THE EFFECT OF ORAL CONTRACEPTIVE AGENTS ON COPPER AND ZINC BALANCE IN YOUNG WOMEN

by

Michael Glen Crews

Dissertation submitted to the Graduate Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Human Nutrition and Foods

APPROVED:

S. D. Ritchey, Chairman

R. E. Webb

C. E. Polan

J. A. Phillips

P. E. Bowen

August, 1978

Blacksburg, Virginia

ACKNOWLEDGEMENTS

The author wishes to express his grateful thanks to Dr. S. J. Ritchey, committee chairman, for his patient advice and encouragement throughout this project. Appreciation is also expressed to the young women who endured the study with so much grace and good humor. Special thanks are expressed to A. G. Dickinson without whose able help the project would have been impossible to complete. Thanks are expressed to Dr. Michael McGillard for his help in statistically analyzing the data. Appreciation is expressed to Dr. Ryland Webb, Dr. Jean Phillips, Dr. Carl Polan, and Dr. Phyllis Bowen for their excellent advice and suggestions during the study. Thanks is expressed to Mrs. Jean Phlegar for her management of the dietary kitchen and help in the drawing of blood samples.

Special appreciation is expressed to Associate Professor Nelson O. Price who so graciously shared his vast trace element experience with the author and for his persistant encouragement throughout this study.

Thanks are expressed to the author's wife for her kind understanding and continual encouragement during this study.

TABLE OF CONTENTS

TABLE OF CONTENTS - continued

LIST OF TABLES

CHAPTER I

INTRODUCTION

With the introduction of the atomic absorption spectrophotometer, trace element research underwent a virtual revolution. New instrumentation and analytical techniques have greatly increased the speed, accuracy, and precision of trace mineral analysis. Accurate analysis of trace elements in concentration orders of magnitude below what was considered possible 15 years ago are now routine. These new laboratory methods have allowed the discovery of the essential need of very small concentrations of some trace elements. This new information when coupled with the ever changing dietary habits and lifestyles of the human population provide an ever expanding need for research in trace element metabolism.

Copper and zinc are two of the most studied trace minerals. Copper, atomic number 29 and zinc, atomic number 30 are members of the first transition series of the periodic chart, and like most of the members of the series have the capability to form strong complexes with organic ligands (30). These complexes of copper and zinc, as well as other members of the series like cobalt, iron, manganese, and chromium may be incorporated into biological systems.

The essentiality of copper was demonstrated in 1928 for rats (72), and six years later in 1934 for zinc in the rat (149). This early work in small mammals implied that both copper and zinc would be found essential for man. The ubiquitous nature of both copper and

zinc seemed to make the likelihood of a frank deficiency state of dietary origin unlikely (107,151).

Studies in Egypt (63) and Iran (117) have since demonstrated that dietary deficiencies of zinc do occur, particularly where diets contain large amounts of phytate or fiber. One genetic disorder has been found, acrodermatitis enteropathica, which results in severe zinc deficiency due to zinc malabsorption (70).

Deficiency states have been shown for animals grazing on copper depleted soils (42), but as yet no uncomplicated deficiency state has been observed in man. Two genetically linked disease states are known for copper, one of which produces severe copper deficiency and the other copper toxicity (42).

Oral contraceptive agents were introduced in the early 1950's (133). Since their introduction there has been a steady increase in the number of women of childbearing age using oral contraceptive drugs so that now it is estimated that one woman of every six in the United States and fifty million women worldwide are currently taking an oral contraceptive agent (122,133). The most common "pill" in use is the combined type with 0.05 mg estrogen and 1.0 mg progestogen (133).

The exact metabolic effects under the pseudo-pregnant state have yet to be fully elicited, but the current research indicates changes in circulating proteins, elevation in serum vitamin A, increased plasma copper, abnormal tryptophan metabolism, decreased plasma levels of vitamin B_6, lowered serum zinc, and deranged plasma lipid patterns

(102). Scientific studies to assess the interactive nature of the effects of combined oral contraceptive agents on copper and zinc metabolism are few (78,96). In most cases the subjects' dietary regime or the level of zinc and copper in the diet have been so altered as to have little practical value in assessing the "real life situation". At least one researcher reported a zinc balance study and ignored both the urinary and integumental loss of zinc (96). The question of what effect oral contraceptives have on a free living population of young women with "normal" intakes of copper and zinc, still remains unanswered.

