.
0.138 ± 0.01% in nondiabetic). Insulin appears to protect against
excess chromium loss and prevents further enhancement of glucose
intolerance.

1.5.4.2. Other effects of chromium deficiency in animals

Poor growth is observed in rats on chromium free low protein

soya diets (Roginski & Mertz, 1969). Addition of 2 ug/mL of Cr^{+3}

in their drinking water mildly stimulates growth with a proportional

increase in tissue protein. Roginski & Mertz (1969) studied the

uptake of alpha-aminoisobutyric (AIB) acid, a nonmetabolizable amino

acid, by the chromium deficient heart. Insulin dependent uptake of

the amino acid was significantly increased (P < 0.05) upon Cr^{+3}

supplementation (AIB tissue/AIB plasma: 0.82 in unsupplemented vs.

3.0 in supplemented tissue).

Insulin increases the protein synthetic process in the ribosomes.

In response to chromium supplementation, insulin administration increases

the incorporation of glycine, methionine and serine by 30% in the

deficient animal's heart and liver. Incorporation of other amino acids,

such as phenylalanine, lysine, was not significantly altered. The

selective effect of insulin on some amino acids (Roginski & Mertz, 1969)

contradicts the *in vitro* studies which suggest that all amino acids are

sensitive to insulin (Krahl, 1961).

Schroeder & Balassa (1965) studied the effect of chromium on

cholesterol metabolism in male and female rats with mild chromium

deficiency. Supplementation with 5 ug/mL of chromium for 17 months

reduced the circulating cholesterol in the males (108 mg/100mL in the

controls vs. 77mg/100mL in the supplemented males) and increased it in

the females (80mg/100mL in the controls vs. 101mg/100mL in supplemented

females). In a more recent study, Schroeder (1968) observed a lowering

of serum cholerterol in both males and females. A more uniform effect

of chromium deficiency is an increased incidence of aortic plagues in

the chromium deficient rats. Chromium supplementation decreased the

incidence of the plaques from 11% in the unsupplemented animals to

2% upon chromium supplementation. In a more recent study, Preston

(1976) found no correlation between chromium supplementation, cholesterol

metabolism and incidence of aortic plaques in chromium deficient guinea

pigs. This suggests that chromium response may be species dependent.

Schroeder and his group (1963, 1965) studied the effect of chromium

supplementation on survival and growth of rats and mice. There was a

24% increase in survival of male mice at 12 months and continuation of

the experiment increased the life span of all experimental animals by 10%.

Chromium supplementation also increased survival of animals in stress.

The apparent effect of chromium on survival and growth is small and

not as striking as those observed with supplementation of other metals,

like zinc and copper. The symptoms of chromium deficiency precedes or

are accompanied by severe abnormalities in carbohydrate metabolism,

therefore, the impairments fall within the framework of impaired

carbohydrate metabolism.

1.5.5.   Chromium Deficiency in Man

1.5.5.1.   Carbohydrate metabolism

Impaired carbohydrate metabolism is frequently observed in diabetes

mellitus, malnourishment and old age. Based on the data in animal studies,

the effect of chromium supplementation has been evaluated in the above

conditions.

Short-term (1-7 days) supplementation of 7 adult overt diabetics

with 1 mg of chromium chloride had no effect on glucose metabolism

(Glinsman & Mertz, 1966). The supplementation was extended to 10 months

in four of these patients at 180-1000 ug $CrCl_3$/day and it resulted in

improved oral glucose tolerance test (OGTT). Schroeder (1968) in two similar experiments had 33% responders, i.e. patients with improved OGTT after chromium supplementation. Sherman *et al.*, (1968) carried out a double blind study with 4 normals and 10 diabetics supplemented with $CrCl_3$ for 16 weeks. No improvement of OGTT and fasting glucose level was found. Doisy *et al.*, (1976) supplemented five insulin-requiring diabetics with 4-8 g/day of brewer's yeast (GTF) for 1-2 months. The insulin requirement of these participants was reduced from 60-130U/day to 20-45U/day. However, the author observed a hypoglycemic response when initiating chromium supplementation. Wise (1978) supplemented nine hospitalized hyperglycemic patients with 1 mg/day $CrCl_3$ for 6 days. His patients had a hyperglycemic response, 5% to 34% increase in fasting glucose level. Doisy *et al.*, (1976) supplemented siblings and offsprings of diabetics with mildly impaired OGTT with 4-8 g/day of Yeastamin daily for 8 months. Their glucose tolerance tests were normal after chromium supplementation treatment.

Levine *et al.*, (1968) supplemented 10 old age subjects, with abnormal GT, with 150 ug $CrCl_3$ for 61-117 days and four of the patients responded favourably to the treatment. Six of the nonresponders had severe impairment of GT and were suspected to have developed a metabolic impairment irreversible with chromium. Hopkins & Price (1968) carried out a similar experiment and had 40% responders. He noted that the non-responders were obese and obesity is known to produce abnormal GT (Gabbe, 1977). Doisy's *et al.*, (1976) study with elderly subjects and yeast supplementation met with a more favourable response.

Impaired carbohydrate metabolism, as indicated by hypoglycemia, and abnormal GTT is generally associated with Kwashiorkor & Marasmus

malnutrition. Hopkins *et al.*, (1968) supplemented malnourished Nigerian and Jordanian children with $CrCl_3$ for 1-2 days. They found significant improvement in the glucose removal rate (GRR) (0.6%/min. before supplementation to 2.9%/min. after supplementation). Gurson & Saner (1971, 1973) reported similar findings in malnourished Turkish children. In a similar study on kwashiorkor children from Cairo, Carter *et al.*, (1968) observed no improvement of the abnormal GTT and GRR.

Improvement of impaired GTT and GRR with chromium supplementation is faster in infants than in elderly or in diabetics. This is believed to be due to the infant's ability to convert Cr^{+3} into GTF at a faster rate than either the elderly or the diabetic (Doisy *et al.*, 1976).

In hemochromatosis, an iron storage disease, there is a high incidence of diabetes - 11 to 87% - inspite of a functional pancreas Dymock & Williams, 1971). Ill *et al.*, (1979) administered ^{51}Cr to such patients and found them to have a significantly lower retention capacity for ^{51}Cr. He suggested that reduced chromium may be responsible for impaired carbohydrate metabolism.

Pekerak *et al.*, (1975) found that in acute stress there is a decrease in serum chromium, resulting in abnormal GTT. Jeejeebhoy *et al.*, (1977) and Freund *et al.*, (1979) similarly reported decreased serum chromium, abnormal GTT and neuropathy in patients on prolonged total parenteral nutrition. Chromium supplementation reversed all the above symptoms.

1.5.5.2. Lipid metabolism

Increased cholesterol levels are often associated with abnormal GTT. Schroeder (1968) investigated the tissue chromium levels in 15 subjects who died of coronary occlusion. Aortic chromium level in the

coronary patients were one-tenth of the normals. Other tissue chromium levels were normal. Newman *et al.*, (1978) found that patients with coronary heart disease have one-third the serum chromium when compared to noncoronary patients. Doisy *et al.*, (1976) supplemented 15 normal subjects with GTF for one month and noted a significant (P < 0.01) decline in their fasting serum cholesterol. Based on the animal and human epidemiological studies, it has been suggested that a decreased aortic chromium level may result in abnormal lipid metabolism resulting in arteriosclerosis. This condition could be further aggravated by sucrose, a diabetogenic and lipogenic compound (Schroeder 1968; Boyle *et al.*, 1977).

1.5.5.3. Chromium in pregnancy

Carbohydrate and lipid metabolism is substantially altered in pregnancy (Page *et al.*, 1976). In late pregnancy, peripheral glucose utilization is normally impaired and therefore, tolerance to glucose is decreased. Insulin response to glucose challenge is also greatly exaggerated (Spellacy, 1977). The causative factor(s) resulting in the decreased tissue responsiveness to insulin has not been clearly defined (Spellacy, 1977; Gabbe, 1977).

GTF readily crosses the placenta in rats (Mertz *et al.*, 1969) but nothing is known about the placental transport of chromium in man (Shaw, 1980). Chromium must cross the placenta since it is present in human fetal liver (Widdowson *et al.*, 1972) and in the hair of newborns (Hambidge, 1971; Hambidge & Baum, 1972). The fetus is believed to extract chromium from the mother since the neonatal hair chromium exceeds that of the mother: 974 ng/g in neonates and 382 ng/g in the mother (Hambidge, 1971).

Transfer of chromium to the fetus during pregnancy leads to alterations in the hair chromium concentrations in the mother. There is a significant difference (P<0.01) between the hair chromium of the nulliparous (300-2200 ng/g) and parous women (50-500 ng/g) (Hambidge & Rodgerson, 1969). Gurson (1977) monitored hair chromium through the course of pregnancy. Chromium levels start to decrease in the early stages of pregnancy and is most accentuated during the last months before delivery. This decreasing pattern is believed to correspond to the increasing chromium requirement of the fetus (Seeling et $al.$, 1977; Gurson, 1977). Hambidge & Droegmuller, 1974 found no significant difference between hair chromium in early and late pregnancy. They suggest that the women may have may have had an adequate chromium intake.

Davidson & Burt (1973) studied the effect of glucose load on plasma chromium in pregnant and nonpregnant women. The fasting plasma chromium is lower in pregnant women (2.97 ± 0.11 ng/mL) than in the nonpregnant women (4.70 ± 0.15 ng/mL). The pregnant and nonpregnant women had similar GRR but the plasma chromium response was significantly diminished (P<0.001) and the insulin response was exaggerated in pregnancy. The investigators postulated that the chromium is required for full expresssion of insulin's hormonal activity and the lack of plasma chromium mobility in pregnancy may relate to insulin 'resistance' in late gestation. Schroeder et $al.$, (1961) reported nondetectable levels of chromium in postpartum rats and Davidson & Burt (1973) also found decreased hair chromium in pregnant women at term. The follow-up at six months confirmed the depletion of chromium at pregnancy with subsequent and gradual repletion. Mahalko & Bennion (1976) observed no difference in women who had borne only one child and those who have more

than one. However, hair chromium concentrations increased significantly with the amount of time between pregnancies, especially after four years since the last pregnancy, (94 ± 10 ug/mL at 2 years and 169 ± 18 ug/mL at 4 years).

The knowledge regarding chromium and pregnancy is still very sparse and little or nothing is known about the consequence of maternal chromium deficiency on the fetus (Shaw, 1980). The chromium status before pregnancy, chromium intake during pregnancy and the duration of pregnancy is believed to regulate maternal-fetal chromium homeostatis (Gurson, 1977).

1.6.   Manganese

1.6.1.   Distribution

Manganese is found in all tissues and fluids. This element is highest in the mitochondrial tissues (1.68 ug/g in liver vs. 0.19 ug/g in the ovaries) and 75% of the cellular manganese is in the mitochondria (Maynard & Cotzias, 1955; Tipton & Cook, 1963). Manganese levels in human blood, serum and plasma are presented in Table 4.

The widely varying manganese values for plasma and serum are believed to be due to lack of carefully controlled investigations. Versieck *et al.*, (1980) has strongly suggested that rigorous experimental conditions for sample collection and processing must be employed before any investigations are carried out. At present, there is considerable doubt about the true manganese concentration in the serum or plasma of healthy individuals.

1.6.2.   Metabolism

Little is known regarding manganese metabolism in man; however, it has been well studied in laboratory mice and rats. Only 3-4% of an orally administered dose of radiomanganese is absorbed in rats (Greenberg *et al.*, (1943). It is well absorbed throughout the length of the small intestine.

Table 4. <u>Manganese levels in normal human blood</u> (ug/L ± 1 S.E.M.)

	Level	Reference
Whole blood	9.84 ± 0.4	Cotzias *et al.* (1966)
	8.44 ± 2.7	Papavosilou *et al.* (1966)
	12.2 ± 3.9	Buchet *et al.* (1976)
	23.2 ± 10	Tanaka (1977)
Plasma		
	0.59 ± 0.18	Cotzias *et al.* (1966)
	1.5 ± 0.2	Hambidge & Droegmuller (1974)
Serum	1.42 ± 0.2	Papavoslou *et al.* (1966)
	24 ± 0.7	Mahoney *et al.* (1968)
	1.94	Grafflage *et al.* (1974)
	13.4 ± 6.9	Bek *et al.* (1974)
	2-3	Banta & Maresbury (1976)
	1.02 ± 0.19	D'Amico & Klawans (1976)
	0.57 (0.38-1.04)	Versieck *et al.* (1980)

The uptake is a two step mechanism involving initial uptake from the lumen and then transfer across the mucosal cells to the body. Both steps in the uptake mechanism operate simultaneously. (Thomson et al., 1971).

Manganese absorption in rats is decreased in presence of large quantities of iron and copper. This decrease in manganese absorption is due to competition for common binding sites in the gut (Thomson & Valberg, 1972). Excess dietary calcium also decreases manganese uptake by reducing the availability of soluble manganese (Wilgus et al., 1936). Body manganese status also affects mineral absorption from the gut. There is increased absorption of the metal in low manganese states and decreased absorption in manganese sufficient states (Howes & Dyer, 1971)

The absorbed manganese is almost totally excreted via the gut by several routes. These routes are interdependent and combine to provide an effective homeostatic mechanism to regulate tissue manganese levels (Papavosilou et al., 1966). The primary route of excretion is the bile and pancreatic juice. The auxiliary routes are via the duodenum and the jejunum. Kent & McCance (1941) and Maynard & Cotzias (1955) found very little urinary excretion of manganese except in presence of chelating agents.

1.6.3.   Manganese deficiency in animals

1.6.3.1.   Skeletal disorders

Wilgus et al., (1936) investigated the nutritional cause of perosis or 'slipped tendon' in poultry. Perosis is characterized by enlargement of the hock joints and was suspected to be due to calcium deficiency. The animals were inadvertently supplemented with two different grades of calcium salts. The investigators found that the high grade calcium salt

aggravated perosis while the laboratory (low) grade salt alleviated the problem. Spectrophotometeric analysis of the salts revealed the absence of manganese in the high grade calcium salts. Addition of 1-2% manganese in the diet alleviated perosis in the chicks. Since that time, manganese supplementation has been found to prevent similar skeletal abnormalities involving the radius, ulna, tibia, and fibula in several species of animals: rats (Amdur *et al.*, 1945), swine (Plumlee *et al.*, 1956) and cattle (Rojas *et al.*, 1965). A similar defect in bone development has also been reported in the offspring of manganese deficient rats (Hurley *et al.*, 1961). The bone volume, bone ash composition, x-ray examination and $AgNO_3$ staining is normal in bones of manganese deficient animals (Caskey *et al.*, 1939; Amdur *et al.*, 1945; Parker *et al.*, 1955). However, the epiphyseal cartilage cell sequence, including proliferation and growth of cells, matrix formation and maturation is retarded in deficient bones (Wolbach & Hegstead, 1953); therefore, manganese deficiency affects chondrogenesis rather than osteogenisis.

In view of the above conclusions, Leach & Muenster (1962) demonstrated that manganese deficient chicks have an impaired ability to synthesize mucopolysaccharide, i.e. organic matrix of the cartilage. Manganese deficient cartilages had reduced radiosulphate uptake and decreased total concentration of hexosamines and hexouronic acids. The most affected mucopolysaccharide was chondroitin sulphate. Subsequently Schrader & Everson (1967); Tsai & Everson (1967) also observed a reduced concentration of acid mucopolysaccharides in ribs and epiphyseal cartilages of newborn guinea pigs.

The biochemical lesion in impaired mucopolysaccharide synthesis

is decreased activity of a mano-metalloenzyme, glycosyl transferase
(Leach *et al.*, 1969; Leach, 1971; Morrison & Ebner, 1971). Glycosyl
transferases are involved in transfer of sugar to a variety of
acceptors and is therefore important in sythesis of polysaccharides
and glycoproteins. In chondroitin sulphate synthesis in the cartilage,
there are two manganese dependent enzyme systems: 1) polymerase enzyme,
which is responsible for the polymerization of UDP-N-acetylgalactosamine
and UDP glucuronic acid to form a polysaccharide, 2) galactotransferase
which incorporates galactose into galactose-galactose-xylose unit,
the cross-link between polysaccharide and the protein.

Other manganese dependent mucopolysaccharide deficiency skeletal
disorders are nutritional chondrodystrophy and poor egg-shell matrix
(Leach, 1976).

1.6.3.2. Neurological disorders

Hurley *et al.*, (1961, 1963) observed that ataxic or nonataxic
offspring of manganese deficient mothers were more susceptible to
electroshock than the supplemented controls. The investigators
postulated that manganese is required for normal function of the brain.

Cotzias *et al.*, (1972) found that pallid mice have a defect in
transportation of manganese coupled with slow transportation of neutral
amino acids, levodopa and tryptophan. Cotzias & collaborators, (1976)
then studied the interaction of manganese with catecholamines in young
mice. These investigators found that brain manganese level in the one
and one half months neonate is dependent on their manganese intake,
4.16 ± 0.14 ug/g in brain of supplemented mice vs. 0.98 ± 0.03 ug/g
in unsupplemented mice. The normally occurring postnatal increase in
cerebral manganese is also accompanied by an increase in cerebral

dopamine and the dopamine levels are susceptible to changes in manganese intake. However, the functional relationship between manganese and dopaminergic apparatus remains to be defined.

1.6.3.3. Reproduction and pregnancy

Defective ovulation, testicular degeneration and high infant mortality were observed in the earliest studies on manganese deficiency in rats and mice (Kemmerer *et al.*, 1931; Orent & McCollum, 1931). In females, two different levels of manganese deficiency can be recognized. Deficiency during growth and development of young females disrupts the estrous cycle (Kemmerer *et al.*, 1931). Manganese deficiency during gestation results in ataxic progeny (Caskey *et al.*, 1939; Hurley 1958). Manganese deficiency in male rats is associated with seminal tubular degeneration, lack of spermatozoa, and therefore sterility (Orent & McCollum, 1931). Decreased fertility due to manganese deficiency has also been reported in cattle. However, experiments by Hartmans (cited by Underwood, 1977)., with identical cattle twins fed low manganese ration from age 1-2 months, revealed no differences in fertility when compared to manganese supplemented controls.

The precise biochemical role of manganese in reproduction has not yet been established but Doisy (1972) suggested that the lack of manganese inhibits synthesis of cholesterol and sex steroids and thereby affecting reproduction. *In vitro* studies on cholesterol synthesis have shown that manganese is required at two different loci of the synthetic pathway: in condensation reaction to form mevalonate and farnesyl pyrophosphate (Underwood, 1977). The effect of manganese deficiency on cholesterol in the living animal does not appear to have been directly investigated (Leach, 1976).

Neonatal ataxia has been reported in a number of species: guinea pigs (Everson *et al.*, 1959), rats (Shils & McCollum, 1943), and pigs (Plumlee *et al.*, 1956). Ataxia is characterised by incoordination, lack of equilibrium, retraction of the head and delayed development of body-righting reflexes. Studies on the critical period for the production of ataxia revealed that ataxia occurred between days 14 and 18 of gestation in the rat (Hurley & Everson, 1963). The otoliths of the inner ear were defective or absent in the ataxic animals (Hurley *et al.*, 1960; Aisling *et al.*, 1960). The biochemical lesion in abnormal otolith development was faulty mucopolysaccharide synthesis (Hurley *et al.*, 1960; Schrader *et al.*, 1973). The otic capsules of the fetuses were examined and the manganese deficient fetuses had no incorporation of radiosulphate into the macular cells of the inner ear resulting in impaired mucopolysaccharide synthesis and abnormal otoliths.

The ataxic condition of manganese deficient fetuses is also observed in mice homozygous for a certain mutant gene called 'pallid'. The offspring of 'pallid' mice supplemented with 1500 ug/mL of manganese were nonataxic. The 50 times the normal level of manganese in the diet offset the effect of the mutant gene. The offspring of the nonataxic progeny were ataxic; therefore, the genetic constitution was unchanged but the expression of the mutant gene was prevented with supplementation (Erway *et al.*, 1966, 1971; Schrader & Everson, 1973). Cotzias *et al.*, 1972 studied the effect of 'pallid' gene on Mn metabolism. The transportation of 54-Mn, levodopa, and tryptophan was slower through the tissues of the pallid mice than the controls.

1.6.3.4. Other effects of manganese deficiency

Bell & Hurley (1973; Hurley *et al.*, 1963) observed abnormalities in cell function and ultrastructure of manganese deficient tissues. In the

mitochondria there are alterations in the integrity of the cell membranes, the endoplasmic reticulum are swollen and there is an increase in the lipid content of the cells. These structural changes are believed to decrease oxadative phosphorylation in the liver mitochondria.

Prothrombin, a glycoprotein, formation is controlled by Vitamin K. The major function of Vitamin K is the addition and completion of the glycosyl portion of the prothrombin (Pereira & Couri, 1971). Doisy (1972) found that manganese deficient chicks had reduced clotting response to Vitamin K and he postulated that manganese may be necessary for the full activity of uridine diphosphoglycosyl transferase in the synthesis of preprothrombin. Leach (1976) has been unable to reproduce the above phenomenon.

Schrader & Everson (1968) observed aplasia or hypoplasia of the pancreatic tissue in the congenital manganese deficient guinea pigs. The animals that survived to an adult age exhibited diabetic-like glucose tolerance curve and Mn supplementation reversed this phenomenon. The mechanism of action is not known.

Manganese can activate a large number of kinases, decarboxylases, hydrolases and transferases (Vallee, 1964). In manganese deficiency, most of the enzymes are activated by magnesium or other divalent cations with no decrease in their functional capacity (Scrutton et $al.$, 1966, 1972). At present, glycosyl tranferase is the only known mano-metalloenzyme in mammals (Leach, 1976). Avimanganin in avian liver and bacterial superoxide mutase have also been identified as mano-metalloenzyme (Utter, 1976).

1.6.4. Manganese deficiency in man

Manganese deficiency in man has not been clearly demonstrated. Manganese is present in all animal and plant foods (Burch & Hahn, 1979). Doisy (1972)

presented a single case of accidental manganese deficiency in man. The
patient exhibited poor clotting, weight loss, dermatitis and slow growth
of hair and beard. Experiments on manganese deficiency under controlled
conditions are considered unethical because of its suspected sterilizing
effects.

Tardive dyskinesia, a prolonged and sometimes permanent extrapyramidal
syndrome, occurs in patients on prolonged treatment with neuroleptic agents.
Anticholinergic and antiparkinson agents have no beneficial effects. Kunin
(1976) supplemented such patients with manganese and had a favourable
response in 14 out of 15 patients. The rationale for the treatment was
that the neuroleptics, like phenothiazines, are potent manganese chelators.
Manganese is present in high concentrations in the extrapyramidal systems
and these patients were manganese deficient.

Dr. Y. Tanaka monitored manganese levels in pediatric patients with
convulsive disorders. The blood manganese level in convulsive patients
was considerably lower than the controls, 6.0 - 8.5 ng/mL in the patients
vs. 10.8 - 40.4 ng/mL in the controls. He found similar decreases in
adult convulsion patients.

Hambidge & Dreogmuller (1974) monitored manganese levels during
pregnancy and found a significant decline in hair manganese from the 16th
to the 38th week of gestation. No change in plasma manganese levels were
observed. Hair manganese in small-for-gestational age and normal neonates
were not significantly different (Gibson & deWolfe, 1979).

SYNOPSIS OF EFFECTS OF TRACE ELEMENT DEFICIENCIES : Table 4a

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Iron (Man)	1) Anemia	McFee 1979	: decreased hemoglobin synthesis	
	2) Poor resistance to infection		: decreased synthesis of leukocyte myeloperoxidase	Prasad,J 1979
	3) In pregnancy: mother : maternal anemia : inability to tolerate blood loss : toxemia : premature labor	Beischer *et al.* 1970 Chaudhri 1970 McFee, 1973	: decreased hemoglobin synthesis and impaired circulatory adjustments in pregnancy	Page *et al.* 1976
	4) In pregnancy : neonate : low birthweight : postnatal anemia : poor resistance to infection : impaired behaviour	Bogden *et al.* 1978 Saarinen 1978 Baggs & Miller,1973 Oski & Honig 1978		

Table 4a continued...

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTION	REFERENCES
Zinc (Animal)	1) Growth retardation	Todd *et al.* 1934	: decreased activity of thymidine kinase and therefore, impaired DNA synthesis and cell division	Mills *et al.* 1967
	2) Poor wound healing	Sandstead *et al* 1970		
	3) Delayed sexual maturation	Todd *et al.* 1934	: reduced gonadotropin production and failure of spermatogenisis	Miller *et al.* 1958
	4) Anorexia	Somers & Underwood 1969		
	5) Hypogeusia	Sandstead 1976	: decreased production of gustin	Henkin *et al.* 1973
	6) Parakeratosis	Macapinlac *et al.* 1967		
	7) Poor skeletal development	Hickory *et al.* 1978	: decreased osteoblastic activity at the epiphyseal plate and decreased bone alkaline phosphatase activity	Mills *et al.* 1967

ELEMENT	SIGN OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTION	REFERENCES

Zinc (Animal)	8) Changes in carbohydrate metabolism
	
	9) In pregnancy : mother
	  : increased abortion
	  &resorption of implantation sites
	  : difficult parturition
	
	10) In pregnancy : fetus
	  : decreased birthweight
	  : decreased litter size
	  : congenital malformations
	  : impaired behaviour
		Quaterman et al. 1966
		
		Apgar 1977
		
		Hurley & Swenerton 1966
			: decreased serum Insulin-like activity
			
			: decreased activity of thymidine kinase ?
				Huber and Gershoff 1973

Zinc (Man)	1) Growth retardation
	
	2) Delayed sexual maturation
	3) Anorexia
	
	4) Poor wound healing
	
	5) Decreased immune response
		Prasad et al. 1963a
		
		"
		
		Hambidge et al. 1972
		
		Pories et al. 1971
		
		Beisel 1976
			General biochemical changes: decreased enzyme activities
			in : alkaline phosphatase
			  : lactate dehydrogenase
			  : deoxythymidine kinase in sponge connective tissue
				Prasad et al., 1978

Table 4a continued...

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Zinc (Man)	6) Changes in taste and smell perception	Henkin *et al*.1971b	: ? decrease in gustin	
	7) Abnormal dark adaptation	Morrison *et al*.1978	: decrease in Vit. A mobilization	
	8) In pregnancy : mother			
	: difficult parturition	Jameson, 1976		
	: threatened abortion			
	: atonic bleeding			
	: increased bacterial growth in the amniotic fluid	Tafari *et al*.1977		
	9) Genetic disorder - Acrodermatitis Entero-pathica	Moynahan 1974		
Copper (Animal)	1) Anemia	Beck 1941	: Impaired ability to to absorb and mobilize iron	Osaki *et al*. 1966

Table 4a continued...

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Copper (Animal)	2) Poor bone mineral-ization	Baxter 1953	: cessation of osteo-blastic activity	Rucker & Murray 1978
	3) Swayback	Bennetts 1932	: myelin aplasia	Howell 1964
	4)		: decreased cytochrome oxidase a_3 activity and phospholipid synthesis	Gallagher & Reeve 1971
	4) Falling disease - sudden cardiac failure	"	: decreased activity of lysyl oxidase: impaired synthesis of elastin : deranged aortic elastin tissue	Rucker & Murray 1978
	5) Depigmentation and im-paired keratinization	"	: impaired tyrosine to melanin conversion	O'Dell 1976

	6) In pregnancy : mother
	   : fetal death and resorption
	   : placental necrosis
		Howell & Hall 1969		

Table 4a continued...

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Copper (Animal)	7) In pregnancy : neonate : neonatal ataxia : anemia : retarded development	Howell & Hall 1969		
Copper (Man)	1) Anemia - unresponsive to iron, Vit. B_{12} and folate therapy	Cardano *et al.* 1964	: Decreased level of cerulo-plasmin	Osaki *et al.* 1966
	2) Bone lesion - rarefraction and weakness of the bone	Al-Rashid & Spangler 1971	:	
	3) Developmental delay and failure to thrive	Dauncey *et al.* 1977		
	4) Genetic - Menkes' Kinky Hair syndrome	Menkes *et al.* 1962		
	5) In pregnancy : mother : threatened abortion : miscarriage	Heijkenskjold & Hendenstedt 1962		

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES

Copper (Man)	5) In pregnancy : mother
	   : preclampsia
	   : eclampsia
	   : placental insufficiency
		Jameson, 1976
		Friedman *et al.* 1968		

Manganese (Animal)	1) Perosis 2) Neonatal ataxia 3) Increased sensitivity to electroshock 4) Decreased prothrombin synthesis 5) Sterility 6) In pregnancy : neonate : Ataxia	Wolbach & Hegsted, 1953 Erway *et al.* 1966 Hurley, 1976 Doisy, 1972 Doisy, 1972 Erway *et al.* 1966	: impaired mucopolysaccride synthesis : defective otolith development : decreased glycosyl transferase activity : ?decreased synthesis of sex steriods	Leach & Muenster, 1962

Table 4a continued...

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Manganese (Animal)	7) Abnormalities in mito-chondrial ultrastructure	Bell & Hurley 1973		
Manganese (Man)	1) ? impaired Vit. K metabolism 2) ? impaired cholesterol metabolism 3) Epilepsy (?)	Doisy, 1972 " Tanaka, 1975		
Chromium (Animal)	1) Diabetogenic glucose tolerance response in presence of sufficient insulin in genetic or induced diabetic animals	Mertz, 1969	: Glucose tolerance factor enhances insulin activity	

ELEMENT	SIGNS OF DEFICIENCY	REFERENCES	KEY BIOCHEMICAL FUNCTIONS	REFERENCES
Chromium (Man)	1) ? diabetogenic response in : malnourished infants old age diabetics 2) In pregnancy : mother : ? gestational diabetes	Hopkin *et al.* 1968 Levine *et al.* 1968 Sherman, 1968 Doisy *et al.* 1976 Davidson & Burt, 1973		

1.7. Analytical instruments for trace element analysis

The analytical challenges faced by the trace element investigators
are: 1) the very low concentrations; 2) problems of contamination from
the environment, reagents used in the processing and the analytical
instruments; 3) the form and chemical combination in which the trace
element occurs in nature (Underwood, 1976). The most widely used
analytical instruments for trace element analysis are neutron activation
analysis, emission spectroscopy, and atomic absorption spectrometery.
The instrumental improvements, in the past two decades, have greatly
increased the analytical sensitivity and applicability of these instruments
to a wide range of elements in a variety of sample matrices (WHO, 1973).

No single technique currently provides sufficiently high sensitivity
for all elements now known to be important in the biological systems
(Hieftje & Copeland, 1978). Neutron activation offers great sensitivity
for most elements but the lengthy procedures and expensive instrumentation
are serious drawbacks for routine trace analysis (D'Amico & Klawans, 1976).
Detection limits in emission spectroscopy are poor if the elements are
present in a complex matrix because of spectral interferences, i.e., the
lack of elemental specificity and high background emission from the
flame and sample matrix (Welz, 1976). Flame atomic absorption spectrometery
is applicable to a large variety of sample matrix and elements, but often
requires sample modifications and is not very sensitive for elements in
very low concentrations, e.g., chromium in serum at 1-3 ng/mL.

Instrumental modifications of emission spectroscopy and atomic
absorption spectrometery in the past decade has introduced two new and
more sensitive analytical instruments. The inductively coupled plasma
torches (ICP torch) in emission spectroscopy provides high sensitivity

low matrix interference and multielement analysis capabilities for samples
in solution. However, the ICP system is very expensive to buy and
operate, is bulky and inconvenient. It also suffers from subtle interelement
effects which have not been fully overcome (Hieftje & Copeland, 1978).

The introduction of electrothermal atomization in AAS, has greatly
improved the analytical sensitivity with acceptable accuracy and
precision for analysis of a large range of elements. It usually requires
a very small volume (5-50 μL) of sample and most analysis can be done
without any sample preparation. The low cost, high sensitivity, and
small sample size provides the best compromise for routine analysis
of pediatric samples (Welz, 1976). In flameless atomic absorption
spectrometery, a fixed sample volume is introduced into the furnace
and after thermal pretreatment, it is rapidly atomised. Atomization breaks
up the chemical bonds between the molecules in the sample, freeing
individual atoms. These atoms absorb ultraviolet or visible radiation
emmitted from a hollow cathode lamp at a specific wavelength band. This
results in a high, time-dependent absorption signal whose height or area
is proportional to the quantity of the element under study.