Therefore the objective of this study was to examine two groups of female subjects living under normal conditions, consuming a diet selected from common foods with copper and zinc levels approximating levels normally consumed. One group of the subjects served as controls and the second group was on oral contraceptive therapy. It was hypothesized that there is a difference in metabolic balance of copper and zinc between the two groups of subjects.

CHAPTER II

REVIEW OF LITERATURE

Metabolic Action of Zinc

The essentiality of zinc for a living organism was first demonstrated in 1869 in aspergellus niger by Raulin (67). Bertrand and deWolf (67) later demonstrated that zinc was necessary for bacterial synthesis of amino acids as well as several enzymes. The essential nature of zinc in plants was demonstrated by Sommer and Lipman (67) in 1919.

The mammalian need for zinc was first demonstrated in the rat by Todd et al. (149) in 1934. The first naturally occurring zinc deficiency with a proven response to supplemental zinc for a mammal was in swine where Tucker and Salmon (151) demonstrated that swine parakeratosis was indeed a disease of zinc deficiency. Work by O'Dell and Savage (108) demonstrated the essential nature of zinc in birds.

Prasad and Halsted (63,117) working in Egypt and Iran were the first to describe the state of zinc deficiency in man. The first observations of zinc deficiency in man produced a complicated syndrome of retarded growth and sexual development with enlargement of the liver and spleen and an attendant severe anemia (61,63,64,117). Supplementation with zinc and a diet adequate in calories produced growth spurts in young men as well as onset of sexual maturation (61,64,132). Carter et al. (24) raised some questions as to the validity of the original work but the differences in supplementation

levels of zinc and protein-calorie levels make their work not comparable with other studies.

The total body content of zinc was calculated by Lutz (17) to be 2.2 g, work by Widdson (67) places body zinc content in the range of 1.4 to 2.3 g. Zinc is found in all body compartments with zinc levels thought to fall in the following ranges: liver 141-245 mg/kg; muscle 197-226 mg/kg; heart 100 mg/kg; bone 218 mg/kg; prostate gland 520 mg/kg; blood plasma 80-120 µg/100 ml (67). These values are somewhat questionable because most of the samples were autopsy material or from patients hospitalized for medical problems, thus possibly not truly representative of normal values.

Zinc Deficiency

Zinc deficiency has been observed in rats, mice, swine, chickens, turkeys, goats, lambs, dogs, Japanese quail, rabbits, squirrel monkeys, hamsters, guinea pigs, and man (17,63,67,117,149,151). The symptoms vary with the specie but include: dermatitis, emaciation, alopecia, ocular lesions, testicular atrophy, retarded growth, breeding failure, anorexia, and sensory disturbances of taste and smell (17, 67,77,83). In addition to the deficiency symptoms in infant or adult animals zinc deficiency, at least in the rat, has proven to be teratogenic (83,85). Hurley et al. (83,85) working with zinc deficient female rats has shown as high as 99 percent teratogenicity with 54 percent resorption of fetuses and 45 percent congenital anomalies. Behavioral impairment and sensory anomalies occur in offspring of zinc deprived female rats (85). The birth anomalies

most commonly seen in pups born of zinc deficient rats are short or missing mandibles, clubbed feet, fused or missing digits, cleft palate, brain abnormalities, and urogenital tract malformations (83,85).

Deficiency of zinc in man produces symptoms or syndromes of severe iron deficiency anemia, hepatosplenomegaly, retarded growth, infantile testes, open epiphyses, spoon nails, rough skin, and hyperpigmentation (67,68). Additional symptoms associated with deficient or marginal zinc nutriture are sensory anomalies described by Henkin et al. (74,77) where decreased or altered taste and smell acuity occur or where wound healing is impaired (27,116). Barcia (6) found a lack of acceleration of wound healing with zinc supplementation, but later work verified the acceleration of wound healing in persons of suboptimal zinc status (67). The zinc deficient state produces endocrine abnormalities associated with hypopituitarism on the basis of poor growth, hypogonadism with decreased corticotropin reserves, abnormal glucose tolerance curves (flattened and delayed), and an increased sensitivity to intravenous insulin (135).

Not as well studied as the severely deficient state but probably of larger consequence is suboptimal zinc nutrition, whether from dietary shortfall or causes secondary to other nutritional deficiencies, medical conditions, or genetic states. Hambidge (68) in surveying children in Denver, Colorado detected about 3 percent of children with: poor appetite, heights below the 10th percentile, and lowered taste acuity, all of which improved clinically with zinc supplementa-

tion. Symptoms associated with such diverse states as spoon nail in beriberi, loss of taste and smell with respiratory infection, anorexia associated with d-penicillamine therapy may all arise from secondary zinc deficiency brought about by either dietary shortfall or secondary to malabsorption brought on by inflamatory bowel disease or other malabsorption states (68). One genetic malabsorption syndrome associated with zinc has been identified, acrodermatitis enteropathica in which zinc is not absorbed efficiently enough to meet body needs with symptoms of zinc deficiency such as chronic skin lesions, diarrhea, alopecia, anorexia, severe growth retardation, and frequent infections (70).

Observations of the teratogenicity of suboptimal zinc status have been made in pregnant women (chronic alcoholics) where 8 children born of women with high caloric intakes of alcohol all showed the same basic patterns of birth anomalies as have been observed in experimental animals (84,88). The infants showed the following anomalies: low birth weight (8/8), short reclining length (8/8), microcephaly (7/8), and bone and joint anomalies (5/8) (88). In a similar study Jameson (86) examined serum zinc concentration in the early weeks of gestation and found congenital defects in six infants whose mothers showed the lowest serum zinc levels. Of three female patients with acrodermatitis enteropathica who reached childbearing age one could not become pregnant and the other two produced offspring with lethal congenital defects (70). Sever and Emanuel (143) examining geographic areas in which problems in zinc nutriture and birth anomalies occur, found

that of the four areas of the world with the highest incidence of zinc dietary problems two of the four have the highest incidence of congenital anomilies of the central nervous system.

Zinc Absorption

The absorption of zinc from the gut presents a problem similar to iron in that the gastrointestinal tract is both the absorptive organ and the major route of excretion of zinc (67). It was originally thought that only a small percentage of zinc was absorbed but Schwarz and Kirchgessner (142) found in rats that intake and absorption followed an inverse relationship with high (90-100 percent) absorption taking place at low levels of intake and lower (34 percent) absorption taking place at high dietary zinc levels. In rats it was further shown that endogenous fecal excretion of zinc exhibited the greatest homeostatic response in the range of optimum zinc intake (142). Radiotracer work with ^{65}Zn in humans show from 20-40% (17) of zinc absorbed from the gastrointestinal tract.

Pearson *et al.* (113) working with everted gut sacs from rats found that zinc is absorbed more efficiently from the distil gut segments. Methfessel *et al.* (100) using the same technique found the duodenum more effective than the distal segments of the gut. Data on the site of maximum absorption in man and on the mechanism(s) of absorption are meager (67), but the rapid appearance of ^{65}Zn in the blood after oral ingestion of the tracer would seem to favor the stomach, duodenum or upper small intestine (17).

The actual mechanism of absorption of zinc is still not completely understood but seems to have aspects of active, facultative, and passive transport (67). A zinc binding ligand has been detected in pancreatic secretions of rats, dogs, and cows (43). A similar ligand is found in milk of rats but not in the intestinal tract of rat pups (84). The structure of the new ligand appears to be the same as prostaglandin E_2 (PGE_2) since it possesses both the optical properties and reacts with antisera for PGE_2 (43). Aspirin is a known inhibitor of prostaglandin synthesis. Rats were treated with aspirin before dosing with ^{65}Zn and demonstrated a marked reduction of zinc absorption. The zinc attached to this ligand appears to be more readily transported into the mucosal cells (43). Zinc can also be sequestered by metallothionein in the muscosal cells either for later transport to the blood or eliminated when the mucosal cells slough off (31). Synthesis of this zinc-metallothionein appears to be governed by serum zinc content with increasing serum zinc causing increased synthesis of the chelator protein which sequesters zinc and causes increased endogenous loss via the gut (31). The changing metallothionein levels function as a homeostatic control in zinc absorption (127). Hoekstra *et al.* (79) studied degradation *in vivo* of copper-induced thioneins in rats and found that zinc protects copper-thioneins from degradation, thereby making homeostasis a multifaceted control of relative links or ratios of metals.

The type of food source of zinc and the meal makeup in regard to protein, fiber, phytate, and levels of other competing trace metals

all affect the amount of zinc absorbed from the food (73,128,134,138). Phytic acid (inositol hexaphosphate) significantly reduces absorption of ^{65}Zn in humans (73). House et al. (81) in an experiment with rats found decreased absorption also, but a concomitant drop in endogenous zinc loss indicating homeostatic control. The addition of protein of a dairy origin to high phytate bread meals increased the apparent absorption of zinc from the bread (134). The typical western diet is considered to be low in phytate but still produces significant drops in absorption of zinc when fed with the zinc or if zinc is administered within 45 minutes after a meal (138).