Figure 2 is a diagrammatic representation of the working principles
of an atomic absorption spectrophotometer.
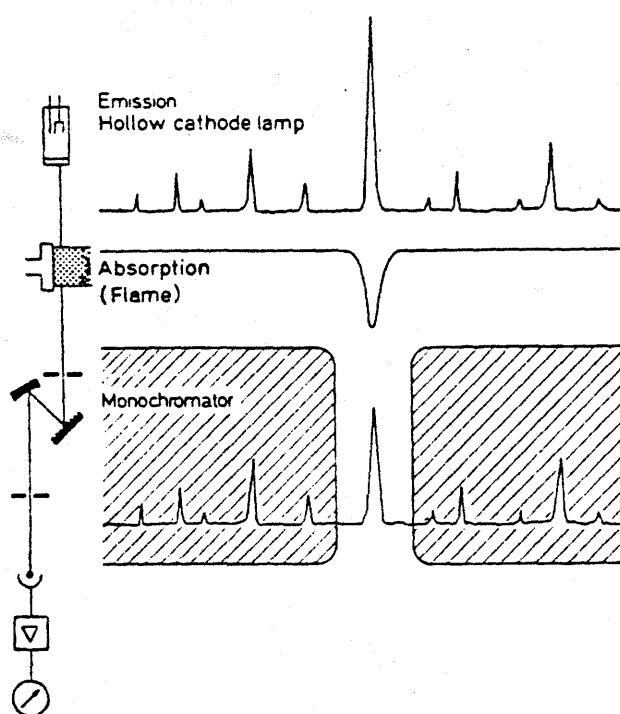
FIGURE 2:   Working principle of atomic absorption spectrophotometer
The spectrum of the element under study is emitted from
a hollow cathode lamp. In the flame or graphite furnace
a portion of the resonance line, corresponding to the
concentration of this element is absorbed. Lines that do
not occur in absorption are not attenuated. After dispersion
of the light in a monochromator the resonance line is
seperated by the exit slit and all other lines are masked.
The detector 'sees' only the resonance line, whose
attenuation is then displayed. (Reproduced with the
permission of Verlag Chemie, Florida) Welz, 1976.

1.8. Objective of the study

Normal fetal growth and development depends on the maternal uterine environment and the genetic inheritance. The fetal demand for nutrients and growth stimulating factors and their supply by the maternal environment and the placenta is in a critically delicate balance. The integrity of this balance determines the normal evolution of the conceptus. The fetal transition to neonatal life and its further development can also be jeopardized by the stress and complications of parturition.

Nutritional deficiencies of trace metals (chromium, copper, iron, manganese, and zinc), in pregnant animals, results in impaired fetal and neonatal development and difficult parturition. Similar nutritional deficiencies in human pregnancy have not been well studied (Table 4a). This project was designed as a pilot study into the occurrence and effects of trace element deficiencies in pregnant Saskatchewan women. The normal serum trace element (chromium, copper, iron, manganese, and zinc) values in nonpregnant women and the fetal umbilical cord blood was established. In the pregnant women, the pattern of changes with advent and course of pregnancy was monitored. Correlations between trace element levels and status of pregnancy, parturition and fetal development was investigated.

The trace elements were analyzed in the Perkin-Elmer Spectro-photometer 603-HGA 2200. The methodologies for analysis of trace elements in serum and urine were also developed.

2. Materials

2.1. Sample selection

The project to evaluate serum and urine trace element levels in

pregnant women was approved by the President' s Advisory Committee on

Ethics in Human Experimentation and the Medical Advisory Boards at

University Hospital, City Hospital and St. Paul's Hospital.

Information regarding the project was distributed through the physicians'

offices in Saskatoon and Yorkton (Appendix 1). All the participants in

the study signed consent forms. The blood and urine specimens from the

pregnant women were collected at the end of the first trimester (12-16 weeks),

second trimester (24-26 weeks), at labor, 3-days postpartum and 7-12

weeks postpartum. Fetal umbilical cord blood samples were obtained at

delivery. This project was a cross-sectional study, rather than a longi-

tudinal one, i.e. the same pregnant women were not monitored through the

full course of pregnancy. The controls for this project were healthy,

non-pregnant Saskatoon women between ages 17-45.years.

Table 5 summarises the number of blood samples analysed in each

category and the participating institutions.

2.2 Demographic and medical information

The medical information on the pregnant women was obtained from

their physicians' reports, and the nonpregnant women were requested to

fill out a card regarding their medical history. The type of information

obtained from the medical reports was: age, parity, preparous

health, previous complicated pregnancies, physician's nutritional assess-

ment, medications, course and history of present pregnancy and fetal

health. The nonpregnant group gave information regarding their age, parity

TABLE 5: Number of participants in the study

Category	Number of women	Institution
1. Nonpregnant women	102	Kelsey Institute University of Saskatchewan General city population
2. Pregnant women		
a) 10 weeks of gestation	36	Selective Test Centre, University hospital
b) 11-16 weeks	65	Selective Test Centre Alvin Buckwold Centre study
c) 24-26 weeks	51	Alvin Buckwold Centre study
d) Term (Labor)	174	St. Paul's Hospital Yorkton Union Hospital
e) Fetal Cord	177	St. Paul's Hospital Yorkton Union Hospital University Hospital
f) 3-days postpartum (lactating)	28	St. Paul's Hospital University Hospital
g) 6 weeks postpartum (lactating)	30	Alvin Buckwold Centre Study

health, menstrual history, nutrition, contraception and medications.

2.3. Equipment

Perkin-Elmer model 603-HGA 2200 flameless atomic absorption spectro-photometer (FLAAS) was used for elemental analysis. The instrument was also equipped with deuterium background corrector and a microprocessor to calculate and correct any deviations in the calibration curve. The glass columns with porous polyethylene bed support for ion-exchange chromatography were purchased from Bio-rad Laboratories. The heating block for hot acid digestion was temperature-controlled Tecam-Dri Block DB-3H from Fischer Scientific Co..

The control of contamination is a very important factor in trace element analysis. The blood was collected in special trace element free, Becton-Dickinson #6526 siliconized vacutainers (Williams, 1979; Reimold & Besch 1978; Lin *et al.*, 1978). The disposable pasteur pipettes (Medical Stores) and polypropylene bottles and tubes (Belarts Products and CanLab) used for processing and storing the blood and urine specimens were washed with 30% nitric acid. The polystyrene conical tubes used for random urine collection, at delivery at St. Paul's Hospital, were not acid-washed. Gibson pipettors, P-20 (0-20 ul) and P-200 (0-200 ul) with polypropylene tips (Mandel Scientific Co.) were used for dilutions and injections of the samples.

2.4. Experimental reagents

The water used for cleaning and processing of samples was of an ultrapure quality. Ultrapure water was produced by reverse osmosis through Millipore R.0 40 unit followed by deionization through a Milli-Q unit.

The acids for dilutions and processing of urine samples were: concentrated ultrapure nitric acid, acetic acid, ammonium hydroxide, perchloric acid and sulphuric acid from J. T. Baker. Triton X-100, a nonionic detergent was purchased from Sigma Chemical Co. and the Chelex-100 mesh size 200-400, sodium form, was from Bio-rad Laboratories.

2.5. Reference materials

Zinc, copper, iron and manganese atomic absorption standards at 1 mg/mL in dilute nitric acid were purchased from Fischer Scientific Co.. Chromic chloride ($CrCl_3.6H_2O$) was purchased at Fischer Scientific Co. and a standard solution, 0.5 mg/mL in 2% (v/v) nitric acid, was prepared in the laboratory. Isolated glucose tolerance factor (GTF) from brewer's yeast was provided courtesy of Dr. D. Shapcott, University of Sherbrooke, Quebec.

Calibrated serum substitute, Cation-cal, was obtained from Dade. The concentration of proteins and major ions (Na^+, Ca^+, K^+, and Cl^-) was equivalent to that of natural serum. The reported levels trace element levels were measured by chemical and atomic absorption methods. Appendix 2 gives the detailed composition of Cation-cal.

3. Methods

3.1. Sample collection

3.1.1. Sera

After an overnight fast, venous blood was collected in siliconized vacutainers. The blood samples from women at first and second trimester of pregnancy were routinely taken between 7:00 and 10:00 AM at their homes. The samples from the Selective Test Centre were collected between 10:00 AM and 4:30 PM. The blood samples obtained at labor were taken at "active labor", by the attending physician or nurse. The fetal venous, umbilical cord blood, was also taken on the ward.

The blood was allowed to clot at room temperature and then centrifuged at 1500 rpm for 20 min. The serum was removed with pasteur pipettes into polypropylene tubes and frozen at -20°C.

3.1.2. Urine

Fasting morning urine was collected in polypropylene bottles. The sample was then aliquoted into a 10 mL portion for elemental analyses and a 1 mL portion for creatinine. The samples were frozen at -20°C. Random urine, at term, was collected on the ward in polystyrene tubes and frozen at -20°C.

3.2. Sample processing for elemental analysis

3.2.1. Sera

The serum was thawed and appropriately diluted with a solution of 0.01% (v/v) Triton X-100.

3.2.2. Urine

Urine was processed by three different methods: 1) direct dilu-

tion; 2) hot-acid digestion; and 3) ion-exchange chromatography with Chelex-100.

3.2.2.1. Direct dilution

The urine sample was thawed and then warmed to dissolve some of the precipitate. An even suspension of urine was diluted 1:2 with 0.2% (v/v) nitric acid.

3.2.2.2. Hot-acid digestion

Hot-acid digestion degrades the urinary organic compounds thus improving the charring phase (Vesterberger & Wrangskogh, 1978). An aqueous standard solution of 0.1 ug/mL of Zn^{+2}, Fe^{+2}, Mn^{+2} and 0.25 ug/mL chromium (Cr^{+3}) was prepared in 0.2% (v/v) nitric acid. A mixture of 1 mL of standard solution or urine, 3 mL of concentrated nitric acid and 1 mL of 30% perchloric acid in a pyrex test tube was digested at 95°C for 2 hours and then at 170°C for 5 hours in a temperature-controlled heating block. The digested sample was evaporated to 1 mL and the trace element was determined in the FLAAS. Water blanks, digested mixtures of 1 mL water, 3 mL concentrated nitric acid and 1 mL 30% perchloric acid, were run to determine the level of contamination.

3.2.2.3. Ion-exchange chromatography with Chelex-100

Chelex-100 resin selectively binds to transition divalent cations and removes it from the sample matrix (Kingston et al., 1978).

The glass columns, with porous polyethylene bed support, were precleaned with 30% nitric acid and ultrapure water. A slurry of Chelex-100, 200-400 mesh size, sodium (Na^{+}) form, was poured to make a bed volume of 2.2 cc.. The resin was washed with three 5mL aliquots of 2.5M nitric

acid and rinsed with two 5 mL aliquots of water. The resin was transformed from Na^+ to NH_4^+ form by washing it with three 5 mL aliquots of 2.0 M ammonium hydroxide. Excess base was removed by two water washings of 5 mL each.

A standard solution of 0.01 ug/mL Zn^{+2}, Cu^{+2}, Fe^{+2}, and Mn^{+2} was prepared. Cr^{+3} at 0.012 ug/mL was added to 100 ml of the standard solution and 0.010 ug/mL of GTF to another 100 mL. The urine was acidified with nitric acid to pH 5.0-5.5. 5 mL of urine or standard solution was gradually applied to the prepared resin and allowed to run through at approximately 2.0 mL/min. Selective elution of Na^+, K^+, Mg^{++}, Ca^{++} and organics was carried out with four 10 mL washings with two 5 mL of water. The trace elements were then eluted with 5.0 mL of 2.5 M nitric acid into polypropylene tubes and analyzed in the FLAAS.

3.3. Determination of the analytical parameters in the FLAAS

The following sequence of experiments were done to determine the optimal operating parameters for each element. The optimum wavelength, slitwidth, hollow cathode lamp current and argon flow rate for each elemental analysis were obtained from the instrument manual. The recorder was used to trace all atomization signals.

3.3.1. Standard copper

An aqueous copper standard, at 0.05 ng/ul was prepared with 0.2% (v/v) nitric acid.

3.3.1.1. Drying parameter: Copper

Twenty microliters of the aqueous copper standard was injected into the furnace and dried at 100° C for 30 secs. The drying process was observed through the quartz window.

3.3.1.2. Atomization parameter: Copper

With drying temperature ($100°C$/30 secs) and charring temperature ($200°C$/10 secs) held constant, 20 uL of copper standard was atomized at $1500°C$ and at successively higher temperatures. The atomization process was carried out with two different modes of electrothermal heating: 1) conventional voltage-controlled heating; and 2) temperature controlled maximum power heating. The furnace was cleaned after each analysis by rapid heating to ensure no "carryover" to the next determination. The best atomization temperature was then determined from a graph of atomization signal vs temperature.

The time required to complete the atomization peak on the recorder determined the duration of the atomization process.

3.3.1.3 Charring parameter: Copper

With drying temperature ($100°C$/30 secs) and atomization temperature ($2700°C$/7 secs) held constant, 10 ul of copper standard was charred for 60 secs at $200°C$ and successively higher temperatures in $100°C$ increments. From the plot of atomization signal vs charring temperatures, the optimum charring temperature for copper was determined.

3.3.2. Serum: Copper

The serum was diluted at 1:5 in H_2O and 0.01% Triton X-100

3.3.2.1 Drying parameter: Copper

Twenty microliters of diluted serum was dried at $90°$, $95°$ and $100°C$. The drying temperature and time was determined as in Section 3.3.1.1.

3.3.2.2. Charring parameter: Copper

Since serum has a complex organic and inorganic matrix, non-specific or broadband absorption as a function of charring temperature was determined.

The deuterium arc energy was aligned with the primary source energy (copper hollow cathode lamp) and when they were approximately the same, the hollow cathode lamp was removed. This allows one to measure the broadband absorption in a serum sample.

Twenty microliters of serum diluted in water and in Triton X-100 was used to determine the broadband absorption. With drying (90°C/50 secs) and atomizing (2700°C/7 secs) temperatures held constant, broadband absorption during atomization was determined at a series of increasing charring temperatures. The copper hollow cathode lamp was then re-inserted and charring temperature was determined as in sec. 3.3.1.3.. The deuterium lamp was used to supress nonspecific absorption.

A graph with serum charring temperatures and broadband absorbance was used to determine the optimum char temperature.

The charring time was determined by measuring broadband absorption at the optimum charring temperature with different times.

3.3.2.3. Atomization temperature

Twenty microliters of diluted serum was dried at 90°C/50 secs, charred at 700°C/50 secs and the atomization temperature determined as in Section 3.3.1.2.

3.4. Contamination

Sources of contamination were evaluated by measuring trace element levels in ultrapure water washings of all the equipment.

3.5. Statistical Analysis

The statistical analysis of the clinical data was done with the Discriminant Analysis (Klecka, 1975) and Multirange Analysis of Variance (Kim & Kohout, 1975) subprogram packages from the Statistical Programs for the Social Sciences (SPSS package)

Discriminant analysis statistically distinguishes between two or more 'groups' of cases. These 'groups' are defined by the particular research situation. To distinguish between all groups the researcher selects a collection of discriminating variables that measure characteristics on which the groups are expected to differ. The discriminating variables are used in some fashion, so that the groups are forced to be as statistically distinct as possible. Discriminant analysis attempts to do this by forming one or more linear combinations of the discriminating variables. These discriminating functions are then used to pursue the two research objectives; analysis and classification.

The analysis aspect of this technique provides several tools for the interpretation of the data. Among these are statistical tests for measuring the success with which the discriminating variables actually discriminate when combined into the discriminating function.

The classification technique comes after the initial computation. Once a set of variables is found which provides satisfactory discrimination for cases with known group memberships, a set of classification functions can be derived which will permit classification of new cases with unknown memberships (Klecka, 1975).

4. Development of the analytical method

4.1 Results and discussion *

The operating parameters for the HGA requires rigid control and must be optimized for each type of biological specimen and element. Therefore, the analytical parameters - drying, charring and atomization - were optimized for each element in serum and urine.

4.1.1 Aqueous standards and serum

The drying temperatures for aqueous standard and serum solutions are selected on the basis of the boiling point of their major liquid component. Since water is the main liquid component of both solutions, the drying temperature selected was $100^{\circ}C$. Attention was given to ensure that rapid evaporation occurred without boiling.

The charring phase is to volatilize selectively, as completely as possible, any "smoke" producing components without loss of the element of interest. These "smoke" peaks are known as broadband absorption and are characterized by their nonspecificity of absorption wavelength. The Broadband absorption determines the level of interference occuring during atomization.

The optimum charring temperature for a pure aqueous solution was the highest temperature at which there was no loss of the analyte of interest (Fig. 3., 4). The time for charring was set at 10 secs, because the aqueous standard was in water matrix and therefore, had no broadband interference. Unlike the aqueous standard, the serum matrix is composed of organic (i.e. proteins, carbohydrates, organic acids, amino acids) and inorganic (sodium, potassium, chloride) compounds and "smoke" from their volatilization

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* The results and discussion of the 'development of the analytical method' are presented together to avoid unnecessary repetition of the text.
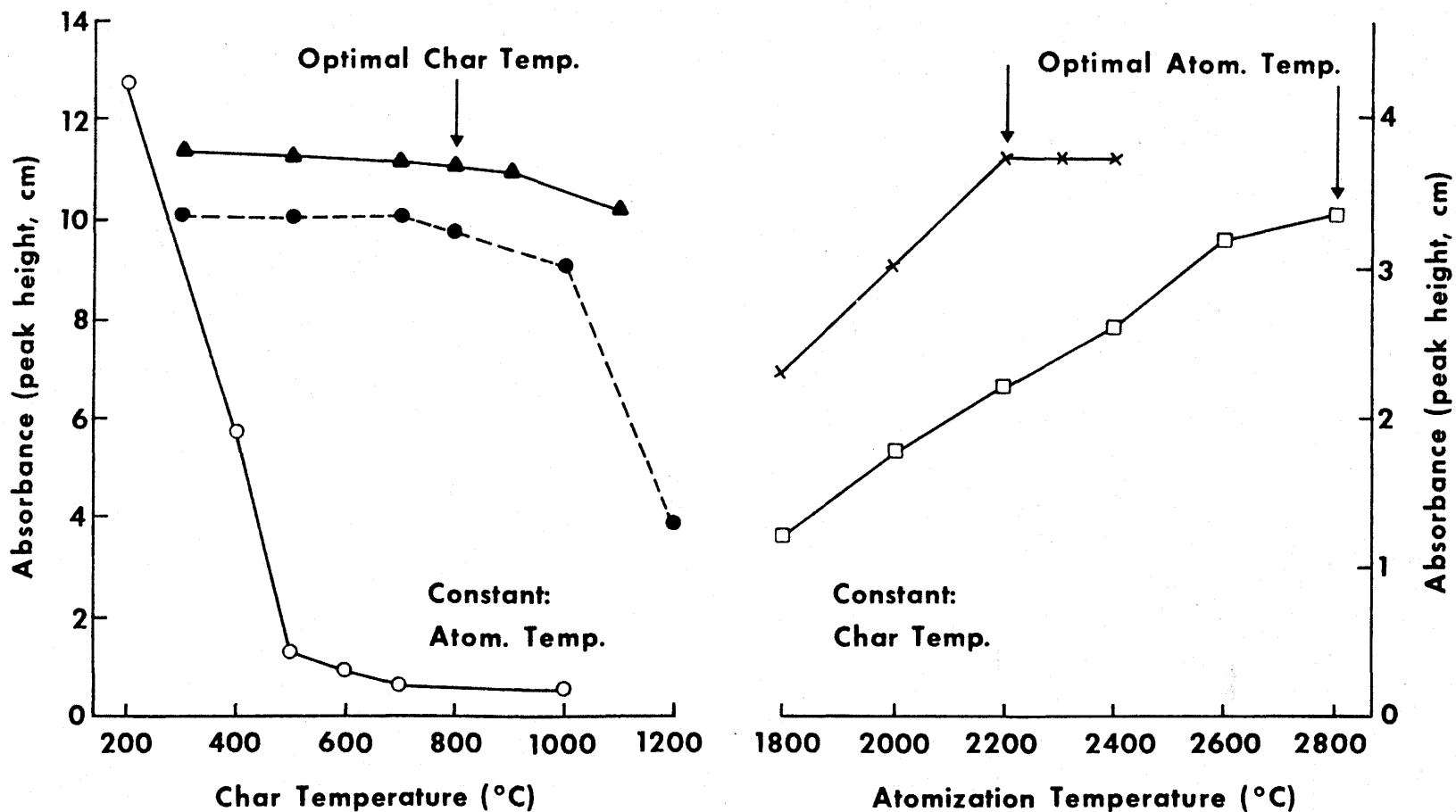
FIGURE 3: Optimal parameters for serum and aqueous copper analysis by the flameless atomic absorption spectrophotometer. The optimum charring temperature was 700°C and the optimum atomization temperature was 2200°C with maximum heating power.

▲————▲ serum solution (1:2); ●— —— —● aqueous standard; O————O Background absorption for serum; x————x Temperature-controlled maximum power heating (aq. standard); □————□ Voltage-controlled heating power
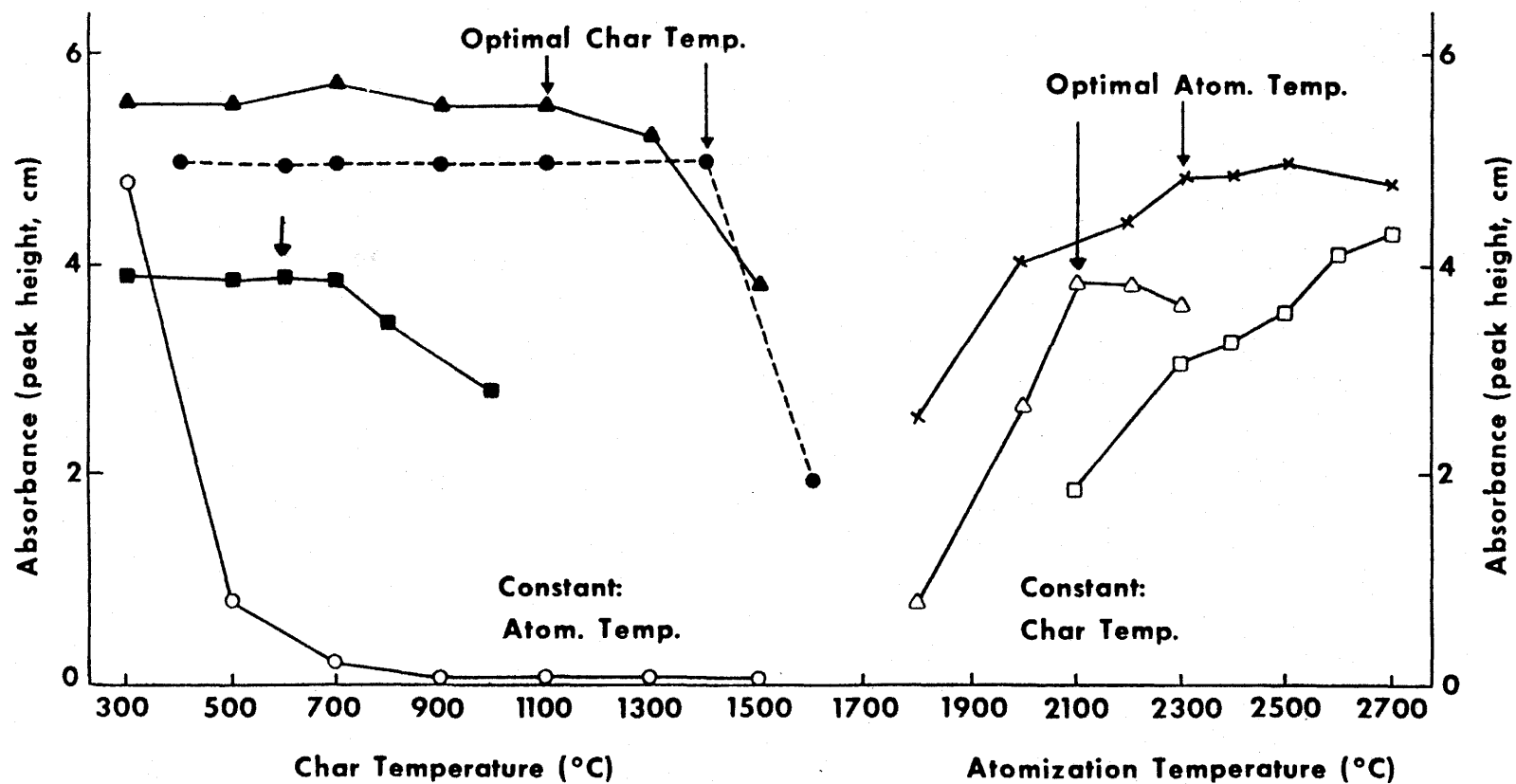
FIGURE 4: Optimal parameters for serum and aqueous chromium using the flameless atomic absorption spectrophotometer. The optimal charring temperature for the organic chromium (GTF) was 600°C and the optimal atomization temperature was 2300°C with the maximum heating power.

▲———▲ - serum solution (1:2); •– – – • Aqueous standard; ■———■ GTF; o———o Background absorption for serum; x———x Temperature-controlled maximum heating power (aqueous standard); △———△ maximum heating power (GTF); □———□ Voltage-controlled heating power (aq. Standard)

constitutes the broadband interference (Fig. 3, 4). The initial large

broadband absorbance, up to 500°C, is due to volatilization of the organic

material and the second, smaller decline is due to volatilization of

inorganic material (Perkin-Elmer, 1978). Comparison of the broadband

absorbance profiles (Fig. 5) of serum in Triton X-100 and in water shows

that a more efficient charring occurs if the serum was diluted in Triton.

Triton X-100 solubilizes complex proteins and improves the charring

procedure. Charring of Triton-serum at 600°C resulted in 40% less "smoke"

at atomization and there was less char material left in the furnace resulting

in improved reproducibility of the elemental analysis. All serum dilutions

were made in 0.01% Triton X-100. At the optimum charring temperature there

was no loss of the analyte and there was insignificant amounts of

nonspecific absorption (Cu - 700°C, Fig. 3).

The best charring time was the minimum time required to produce

insignificant broadband absorbance at the optimum charring temperature,

e.g. 50 secs at 700°C for copper (Fig. 6).

The detectibility* and sensitivity! of an elemental analysis is

influenced by two factors: the surface of the graphite furnace and the

speed with which the temperature is increased for atomization (Welz *et al.*,

1977). For the carbide forming elements (copper and manganese) sensitivity

(upto 5 times) and detectibility (upto 5-20 times) can be reached

if the furnace is coated with a tight layer of pyrolytic graphite.

Pyrolytic coating decreases porosity and total surface area of the

furnace, resulting in reduction in chemical reactivity and carbide

---

* Detectibility: the concentration of an element which is distinctly
                 detectable above the base line (Perkin-Elmer, 1978)

! Sensitivity: aqueous concentration which will cause an absorption
               of 0.0044 absorbance units (Perkin-Elmer, 1978)
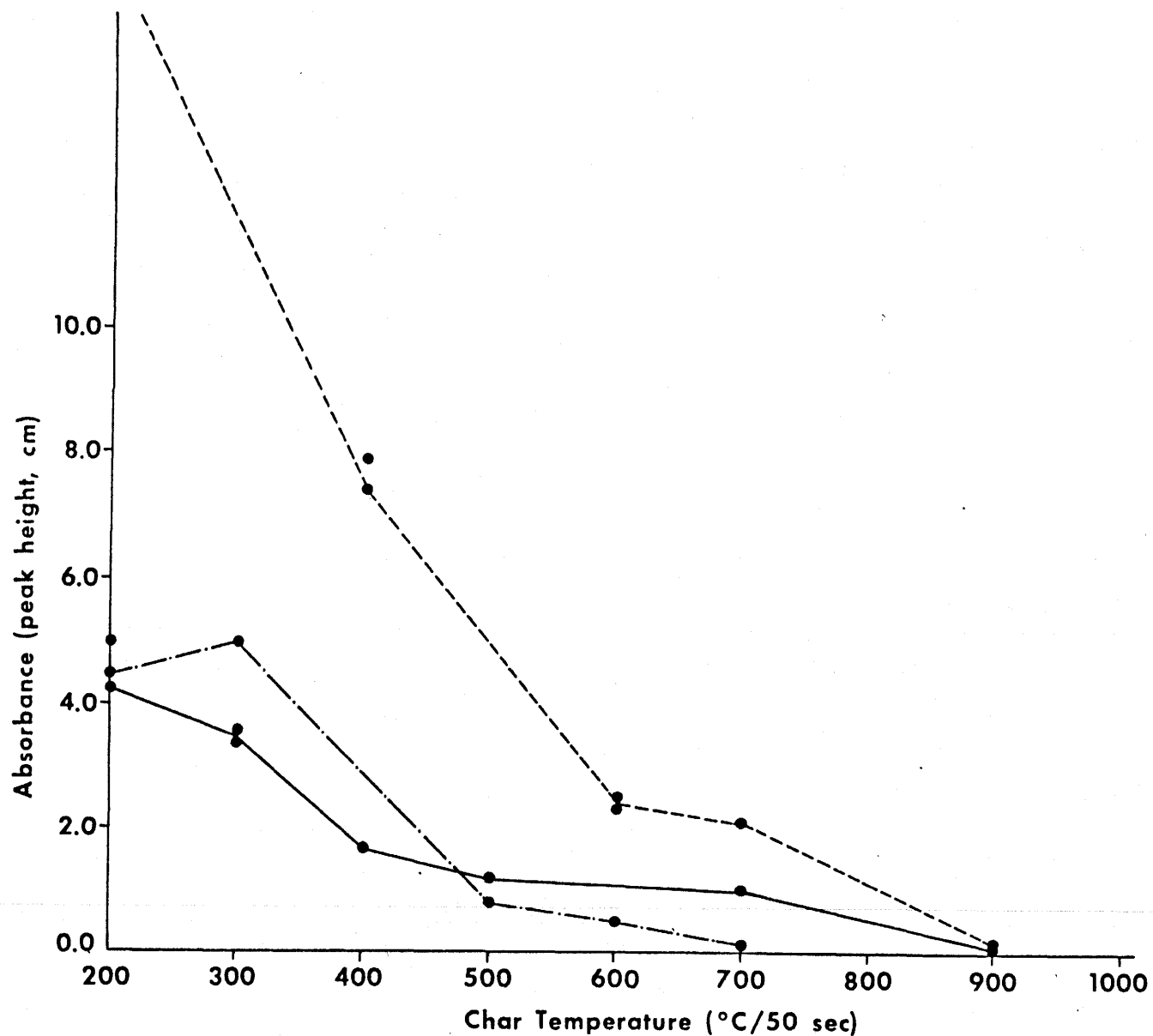
FIGURE 5: Broadband absorption of 1:2 diluted sera at 279.5 nm and 357.9 nm. Sera diluted in Triton X-100 had decreased broadband absorbance and resulted in less accumulation of char material in the furnace giving good reproducibility. •———• Sera + 0.01% Triton at 279.5 nm; •—··—• Sera + water at 279.5 nm; •—·—·• Sera + 0.01% Triton at 357.9 nm.
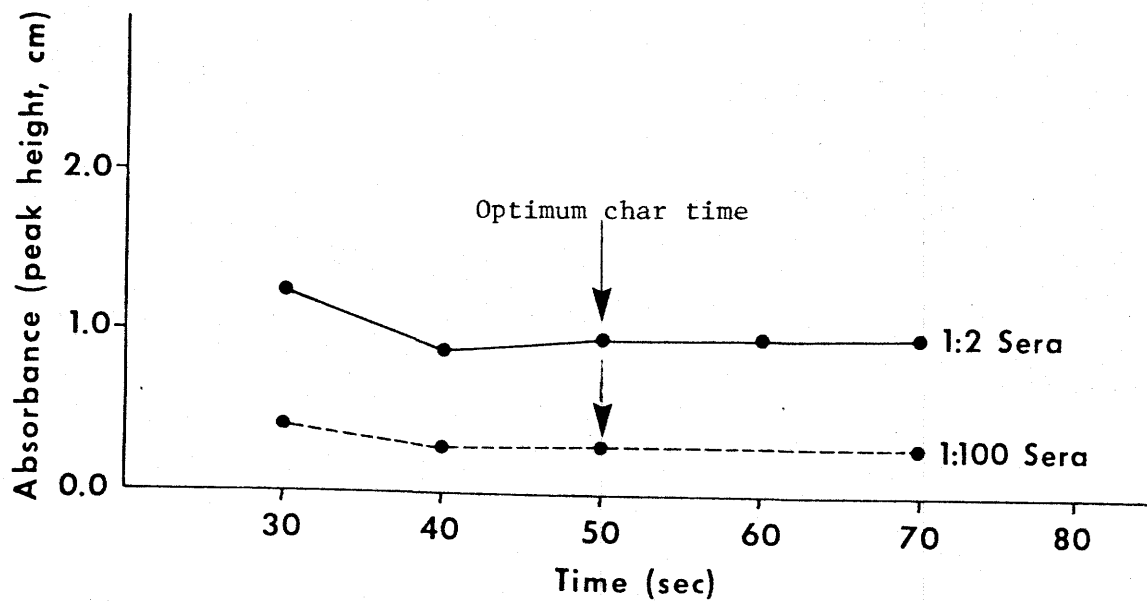
FIGURE 6: Determination of charring time for 1:2 and 1:100 diluted
sera at 600°C. The optimum charring time was 50 seconds.
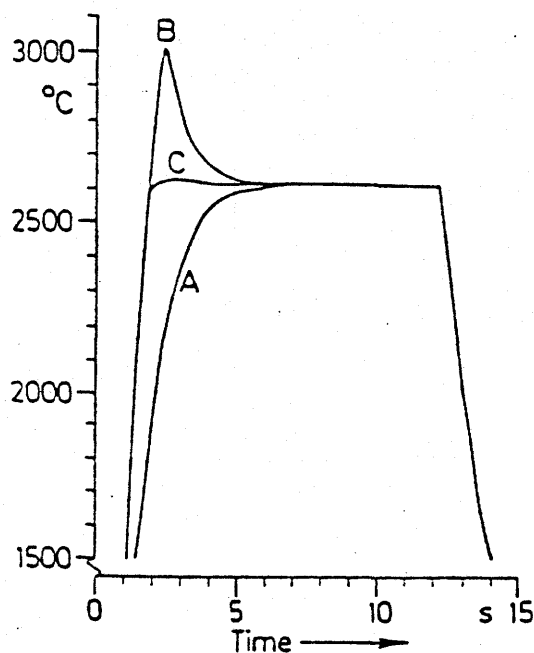
FIGURE 7: Time-temperature profile for the graphite tube heating.
A - normal heating; B - Maximum power heating; C -
Temperature-controlled maximum power heating.
The temperature-controlled maximum power heating results
in rapid rise in temperature without an excessive overshoot
as seen in B. The rapid rise in temperature increases
the efficiency of atomization. (Reproduced with the
permission of Perkin-Elmer Co., Norwalk, Conn.)
Perkin-Elmer, 1978.
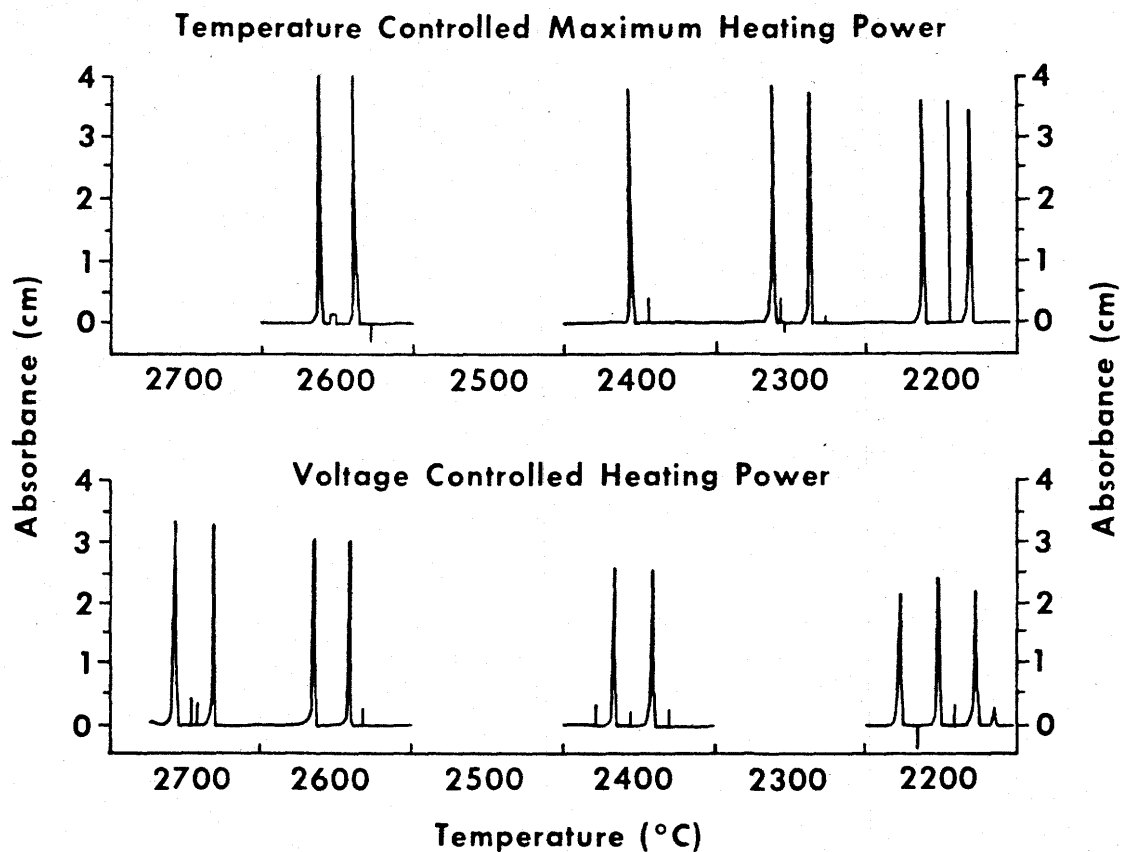
FIGURE 8: Absorbance peaks of copper with two modes of atomization. The maximum heating power mode decreases the atomization temperature by about 400°C and also gives a sharper atomization peaks.