Geophagia has been suggested as a cause of decreased zinc absorption, but in vivo work with rats fed Turkish clay showed the clay to be a life saving source of zinc for zinc deficient rats (67). Feeding of L-histidine or EDTA for removal of heavy metals from the body causes marked diminishment of zinc absorption and extraction of zinc from body stores (67,123).

Zinc is chelated with transferrin, to a small extent by amino acids, and by albumin in the portal plasma (50). Zinc is transported to the liver after which the zinc appears as zinc-α_2 macroglobulin (30-40 percent) and zinc-albumin (50-60 percent) with the remainder of the zinc appearing as simple chelates of histidine, glutamine, threonine, cystine, and lysine (11,20,50,52,112,121,137). The α_2-macroglobulin which contains 30 to 40 percent of the zinc is viewed as the major zinc-metalloprotein in humans (52). The α_2-macroglobulin bound zinc concentration does not vary with changing zinc status as

does albumin bound zinc and zinc amino acid complexes (52). Binding of zinc to albumin appears to be a function of several variables such as hormonal status, circulating free fatty acids, and phospholipid levels in serum (75). The zinc that is excreted via the kidneys and sweat probably arises from the micro-ligand zinc in circulation (160). Albumin-zinc appears to be a readily accessible transport form of zinc for body systems (160).

Biochemical Role of Zinc

The need for zinc to activate a specific enzyme was first demonstrated in erythrocyte carbonic anhydrase by Keilin and Mann (90) in 1940. Vallie and Newroth (129) in 1954 isolated and purified bovine pancreatic carboxypeptidase and demonstrated the need for zinc in the enzyme. By 1969 the list of known zinc enzymes (112) had grown to 18, a recent publication by Vallie and Wacker (154) lists 70 known zinc enzymes. Zinc is known to be essential for the function of or structural integrity of dehydrogenases, aldolases, peptidases, phosphotase, an isomerase, a transphosphorylase, and aspartate transcarbamylase (129). Each of the six categories of enzymes designated by the Commission on Enzyme Nomenclature of the International Union of Biochemistry contains one or more examples of zinc enzymes (129). These enzymes participate in a wide variety of metabolic processes including carbohydrate, lipid, protein, and nucleic acid synthesis or break down (154).

The atomic structure of zinc is probably both the key to broad biological activity and current lack of knowledge of specific biological

activity (129). Zinc is a member of group IIB of the first transition series of the periodic chart (30). Zinc possesses a complete d-subshell and has a valance of 2 (30). The chemically combined form of zinc is always in the +2 oxidation state, with no evidence of biological reduction-oxidation reactions like iron or copper occurring (30). The flexibility of configuration possessed by zinc allows it to complex with two to six ligands (30). The coordination characteristics of zinc are such that there is no ligand field stabilization, therefore the steriochemistry of zinc complexes is largely determined by ligand size and bonding forces (155). This unusual bonding by zinc has been described by Vallee and Williams (155) as an entatic state where unusual bond lengths, distorted geometries, and/or an odd number of ligands can result in more energetic active sites on enzymes. This variable coordination ability allows zinc to serve both in stabilization roles and active site roles in enzymes. The enzyme alcohol dehydrogenase displays zinc in just such a dual role where of the 4 zinc atoms per molecule, 2 zinc atoms stabilize structure and 2 zinc atoms serve as catalytic sites (122). The chelation characteristics and exchange rates in vitro are different for the two types of sites (129).

Zinc lacks probe properties (paramagnetic spectra) needed to detect ligand makeup and configuration which has led to the use of cobalt substituted zinc enzymes for study of these enzymes per se (153). These Co^{2+}-enzymes are still active while possessing spectral properties which allow researchers to study ligand-metal interaction under a

variety of conditions (153). Much of the knowledge of mode of action of zinc enzymes has been derived from the study of cobalt substituted zinc enzymes.