Table 6. <u>Optimal operating parameters for aqueous trace element standards</u>

	Zinc	Copper	Manganese	Iron	Chromium	GTF
Lamp wavelength (nm)	213.9	324.7	279.5	248.3	357.9	357.9
Slit width (nm)	0.7	0.7	0.2	0.2	0.7	0.7
Dry temperature/time °C/secs	100°/25	100°/25	100°/25	100°/25	100°/25	95°/25
Char temperature/time °C/secs	500°/10	700°/10	1100°/10	800°/10	1100°/10	600°/10
Atomization temperature/time °C/secs	2200°/7	2000°/7 maximum power	2400°/7 maximum power	2000°/7 maximum power	2300°/7 maximum power	2200°/7 maximum power
Integration (secs)	4.0	4.0	4.0	4.0	4.0	4.0
Linearity (nanograms)	0.06	.50	.30	.50	1.00	-
Curve correction	yes	yes	yes	yes	yes	yes
Detection limit (picograms)	5	25	25	5	50	-

Table 7. <u>Optimal operating parameters for trace elements in serum and urine</u>

	Zinc	Copper	Manganese	Iron	Chromium
Lamp wavelength (nm)	213.9	324.7	279.5	248.3	357.9
Slit width (nm)	0.7	0.7	0.2	0.2	0.7
Dry temperature/time °C/secs	95°/50	95°/50	95°/50	95°/50	95°/50
Char temperature/time °C/secs	500°/50	700°/50	1100°/70	800°/50	600°/90
Atomization temperature/time °C/secs	2200°/7	2000°/7 maximum power	2400°/7 maximum power	2000°/7 maximum power	2300°/7 maximum power
Integration (secs)	4.0	4.0	4.0	4.0	4.0
Serum dilutions	1:100	1:50	1:3	1:100	1:3
Urine dilutions	1:1	1:1	1:1	1:1	1:1

(1977) found that if GTF was charred at temperatures greater than 500°C, 73% of the analyte was lost. If charred at 500°C, the deuterium (D_2) arc could not adequately compensate for the broadband absorbance, because of poor D_2 emission at 357.9 nm (Fig. 9). Oxidation (Cr^{+3} Cr^{+6}) of GTF in a low temperature asher decreased the volatility of GTF and it could be charred at 1300°C. This alleviated the problem of broadband absorbance. Kayne et al., (1978) oxidised the sample in nitric acid-peroxide medium before analysis. Routh (1979) reduced the nonspecific interference during unoxidized chromium analysis by hydrogen diffusion in the flame AAS. The other alternative is the replacement of the deuterium lamp with a tungsten-halogen lamp because the tungsten-halogen lamp operates in the far ultraviolet range and can adequately compensate for the background absorbance found in Cr analysis (Kayne et al., 1978).

The atomization and charring temperature of isolated brewer's yeast GTF was determined. The maximum charring temperature was 700°C and the atomization (maximum power) was 2200°C (Fig. 4). Accurate analysis of percent recovery of chromium-GTF was not possible since the exact structure is not known. The other problem was poor solubility in water, acidic, basic or alcoholic medium.

The parameters used for serum chromium analysis was 600°C/100 secs char and 2300°C/7 secs atomization with maximum power. The D_2 arc at 357.9 nm absorbed only 60% of the "smoke" peak. Charring time was increased to 100 secs to decrease the "smoke". I believe we were measuring biological Cr but there may be some "smoke" interference even after 100 secs of charring. Present instrumentation limited further monitoring of the smoke interference.

FIGURE 9: The deuterium arc output. The arrows indicate the wavelengths for optimum absorption for each of the elements studied and the corresponding D_2 arc intesity during the elemental analysis. (Reproduced with permission of Perkin-Elmer Co, Norwalk, Conn.) Perkin-Elmer, 1978
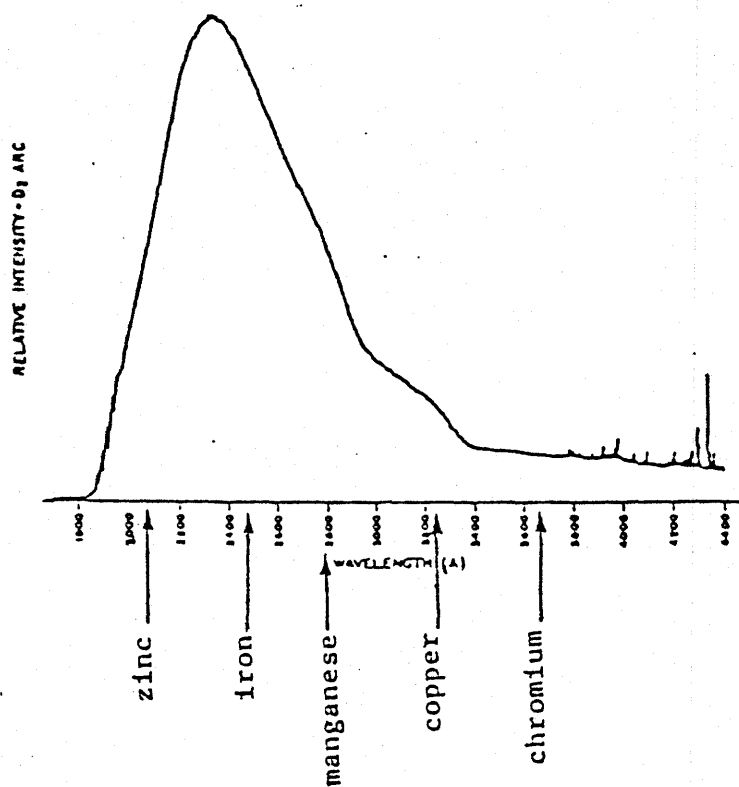
Using the newly developed parameters, a calibration curve was constructed with sucessively higher volumes of a single concentration standard solution. The linear calibration curve obeys the Beer-Lambert Law, i.e., the absorbance is proportional to the thickness through which the light is transmitted and to the concentration of the absorbing substance. Since the range of linearity for all elemental calibration curves was small (Table 6), curve correction was applied to increase this range (Fig. 10). Curve correction is a computer program in the microprocessor of PE 603-AAS, which increases the linear range by correcting any deviations from Beer-Lambert Law. This program can also present the absorbance in the concentration units.

The calibration curves were standardized with a calibrated serum solution, Cation-cal and noncalibrated serum solutions. The elemental levels of noncalibrated serums were also determined by "method of additions". This method is free of matrix interference since it maintains the interference throughout the analysis. The elemental concentration in the Cation-cal and the noncalibrated serum by methods of additions and standard curve had 95% concordance (Table 8). Therefore, serum elemental levels were determined from the standard curves.

4.1.2. Urine

There is a considerable interest in urinary trace element loss in pregnancy and disease states. An attempt was made to develop an analytical method for measuring urinary trace elements.

Fig. 11. shows the broadband absorbance of acidified urine (pH = 4.0) diluted 1:1 with water. The level of "smoke" did not decrease to an appreciably low level until $1100^{\circ}C$. This made the direct analysis of zinc, copper, chromium and iron impossible since their charring
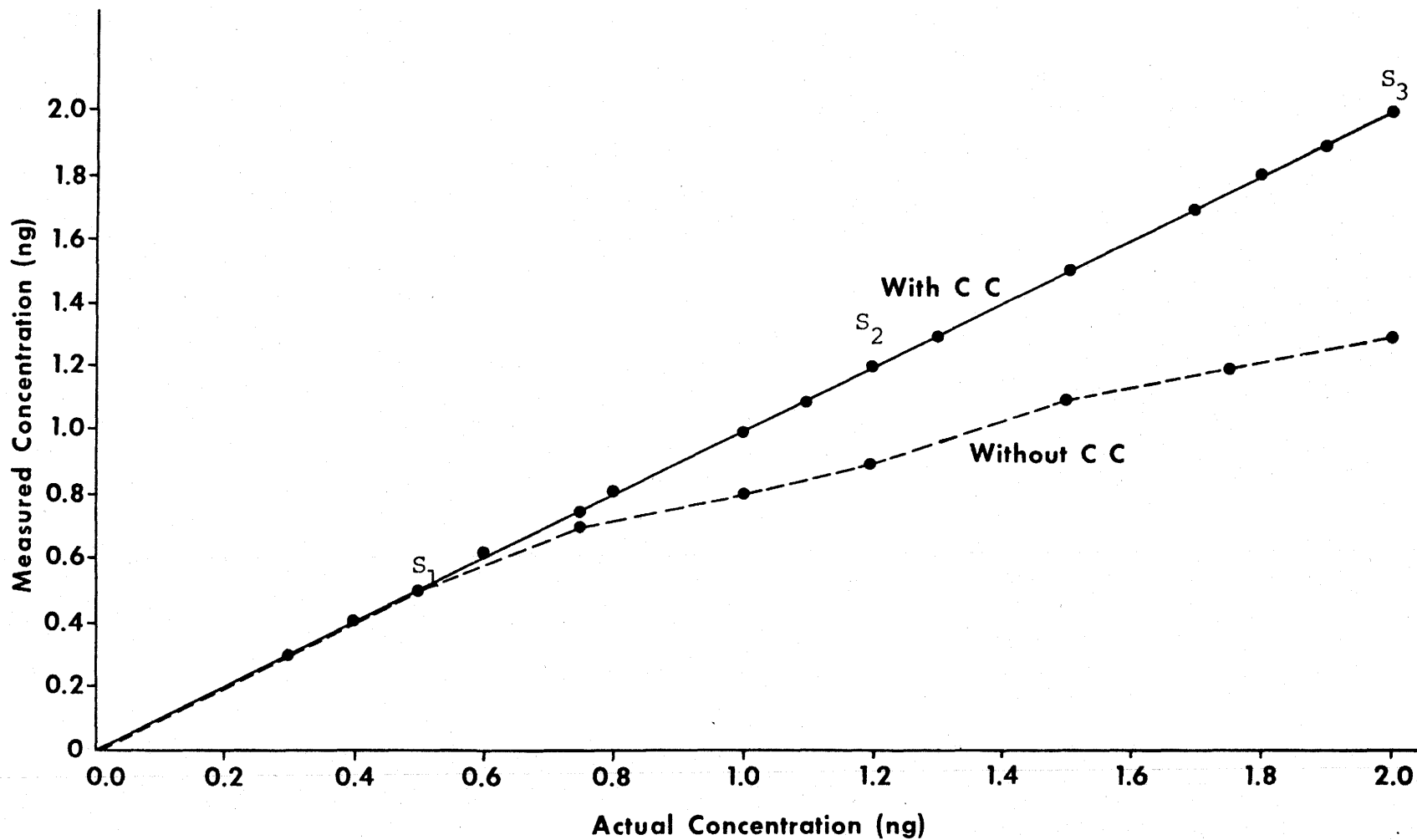
FIGURE 10: Two calibration curves for aqueous copper solution with and without curve correction (CC). Curve correction extends the linearity from 0.5 ng to 2.0 ng. S_1, S_2, S_3 are computational points to maintain linearity.
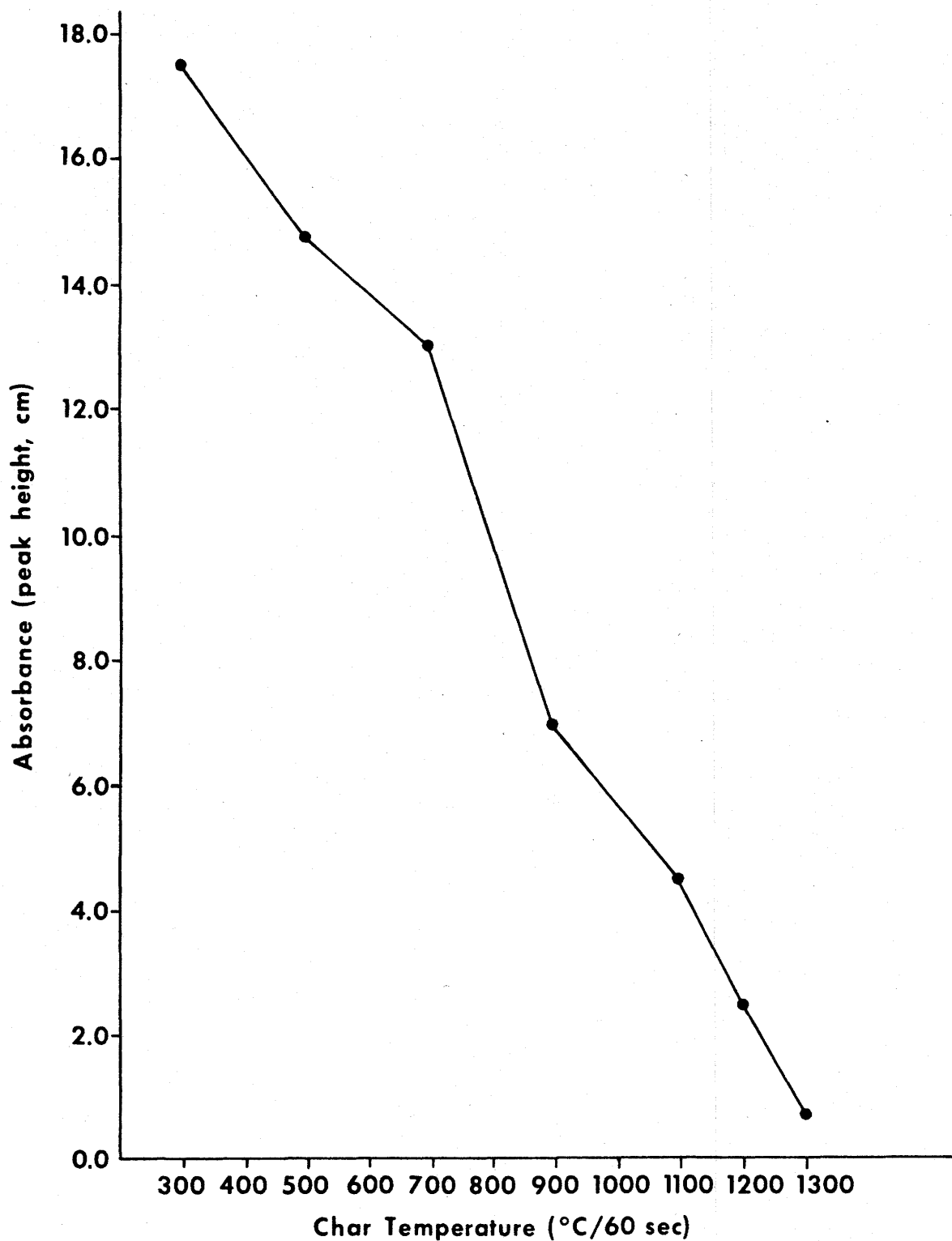
FIGURE 11: Broadband absorbance profile of 1:2 diluted urine at 279.5 nm. Broadband absorbance did not decrease to an appreciably low level until 1100°C.

tried. Fig. 9 shows the working range of the deuterium lamp and only partial control of the background could be obtained for zinc, copper and chromium. Alternative pretreatment methods were tried.

The results from the hot-acid digestion procedure (Table 9) shows decreased recovery (between 70-80%) of zinc, copper and manganese and contamination with iron. The ion-exchange method, Chelex-100 (Table 8) has 95% or better recovery for zinc, copper, manganese and iron, but only 33% recovery of inorganic chromium and 40% for GTF.

Urinary elemental levels were determined after all three pretreatment procedures (Table 10). The urinary levels of zinc, copper, iron and manganese by direct analysis and hot-acid digestion methods were similar. Chromium was 48X higher (677 pg/mL hot-acid digested urine vs 14 pg/mL direct analysis) when hot-acid digestion method was applied. The hot-acid digestion process oxidizes urinary GTF to a less volatile form to chromium (Kayne et al., 1978). Conversion of GTF to a less volatile form is believed to prevent the analytical loss observed during the elemental analysis (Kayne et al., 1978, Shapcott et al., 1977). Therefore, Cr levels found in urine samples following the hot-acid digestion may be the true Cr value. Full investigations into the properties of GTF were beyond the scope of this thesis and therefore, were left unresolved.

4.1.3 Contamination and quality control

Negligible trace element contamination is found in the vacu-tainers and other equipment (Table 8). Ion-exchange process is free of contamination but the hot-acid digestion procedure has a small level of contamination for zinc and copper and is very high for iron.

Quality control was maintained by determining appropriate elemental levels (Zn, Cu, Fe) in Cation-cal after every 15 serum samples. Chromium

TABLE 8:   Contamination and quality control

	Zinc	Copper	Iron	Manganese	Chromium
Vacutainers (n = 10) ng/mL	0.01 ± 0.05	0.06 ± 0.02	0.03 ± .01	0.04 ± 0.02	0.02
Precision of pipetting (n = 20)	99%	96%	99%	95%	90%
Reproducibility (n = 10) Cation-Cal	97%	108%	108%	–	–
Reproducibility (n = 10) Sera	95%	94%	96%	95%	90%

Method of addition and direct analysis

TABLE 9: Percent recovery of trace elements in an aqueous solution after three pretreatment procedures

	Zinc	Copper	Iron	Manganese	Chromium	GTF
Nitric acid dilution	99%	99%	99%	95%	95%	--
Ion-Exchange	95%	95%	96%	90%	33%	40%
Hot-acid digestion	80%	85%	contamination	70%	80%	--

Ion-exchange method is applicable to all trace elements except chromium. Chromium analysis is best after hot-acid digestion.

TABLE 10: Urinary elemental levels after three different pretreatment procedures (mean ± 2 SEM)

	Zinc ug/mL	Copper ng/mL	Iron ng/mL	Manganese pg/mL	Chromium pg/mL
Nitric acid dilution (n = 10)	21 ± 5	38 ± 6	21 ± 8	15 ± 5	14 ± 2
Ion-exchange (n = 10)	–	43	42 ± 10	6 ± 4	1
Hot-acid digestion (n = 10)	19 ± 3	34 ± 10	contaminated	20 ± 10	677 ± 150
Expected values (literature review)	0.5 - 0.8 mg/day	30 ug/day	0.08 mg/day	0.2-1.4 ng/day	200-700 pg/mL

Ion-exchange method is applicable to zinc, copper, iron and manganese and the hot-acid digestion process is best for chromium analysis.

and manganese levels were monitored by redetermining a few of the
previously analyzed samples with each new batch of analysis.

5. Results

5.1. Analysis of demographic data on nonpregnant and pregnant women

The controls in the study were comprised of 100 nonpregnant women. They were healthy, with a mean age of 25 years. Oral contraceptive agents were used by 27% of the women, 8% used an intrauterine devise and 65% were not using any contraceptives (Table 11)

The demographic data on the pregnant group (n = 326) showed that 99% of the women were white Caucasian from the middle socio-economic class. The mean age was 26 years, ranging from 17 to 45 years. No preparous health problems were noted in 92% of the women. Crohn's disease, obesity, epilepsy, renal dysfunction and Marfan's syndrome were observed in the other 8 % in the pregnant women's group. Only 13% of the women smoked during their pregnancy. All the women were assessed to have good nutritional intake and were supplemented with oral iron and vitamins.

The course of the pregnancy was normal in 83% of the cases. The abnormal pregnancies, 17%, consisted of women with toxemia, eclampsia, poor labor history, miscarriage, threatened abortion, anemia, pre- and postmature labor, and postpartum haemorrhage. (Table 12). All the postpartum women in this study were lactating.

The neonates were of normal (38-42 weeks) gestational age in 88% of the cases, 4% were premature and 8% were postmature. The neonatal birth weights were within the expected range for 95% of the deliveries (Table 12).

5.2. Serum trace element profiles

5.2.1. The nonpregnant controls

The serum trace element levels were not significantly affected

TABLE 11: Analysis of demographic data on the women in the
nonpregnant and pregnant groups

Nonpregnant group (n = 100)

1.	mean age	25 (18-45 years)
2.	parity	1 (0-4)
3.	health	good
4.	nutrition	good
5.	contraception	27% oral contraceptive agents
		8% intrauterine device
		65% nonusers
6.	medications	15% on a variety of medications
		85% on no medications

Pregnant group (n = 326)

1.	ethnic background	99% white caucasians
		1% N.A. Indian
2.	economic background	middle socio-economic background
3.	education	cannot assess
4.	mean age	26 (17 - 46 years)
5.	parity	1 (0-12)
6.	nutrition	good
7.	preparous health	8% unhealthy (Crohn's, obesity)
		92% healthy
8.	smoking	17% smokers
		83% nonsmokers

TABLE 12: <u>Outcome of the total number of pregnancies studied</u>*

Mother: course of the pregnancy (n = 326)	85% normal
	15% abnormal
Fetus: gestation (n = 174)	88% normal gestation
	4% premature
	8% postmature
: weight	95% normal birth weight
	5% low birth weight

* This project was a cross-sectional study; therefore, the number of mothers
   and fetuses are not equivalent.

by the menstrual cycle. Women in this analysis were not on OCA.

Significant changes in serum copper and chromium levels were observed in women on OCA. Serum copper level increased (P = 0.05) and the serum chromium level decreased (P = 0.04) (Table 13) in OCA users. The discriminant function coefficient represents the relative contribution of the two most discriminating variables, 0.81 Cr - 0.79 Cu , in distinguishing the two groups, the contraceptive users and nonusers. The two groups are significantly different (P = 0.007), but they are not clearly seperated. The deleterious effect of the overlap is evident in the predictability score, 70.11%. The classification routine was able to identify correctly only 70.11%. The cases as members of the group to which they actually belong. No changes in trace element profile was observed due to parity, age or smoking.

5.2.2. Normal pregnancies

Figure 11a and Table 14 show the changes observed in serum zinc, copper and iron levels before, during and after pregnancy.

The serum Zn declined significantly in the pregnant women at 11-16 weeks of gestation and this decline continued until the 26th week of gestation. The serum zinc, at the end of the second trimester, did not change significantly during the third trimester, 0.93 ± 0.15 ug/mL at 24-26 wk vs 1.03 ± 0.3 µg/mL at labor. In the lactating women serum zinc did not increase to control levels by the end of the 15th week postpartum, 1.88 ± 0.6 µg/mL in controls vs 1.14 ± 0.4 µg/mL in postpartum women (Table 14).

A significant increase in serum copper was observed with onset of pregnancy, and it continued to rise and doubled by the end of pregnancy. The postpartum, lactating women, by the end of the 15th week, had serum copper levels similar to that of the nonpregnant

TABLE 13: The effect of oral contraceptive agents and menstrual cycle on fasting serum trace elements in the nonpregnant controls (mean ± 2 SEM)

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL	Discriminant function coeff.	Predict- ability
Oral contraceptive agents							
a) users (n = 25)	1.88 ± .04	1.38 ± .06	2.17 ± .10	4.00 ± .80	6.20 ± 2.0	.81Cr - .79Cu	70.11%
						$P = 0.007$	
b) nonusers (n = 56)	1.83 ± .04	1.19 ± .04	1.87 ± .06	4.43 ± .07	8.27 ± 2.2		
Probability (discriminant analysis)	0.72	0.05*	0.17	0.32	0.04*		
Menstrual cycle in OCA nonusers							
a) 0-12 days (n = 14)	1.88 ± .36	1.11 ± .22	2.02 ± .58	4.29 ± 1.0	5.87 ± 2.0		
b) 13-17 days (n = 10)	1.79 ± .28	1.81 ± .20	2.10 ± .52	3.81 ± .74	7.43 ± 3.2		
c) 18- days (n = 14)	1.84 ± .26	1.81 ± .24	1.80 ± .46	4.28 ± .88	7.64 ± 2.0		
Probability (discriminant analysis)	0.94	0.88	0.72	0.72	0.53		

Oral contraceptives have a significant effect on the serum copper and chromium levels on OCA users. (* - significant difference).
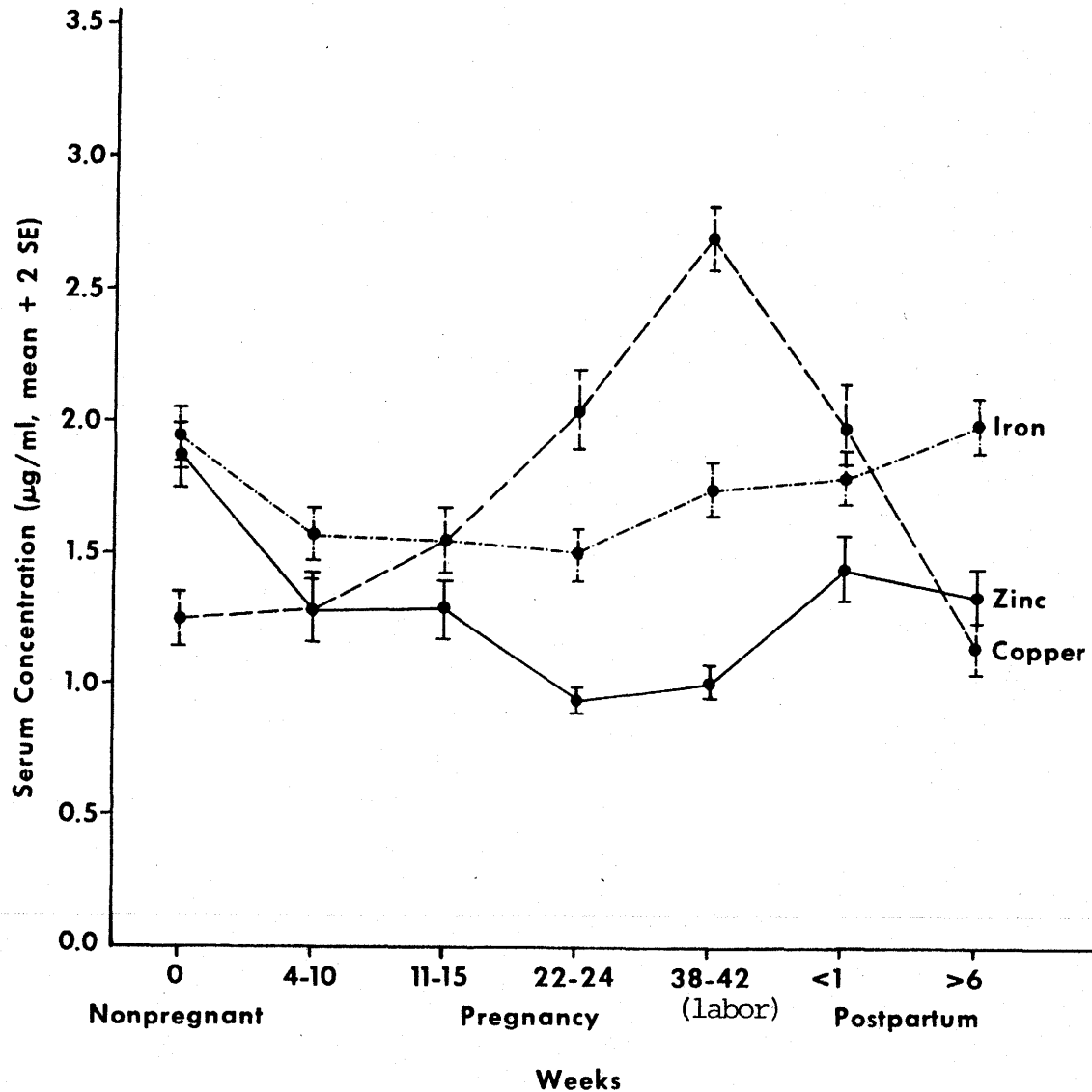
FIGURE 11a: Mean serum zinc, copper and iron in fasting serum of nonpregnant, pregnant and postpartum subjects (mean ± 2SEM). Serum zinc decreases with onset of pregnancy and stabilizes by the end of the second trimester. Some increase in serum zinc is noted in postpartum lactating women. Serum copper increases by 11–16 weeks and doubles by the end of gestation. A sharp drop in serum copper is observed within one week of parturition. Serum iron does not change with the progress of pregnancy.
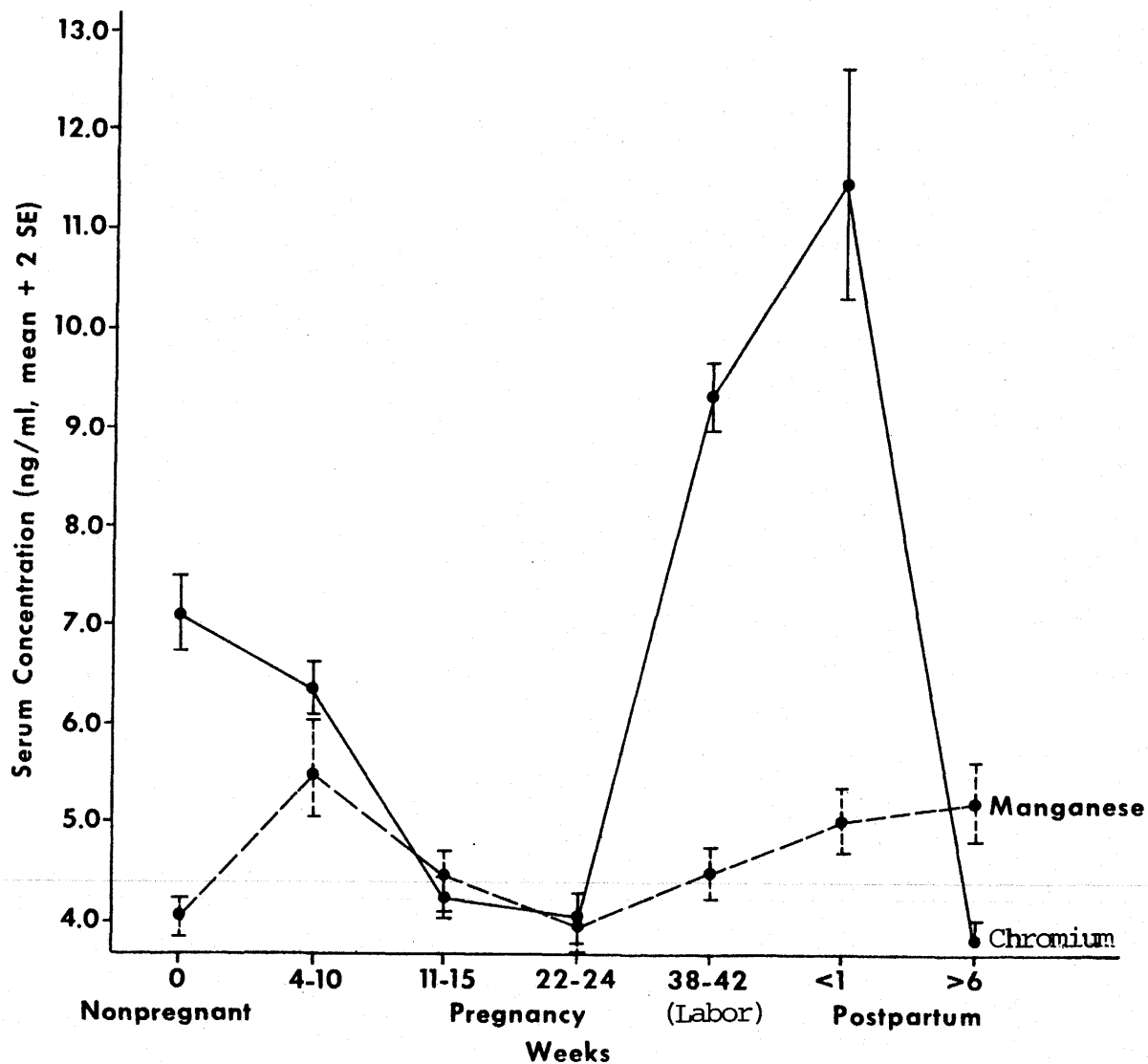
FIGURE 12: Mean manganese and chromium in fasting serum of nonpregnant, pregnant and lactating postpartum subjects (mean ± 2 SEM). Serum manganese does not change significantly with onset of pregnancy. Serum manganese is increased in lactating women. Serum chromium decreases with onset of gestation and rises sharply at labor. Significant drop is again observed in lactating postpartum women.

TABLE 14: Fasting serum trace elements in nonpregnant, pregnant and postpartum women (mean ± 2 SEM)

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
Nonpregnant controls (n=100)	1.88±.12	1.26±.08	1.97±.18	4.33±.38	7.42±.80
Less than 10 weeks of gestation (n=35)	1.29±.13$^+$	1.29±.12	1.58±.22	5.80±.1.0	6.06±1.2
11-16 weeks of gestation (n=64)	1.30±.10	1.55±.12$^+$	1.53±.14	4.73±.32	4.62±.80*
24-26 weeks of gestation (n=51)	0.93±.06*	2.04±.16*	1.50±.16	4.19±.44	4.23±.70
Labor (n=174)	1.03±.06	2.71±.16$^+$	1.76±.12	4.72±.86	8.95±.50*
3-days postpartum (n=29)	1.44±.40*	2.03±.18$^+$	1.80±.34	5.35±2.6	11.50±3.0
<6-weeks postpartum (n=28)	1.14±.14	1.14±.10$^+$	1.97±.30	6.05±1.1	4.02±1.2
5 weeks post miscarriage (n=3)	1.71±.10	1.78±.40	1.84±.2	5.30±1.6	3.90±2.5

The trace element levels of one state have been compared to the levels in the preceding state, i.e. the <10 weeks has been compared to the nonpregnant controls. (* = P 0.05; + = P 0.01)

controls.

Serum iron did not change significantly throughout pregnancy.

Figure 12 and Table 14 show the changes in serum manganese and chromium in pregnancy. Serum manganese did not change during the course of pregnancy but the postpartum, lactating women had a significantly (P < 0.05) higher serum Mn than the controls.

The chromium level dropped with onset of pregnancy, 7.4 ± 1.0 ng/mL in the controls to 4.6 ± 0.8 ng/mL at the end of the first trimester (11-16 weeks). The level was maintained until the end of the 26th week with a sharp rise at labor. The Cr level in postpartum women decreased by the 6th week after delivery. There was an increase in serum chromium at 3 days postpartum but it was not significantly different from the levels at labor because of a large scatter in the data.

5.2.3. Abnormal pregnancies

Discriminant analysis of the data showed that serum trace elements cannot be used as a predictor of abnormal pregnancy (Table 15,). No significant differences were observed between the complicated and uncompli-, cated pregnancies. The discriminant analysis using the most discriminating variables also failed to show any significant differences between the two groups (Table 15). This was reflected in the predictability tests, where the classification accuracy ranged from 51% - 70%. Statistical analysis on serum trace elements in specific abnormalities of pregnancy did not show any significant differences between the normal and abnormal cases (Table 16). No significant differences were observed in women with premature, postmature and normal neonates (Table 17), and also no difference was observed in women with prolonged labor (Table 18).

TABLE 15: Serum trace elements in normal and abnormal pregnancies (mean ± 2 SEM)

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL	Discriminant function coefficient	Predict-ability
10 weeks							
uncomplicated (n=28)	1.24 ± .35	1.34 ± .42	1.52 ± .53	5.95 ± 3.38	5.18 ± 3.97	1.0Cu P = 0.31	51.43%
complicated (n=3)	1.27 ± .29	1.08 ± .29	1.58 ± .84	5.50 ± 1.47	5.37 ± 0.21		
Probability*	0.91	0.31	0.87	0.82	0.73		
1st term							
uncomplicated (n=49)	1.28 ± .37	1.60 ± .53	1.48 ± .46	4.67 ± 2.26	4.64 ± 3.06	—	—
complicated (n=8)	1.21 ± .30	1.55 ± .26	1.66 ± .96	4.90 ± 1.65	3.62 ± 0.59		
Probability*	0.99	0.99	0.99	0.99	0.98		
2nd term							
uncomplicated (n=37)	0.95 ± .21	1.97 ± .58	1.47 ± .55	4.19 ± 1.64	4.33 ± 2.71	0.93Fe - 0.69Zn. P = 0.21	69.39%
complicated (n=7)	0.88 ± .26	2.01 ± .41	1.82 ± .76	4.53 ± 2.05	3.56 ± 0.93		
Probability*	0.45	0.87	0.16	0.63	0.46		

No significant differences were observed between the normal and abnormal pregnancies.
(* - calculated probability of differences between the two groups. The test used
was the discriminant analysis - Wilk's Test).

TABLE 15a: Serum trace elements in normal and abnormal pregnancies (mean ± 2 SEM)

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL	Discriminant function coefficient	Predict-ability
Labor							
uncomplicated (n=91)	1.02 ± .32	2.82 ± 1.15	1.75 ± .86	5.26 ± 2.48	9.15 ± 3.18	0.53Cu⁻ ⁻ 0.72Zn	59.41%
complicated (n=52)	1.11 ± .41	2.61 ± 0.88	1.84 ± .90	4.16 ± 2.12	8.68 ± 3.11	P = 0.20	
Probability	0.12	0.25	0.55	0.31	0.39		

No significant differences were observed between the two groups. (* - Probability: calculated probability of differences between the two groups. Discriminant analysis - Wilk's Test was used).

TABLE 16: <u>Maternal serum trace element levels in specific complications of pregnancy (Mean ± 2 SEM)</u>

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
At labor					
Normal (n=130)	1.05 ± .06	2.78 ± .18	1.81 ± .14	4.85 ± 1.2	8.64± 0.60
Prolonged 2nd stage (n=10)	0.95 ± .17	2.92 ± .40	1.56 ± .40	4.12 ± 1.0	9.33± 3.00
Induced labor (n=19)	0.97 ± .16	2.21*± .22	1.47 ± .26	3.35 ± 0.8	8.95± 1.40
Premature rupture of membranes (n=14)	1.04 ± .12	2.44*± .38	1.54 ± .36	3.40 ± 0.7	9.93± 0.70
Premature labor (n=6)	0.97 ± .20	2.78 ± .10	1.68 ± .10	3.47 ± 1.2	8.28± 2.60
Postmature labor (n=10)	1.04 ± .20	2.52 ± .05	1.38*± .20	3.54 ± 1.1	9.65± 1.60
1st trimester					
Normal	1.28 ± .37	1.60 ± .53	1.48 ± .46	4.67 ± 2.3	4.64± 3.06
Miscarriage (n=3)	1.27 ± .20	1.08*± .20	1.58 ± .10	5.50 ± 1.5	5.37± 1.20
Threatened abortion (n=1)	0.79*	1.05*	1.46	5.7	3.45

* - indicates a trend towards difference from the normal trace element level.

TABLE 17:   Maternal serum trace elements with normal and dysmature neonates (mean ± 2 S.E.M.).

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
Premature neonates (n=6)	0.97 ± .22	2.78 ± .84	1.68 ± .92	3.47 ± 1.2	8.28 ± 2.7
Normal gestational age neonates (n=128)	1.03 ± .06	2.71 ± .18	1.80 ± .14	4.86 ± 1.2	8.90 ± 0.6
Postmature neonates (n=13)	1.04 ± .22	2.52 ± .38	1.38 ± .22*	3.55 ± 1.1	9.65 ± 1.6
Probability – discriminant analysis	0.91	0.81	0.26	0.67	0.66

No significant differences were observed in mothers with normal or dysmature infants but lowering of serum iron (*) may be observed in prolonged gestation.

TABLE 18:   Maternal serum elemental levels at labor (mean ± 2 SEM)

	Zinc u.g/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
0 – 10 hours of labor (n=72)	1.01 ± .06	2.82 ± .24	1.77 ± .18	5.17 ± 2.0	8.9 ± 0.8
11 – 15 hours (n=22)	1.16 ± .16	2.82 ± .48	1.59 ± .24	3.27 ± .60	9.0 ± 1.3
15 – 20 hours (n=8)	0.96 ± .21	2.60 ± .88	1.41 ± .44	4.64 ± 1.2	9.0 ± 2.2
20 hours (n=10)	1.10 ± .36	2.74 ± .69	1.92 ± .94	4.28 ± .88	9.1 ± 2.8
Probability – discriminant analysis	0.23	0.95	0.52	0.78	0.99

No association between the trace element levels and hours of labor was found.

5.2.4. Fetal (venous, umbilical cord) serum trace elements

Fetal serum zinc (P< 0.01), iron (P <0.01), manganese (P < 0.01), and chromium (P < 0.05) were significantly higher than the maternal levels at term. Fetal serum Mn was also significantly higher (P< 0.01) than the serum Mn in the nonpregnant women and serum copper was significantly lower (P < 0.01) than the controls and the maternal levels at term (Table 19). No statistically significant differences in trace element profile were observed between normal and SGA and dysmature neonates (Table 20).

TABLE 19: <u>Trace element levels in the fetus, controls and mothers at term</u> (mean ± 2 SEM)

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
Nonpregnant controls (n=89)	1.84 ± .18	1.24 ± .10	1.97 ± .20	4.26 ± .60	7.39 ± 3.00
Pregnacy at term (n=143)	1.03 ± .16*	2.74 ± .16*	1.78 ± .12	4.86 ± .48	8.98 ± 3.11
Fetus (n=152)	1.38 ± .18[+]	0.89 ± .20*	2.60 ± .24*	11.1 ± 1.8*	5.96 ± 2.11[+]

The fetal trace elements were measured in the venous umbilical cord blood. ($*$ - $P < 0.01$; $+$ - $P < 0.05$).

TABLE 20: Serum trace element levels in normal, dysmature and small-for-gestational age age neonates (mean ± 2 SEM).

	Zinc ug/mL	Copper ug/mL	Iron ug/mL	Manganese ng/mL	Chromium ng/mL
Normal (n=135)	1.36 ± .06	0.91 ± .06	2.84 ± .12	10.1 ± 2.6	7.4 ± 3.0
Premature (n=9)	1.20 ± .16	0.86 ± .08	2.60 ± .70	13.1 ± 0.6	5.7 ± .48
Postmature (n=14)	1.23 ± .14	0.89 ± .14	2.17 ± .40	10.6 ± 1.6	5.6 ± 1.4
Probability - discriminant analysis	0.31	0.93	0.10	0.19	0.24
Small for gestational age (n=4)	1.22 ± .07	0.91 ± .05	2.49 ± .20	19.2 ± 4.0	6.0 ± 1.0

6. Discussion

6.1. Nonpregnant controls

The serum trace element levels in healthy nonpregnant controls are not significantly affected by the hormonal changes of the menstrual cycle. The data on the elemental levels at various phases of the menstrual cycle were obtained from a cross-sectional study. Since both the menstrual cycle and the tissue trace elements are highly varied amongst the human population, a longitudinal monitoring of the serum trace element levels will be more beneficial in concluding the effects of endogenous hormonal changes.

The serum copper is significantly increased in women on OCA. This increase is due to the estrogenic component of the OCA and is manifested as increased serum ceruloplasmin (Briggs et $al.$, 1970; Hambidge & Droegmuller 1974; Margen & King, 1975). The erythrocyte copper content and free copper concentration remains constant (Henkin et $al.$, 1971a). Serum chromium is also changed in women on OCA. The reason for the observed decrease is not known. Studies with insulin have shown that in presence of this hormone the body chromium is redistributed from the serum to the tissues (Kraszeski et $al.$, 1979). It is possible that the sex steriods may be having a similar effect on the blood Cr resulting in a significant decrease in the serum Cr in the OCA users.

6.2. Normal pregnancies

6.2.1. Zinc

Significant ($P < 0.01$) decline in serum zinc is observed with onset of pregnancy and this decline continues until the end of the second trimester. The decline is believed to be partially due to increasing estrogen levels (Deemings & Weber, 1978) and due to dilution

with the increasing plasma volume (Jameson, 1976). The zinc level,
after the 26th week of gestation, does not decline despite a 10-15%
increase in plasma volume, 2-3 fold increase in estrogen level and a
concomitant increase in fetal demand. This maintainence of serum zinc
is most likely due to increased absorption from the gut and possible
zinc mobilization from the tissue stores. However, Hurley et al. 1972
observed no zinc mobilization from the bones of the zinc-deficient
dams, while Evans & Reis (1976) did observe bone zinc mobilization in
zinc-deficient mice. Since no such data are available in humans, one
can only speculate on the possibility of mobilization of zinc from the
maternal stores in the liver and bone. The postpartum, lactating
women did not attain the nonpregnant zinc status even after 15 weeks
and this is probably due to loss of zinc through the maternal milk.

The fetal serum zinc is significantly higher than the maternal
serum zinc, but not as high as those observed in the controls. The fetus
actively accumulates zinc in the latter third of pregnancy (Widdowson,
1974) for the rapid postnatal growth. This active accumulation of
zinc from the maternal zinc stores is reflected in the marked differences
observed between the maternal and fetal serum zinc levels at birth.

6.2.2. Copper

The maternal copper level increases with the gestational age and
is doubled by the end of the pregnancy. The increase is almost
entirely due to increased ceruloplasmin synthesis induced by increased
circulating estrogen (Henkin et al., 1971a; Margen & King, 1975). However,
estrogen is not considered the sole factor in increasing ceruloplasmin
level because adrenal hormones, which increase in pregnancy, is also
known to increase ceruloplasmin synthesis (Evans, 1973; Fuchs & Klopper
1977). The increase in copper level is met by increased hepatic

mobilization (Mason, 1979) and with a compensatory increase in copper
absorption by the G.I. tract. The postpartum and postmiscarriage women
attain the nonpregnant copper levels by 6 weeks and, unlike zinc, it
appears that the lactating women have a positive metabolic balance
of copper, since they maintain their nonpregnant copper status, despite
the loss of copper in the maternal milk.

The fetal serum copper level is only one-third of the maternal
level and two-thirds of the nonpregnant controls. However, the fetal
serum copper is not an indication of the fetal liver stores which is
known to increase with increasing gestational age (Widdowson et $al.$, 1972).
The fetal serum copper is very low because the major cupro-protein,
ceruloplasmin, is not synthesized in the fetal liver until after the
onset of postnatal life (Sass-Korstak, 1965).

6.2.3. Iron and manganese

Serum iron remains constant throughout the course of pregnancy.
The lack of any observable change in the serum iron is due to a more than
adequate intake of iron during pregnancy. All the women in the study
were supplemented with oral iron preparations.

Serum manganese remained fairly constant throughout the
pregnancy. Since manganese is present in a very wide variety of foods,
the women in this study may have had an adequate dietary intake.

The serum cord levels for both elements were also significantly
higher than the maternal levels suggesting active prenatal accumulation
of the metal for postnatal growth and development.

6.2.4. Chromium

Chromium level decreases by the end of the first trimester and is
then maintained at the decreased level until the end of the second

trimester. The decrease in serum chromium is probably due to an increase in fetal demand and is also related to the increased insulin glycemic response observed in pregnancy. The dramatic increase in serum chromium at labor is most likely a phenomenon of labor itself, rather than a characteristic of the third trimester. This hypothesis is further supported by Davidson & Burt's work; they observed a decrease in serum chromium at the 36th week of gestation, prior to labor. The mechanism of labor and the associated metabolic changes are not well defined and therefore, it is difficult to pinpoint the biochemical basis for the increase in serum chromium. It could be due to release of the element from the active myometrial muscle cells or due to changes in the tissue response to the circulating insulin.

The observed decrease in serum chromium in pregnancy has been reported by other investigators: Davidson & Burt, 1973; Gurson, 1977; Mahalko & Bennion, 1976; Hambidge & Droegmuller, 1973. There are some serious questions regarding the accuracy of the absolute value of serum chromium in the nonpregnant controls (Table 3) and in pregnancy (1.5.5.3) At present plasma chromium values range from 0.3 - 6.0 ng/mL and the serum values range from 0.14 - 6.1 ng/mL. Due to this controversy over the absolute value and also over the methodology (1.5.2), I think the pattern of change of serum chromium and the relative differences between the normal and abnormal pregnancies are of greater importance than the absolute concentrations.

The fetal chromium concentration is higher than that of the women at 26 weeks of gestation and nonpregnant controls suggesting active accumulation of chromium by the fetus.

6.3 Abnormal pregnancies

6.3.1. Statistical analysis

Analysis of variance and discriminant analysis of the data on normal and abnormal pregnancies showed no statistically significant differences in the trace element profiles of the two groups. A good statistical analysis requires at least 15 cases in each category, and due to the low incidence of problem pregnancies and small sample size, there were fewer than 15 cases in each specific category of abnormal pregnancy.

6.3.2. Anecdotal analysis   (Table 16)

Three of the women in this study miscarried by the 10th week of gestation. Their blood samples had been obtained 3-4 weeks prior to the unfortunate event and upon analysis it was found that the serum copper was decreased in all three cases, 1.08 ± 0.20 ug/mL vs 1.60 ± 0.53 ug/mL in normal pregnancies. The other trace elements were within the normal expected range. Decreased copper levels prior to miscarriage were also reported by Friedman et $al.$, (1968) and is believed that decreased serum copper is an indicator of impaired placental function and an impending miscarriage." In one case of threatened abortion, low serum zinc and copper were found. Decreased serum copper is also observed in women with premature rupture of the membranes (Artal et $al.$, 1979) and in women undergoing induced labor (Table 16). Reduced iron is also observed in women with induced labor and postmature pregnancy. .

The cause and effect of such deficiencies are not known but they may act as an indicator of complications.

The low birth weight and premature neonates show a slight decrease in serum zinc and iron, suggesting inadequate accumulation of the elements during the latter third of gestation. Low iron is also

seen in postmature neonates. The reason for the observed decrease is hard to explain, since the fetus accumulates iron during its *in utero* life and the dramatic decline is only observed with the onset of postnatal maturation (Dallman *et al.*, 1980).

The effect of pathological disorders such as toxemia, eclampsia, hypertension, and abruptio placentae on serum trace elements, despite adequate nutrition, could not be assessed since there were fewer than five cases of each of the various disorders.

6.4. Limitations of the study

' Trace elements in human pregnancy ' was a clinically oriented study and was fraught with the organizational and interpretative problems unique to clinical studies.

Organizational limitations:

1) poor response from the pregnant population resulted in a small sample size

2) unequal representation from all segments of the Saskatchewan population

3) poor response from the physicians and the hospital medical staff resulted in loss of or improper collection of the blood samples.

Limitations of the data:

1) clinical data were often inadequately available

2) the interpretation of the events surrounding the course of, and termination of pregnancy was very subjective and therefore, lacked uniformity. This lack of uniformity seriously hampered the interpretation of the elemental data.

3) unlike animal studies, blood and urine are the only two easily accessible experimental materials in human experimentation. Interpretation of the data obtained from the blood analysis is greatly limited to the immediate events surrounding the collection of the sample. In this particular study, we were able to determine the elemental state of the woman for that particular day but no information regarding her elemental storage states could be determined. It was also impossible to evaluate the possibility of an impending deficiency.

4) large genetic variations and varied dietary habits usually result in wide variations in the normal levels of the blood constituents. Since this project was a cross-sectional study, some of the differences in the elemental values between the normal and abnormal pregnancies may be lost in the large normal variations in serum trace elements. A longitudinal study will be far more beneficial in determining the subtle differences between the normal and abnormal pregnancy.

7. Conclusion and recommendations

Flameless atomic absorption spectrometry can be used to measure serum zinc, copper, and iron levels accurately. However, a number of questions are raised regarding the methodology for measuring serum manganese and chromium. Versieck *et al.* (1980) has strongly indicated that the manganese analysis is fraught with contaminations, but I was unable to determine any major source of contamination in this study. Serum Cr values in the literature range from 0.14 to 6.10 ng/mL and the level in this study were approximately 7.0 ng/mL. The large variations in the serum chromium can be partially explained by the lack of adequate control on smoke interference encountered during the analysis and the loss of the analyte during the analytical process. The recommendations regarding the methodology are wet-acid digestion process as presented by Kayne *et al.* 1979. The other query regarding serum Cr is the validity of such a test. The experts (sec.1.5.2.) suggest that the serum Cr is a poor indicator of body Cr status and only Cr monitored during a glucose load test are valid. The other alternatives are to measure 24 hour urinary excretion and chromium in hair.

The demographic and medical data on the participants in this study suggests that the study was biased towards the middle socio-economic group. Most of the women were in good preparous health and had good nutritional intake during pregnancy. Each serum trace element has a characteristic profile that changes with the onset and course of pregnancy. Serum zinc declines with onset of pregnancy and plateaus at the new low level by the end of the second trimester. Serum copper increases with pregnancy to to twice the levels observed in the nonpregnant controls. The serum chromium decreases with the onset of pregnancy with a very sharp rise at labor. Serum Fe and Mn levels are maintained

throughout the gestational period. The trace element profile of the postpartum women is similar to their nonpregnant counterparts except for the slightly lower serum zinc level.

The fetus actively accumulated all trace elements during gestation and this is reflected by the significantly higher levels observed in the fetus vs. those observed in the mother at the end of pregnancy. The only exception is serum copper and it is due to decreased synthesis of ceruloplasmin by the fetal liver.

No significant association has been found between the trace element profile of the mother and fetus to the various pathological conditions observed in pregnancy. The nutritional trace element intake of the studied population was most likely adequate, since they all had vitamin and mineral supplements and were on good diet. The good nutritional status would have greatly decreased the incidence of abnormalities attributable to mal- or undernutrition.

Based on this pilot study the serum trace elements cannot be used as an indicator of abnormalities in pregnancy. I believe, that a study comprising about a thousand longitudinal pregnancies will give a better indication of the effect of trace element deficiencies in pregnancy. The study should also deliberately incorporate women from low income strata and those suspected to have poor nutritional habits. Recent survey of typical American diets (Klevay et al., 1979; Kumpulainen et al., 1979) and Canadian diets (Kirkpatrick & Coffin, 1977) have shown that these diets have subnormal levels of copper, zinc, iron and chromium and due to the increased demand for these nutrients in pregnancy, trace element deficiencies would definitely be felt if the woman does not change her dietary habits or is not orally supplemented .

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Appendix 1

A2   Informed Consent Form

<u>A Prenatal Study into the Prevention of Disorders in Child Growth and Development</u>

The Alvin Buckwold Centre Department of Pediatrics, University Hospital, Saskatoon would like to collect specimens from you during your pregnancy for the expressed purpose of research <u>into prevention</u> of disorders in growth and development. We would like to establish a biochemical profile of trace elements or minerals (zinc, copper, iron, manganese, chromium), urinary metabolites, hormones and vitamins. <u>This research project   be of value to future generations but if abnormal biochemical values are found in your specimen, your doctor will promptly be informed</u>. The specimens will be collected in the morning by our own staff at your home with as little inconvenience to the doctor and yourself as possible.

The following samples are required if you agree to participate in the project:

a) End of 3rd month of pregnancy - .   fasting blood samples
                                   - a 24-hr. urine collection

b) End of 6th month of pregnancy - a tube of fasting blood sample
                                   - a 24-hr. urine collection

c) At the time of birth   - a tube of maternal blood
                                   - a tube of cord blood
                                   - morning urine

We also request your permission to correlate the biochemical profile with the clinical assessment made by your doctors of your pregnancy and your newborn baby. Strict confidentiality will be maintained.

You are quite free to withdraw at any time from this study.

If you do not agree to participate in the study, this will not affect in any way the quality of medical care from your doctors.

If more information is required or you wish to visit the Alvin Buckwold Centre and its Laboratoy, we will be glad to arrange a visit at a suitable time (343-5901). We thank you for considering our proposal and whatever is your decision, we wish you the best of success.

Respectfully yours,

F.S. Mendelson, Ph.D.   W.A. Zaleski, M.D., M.R.C.Psych., F.R.C.P.(C)
Assistant Professor of Pediatrics   Professor, Department of Pediatrics
Lecturer in Physiology

I agree to participate in the Alvin Buckwold Centre's prenatal study into the prevention of disorders in child growth and development.

Doctor's name_____   Doctor's Signature_____

Patient's name_____   Signature_____

Address_____

Telephone No.: Work_____ Home_____   SHSP#_____

Return to: Alvin Buckwold Centre, Department of   To Be filled in by Doctor:
           Pediatrics, Room 45, Ellis Hall
           University Hospital, Saskatoon   Last Menstrual Period_____
           Saskatchewan   S7N 0W8
                                                     Expected Date of
           Att'n.: ABC Prenatal Project   Confinement_____

LSM/WAZ/cs

UNIVERSITY OF SASKATCHEWAN

DEPARTMENT OF PEDIATRICS

ALVIN BUCKWOLD CENTRE

UNIVERSITY HOSPITAL
SASKATOON. SASK.
S7N 0W8

DIRECTOR: W.A. ZALESKI. M.D
TEL. 343-5177

Dear Dr.

The Alvin Buckwold Centre is carrying out a combined nutrition, metabolic
and endocrine study of human pregnancy with the cooperation of the
Nutrition Science Bureau of Health and Welfare, Canada. The objective
of this overall project is to prevent disorders of growth and development.
In combining 3 separate projects in one, we hope to minimize patient and
physician contact hours and, hopefully, reap maximum information in preparing
an overall nutrition, metabolic and endocrine profile of an individual
patient. An informed consent form is included which explains the protocol.
The patients may withdraw from the project at any time and will only be
approached with the consent of their physician.

If any biochemical measurements show unusual results, this will be brought
to the attention of the physician.

We would like to get some control samples from your region. The criteria
for selection is that they should be between the age group of 18 - 35,
nonpregnant and fairly healthy. Fasting blood sample is preferable and we
will supply the tubes and vacutainer needed.

I look forward to meeting with you and discussing this project at your
convenience.

Respectfully yours,

I.S. Mendelson, Ph.D.   W.A. Zaleksi, M.D., M.R.C.Psych., F.R.C.P.(C)
Assistant Professor in Pediatrics   Professor, Department of Pediatrics
Lecturer in Physiology

ISM/WAZ/cs
encl.

PRINCIPAL'S ADVISORY COMMITTEE ON ETHICS IN HUMAN EXPERIMENTATION

Dr. I.S. Mendelson (EC#78-3)
_____

Your Project entitled ___"Early Diagnosis of Human Growth and Development Disorders'___

_____

_____

_____

has been approved by the Committee.

1. Therefore you are free to proceed with the following conditions:

    _____

    _____

    _____

    _____

    _____

2. Any significant changes of protocol should be reported to me for the

    Committee's consideration in advance of its implementation.

3. If you are applying for funds for this project to any of the following

    agencies, the required form is being completed by this office and forwarded

    to the agency with a copy enclosed herewith for your record:

                                   - National Institute of Health
                                   - Medical Research Council
                                   - Canadian Heart Foundation

                                   Sincerely,

                                   Dr. T.W. Wilson, Chairman
                                   Principal's Advisory Committee
TWW:paw   on Ethics in Human Experimentation

CATION-CAL™ CALIBRATION REFERENCE

For use in calibration of clinical chemistry procedures utilizing atomic absorption and flame emission equipment

SUMMARY

The use of liquid plasma protein-based calibration reference materials has been well established over the past several years in the clinical chemistry field to provide reproducible, consistent samples which behave like patient samples in the calibration (standardization) of clinical laboratory instruments.

Cation-Cal is a bovine albumin-based product whose constituents have been adjusted during manufacture to provide levels suitable for calibration reference values in atomic absorption or flame emission cation analysis.

Determination of the actual cation levels in Cation-Cal are carried out using well-established reference procedures which are standardized against primary standards. This system of reference method assays allows for an independent determination of constituent levels for each lot of Cation-Cal, so that the assigned values are not influenced by any given instrument, or previous lot of calibration material.

The reference method assay values are then utilized as reference set values for atomic absorption or flame emmission instruments.

The set values as well as the relevant reference methods are as shown under Assay Values.

PRINCIPLE

The use of Cation-Cal is based on the principle that a liquid plasma protein-based product, prepared under controlled manufacturing conditions, and having reliably determined constituent values, will be suitable for the calibration of the cation analyses for which it is intended.

Since this material provides a protein matrix similar to a patient sample, it has similar aspiration and background characteristics in atomic absorption and flame emission instruments.

REAGENT   For In Vitro Diagnostic Use

Cation-Cal Calibration Reference: A liquid product prepared by addition of pure chemicals to a 6% bovine albumin base. Constituent values determined by the reference procedures are listed in the assay. A preservative is present. Store at 2-8C.

Indications of possible deterioration of Cation-Cal: any cloudiness, or inability to recover label values for all constituents assayed.

PROCEDURE

Use in accordance with the instrument manufacturer's instructions for calibration reference (standardization) of

each cation determination as listed. The instrument calibration set values for each constituent are shown under Assay Values.

NOTE: This product is recommended for use with flame emission equipment for analysis of sodium and potassium only.

SPECIFIC PERFORMANCE CHARACTERISTICS

The performance criteria listed below detail the specific performance characteristics of Cation-Cal and reflect the reliability and usefulness of the product in calibration of atomic absorption and flame emission equipment.

VIAL-TO-VIAL CONSISTENCY — Assured by homogeneity of the production pool.

RELIABILITY OF ASSIGNED VALUES — means of single analyses by several analysts using well-established reference methodologies (see assay for details).

SIMILARITY TO HUMAN SERUM — in chemical and physical characteristics.

MEMBRANE FILTERED AND LOW BACTERIAL COUNT.

ASSAY VALUES	Lot No. CATC-109 A,B	
CONSTITUENTS	CONCENTRATIONS*	
Calcium[1]	6.2	meq/liter
Copper[1]	205	µg/dl
Iron[1]	208	µg/dl
Lithium[2]	2.0	meq/liter
Magnesium[1]	6.1	meq/liter
Potassium[3]	6.0	meq/liter
Sodium[3]	155	meq/liter
Zinc[1]	301	µg/dl

ASSAY METHODS WITH REFERENCES

1. Atomic Absorption, Instrumentation Laboratory Inc., G.D. Christian and F. Feldman, "Atomic Absorption Spectroscopy, Applications to Agricultui Biology and Medicine", John-Wiley Inc. New York, 1970

2. Atomic Absorption, Zettner, A., Rafferty, K., and Jarecki, H.J., Aton Absorption Newsletter, 7: 32, 1968.

3. IL Flame Photometer.

*Represents the mean value of a minimum of 60 determinations, performed single analyses by each of 3 to 4 analysts on samples representative of the m ufacturing lot.

This product is warranted to perform as described in its labeling and in DADE's literature, and DADE DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR ANY OTHER PURPOSE, AND IN NO EVENT, SHALL DADE BE LIABLE FOR ANY CONSEQUENTIAL DAMAGES ARISING OUT OF THE AFORESAID EXPRESS WARRANTY.

CAT. NO.	PRODUCT	PKG.
85150-1	Cation-Cal™ Calibration Reference	10 x 10 ml
85100	Lab-trol® Chemistry Control	6 x 3.5 ml
85101	Lab-trol® Chemistry Control	6 x 7.5 ml
85102-25	Lab-trol® Chemistry Control	1 x 25 ml
85110	Patho-trol® Chemistry Control	6 x 3.5 ml
85112-25	Patho-trol® Chemistry Control	1 x 25 ml
85103	Moni-trol I ® Chemistry Control	6 x 5 ml
85113	Moni-trol II ® Chemistry Control	6 x 5 ml