In spite of strides made in the establishment of basic enzymology of zinc, knowledge of *in vivo* roles for zinc are still rudimentary and the biochemical basis of symptoms of zinc deficiency are still of speculative nature. Price and Vallee (124) have worked with the microorganism *Euglena gracilis* which shows growth arrest, depressed RNA and protein synthesis, changes in cell volume, changes in types of protein accumulated in the cell, and increases in intracellular calcium, manganese, and iron when grown in a zinc deficient medium (124). Hsu and Anthony (82) and Prasad and Oberleas (120) working with rats and Prasad *et al.* (123) with men have shown similar changes in rats and men with significant decreases in alcohol dehydrogenase, alkaline phosphatase, and carboxypeptidase in rats and significant decreases in alkaline phosphatase, lactic dehydrogenase, and ribonuclease in men, when both rats and men were made zinc deficient. McClain *et al.* (99) found changes in the type of collagen synthesized in zinc deficient rats with a 60 to 80 percent drop of incorporation of [Me-^{3}H] thymidine into skin DNA of zinc deficient rats when compared to zinc supplemented control rats. Prasad *et al.* (123) working with induced zinc deficiency in men found similar changes in sponge connective tissue with total protein, total collagen, RNA/DNA, and activity of deoxythymidine kinase upon zinc supplementation. Even with animal and microorganism studies the problem still remains that the mechanism(s) involved in the

production of zinc deficiency syndrome in man and animals is still largely unknown.

Burch et al. (17) cite difficulties in extrapolation across species lines since similar enzymes in different species may or may not be zinc enzymes. The rapid onset and decline of symptoms are hard to explain if action on an enzymatic level only is used (17). Chvapil et al. (28) postulate that zinc may exert a protective effect in membrane maintenance that may account for rapid onset and decline of deficiency symptoms. McMahan (101) noted abnormalities in content of copper, magnesium, manganese, and selenium in tissues of zinc deficient animals and suggests that inorganic ions may regulate cyclic nucleotides (Cyclic AMP and GMP) that in turn stimulate enzyme production and thus function as an off/on biological switch. Hambidge (69) questions whether the central role for zinc may not include thymidine kinase, RNA polymerases, and DNA polymerases required for normal nucleic acid synthesis as being the pivotal point in zinc nutriture.

Metabolic Action of Copper

The essentiality of copper in mammalian metabolism was demonstrated over 50 years ago in the rat (72). The biological need for copper in erythropoiesis opened the way for later researchers to elicit the action of many important copper complexes (41,42,49,107). The exact role of copper in iron metabolism is still surrounded by controversy as to which copper enzyme is the true key, but scientific opinion now is that ferroxidase II and ceruloplasmin are both

responsible for changing Fe^{+2} to Fe^{+3} thereby making the iron exchangeable from transport and storage forms of iron to cellular sites of activity (130,150).

Cytochrome C oxidase is a copper dependent cytochrome containing 1 gram atom of copper per mole of heme (107). The action of cytochrome C oxidase is in the terminal transfer of oxygen to cytochrome C the final step in the electron transport chain (34).

Superoxide dismutase catalyzes the decomposition of the superoxide radical and is sensitive to zinc levels (22). The protection of cellular components and membranes appears to be the major function of superoxide dismutase (42,107). The enzyme, known by several names in various tissues: erythrocuprein, cerebrocuprein, hepatocuprein from erythrocytes, brain, and liver, respectively, is now known to be the same enzyme in all components, superoxide dismutase (22). Superoxide dismutase is suggested to play a role in two very important systems: 1) protection of cytochrome C from fixation in the reduced state by the superoxide radical, 2) the oxidation of xanthine by xanthine oxidase (42).

Monoamine oxidase or lysyl oxidase functions in the oxidation of peptidyl lysine necessary for the formation of α-aminoadipic-δ-semialdehyde, the precursor for crosslinking that occurs in connective and elastic tissue (71). The enzyme is inactive without the presence of copper (71). Bone defects have been observed in rabbits, dogs, chicks, and pigs due not to bone mineralization, but to improper collagen matrix formation (152). Heart lesions and aneurysms in the

aorta and other large vessels have been reported in rats, chicks, and pigs with sudden cardiac failure or rupturing of the aorta or other large vessels associated with defective elastin formation in copper deficient animals (42,71,107,152).

Dopamine-β-hydroxylase is responsible for hydroxylation of DOPA to form norepinephrine in catechlolamine synthesis (107). The effects of copper deficiency on Menkes' disease and nervous disorders in several species, including pigs, sheep, guinea pigs, and rats are postulated to be due to disruption of catecholamine synthesis by the shortage of copper that has a depressing effect on the action of dopamine-β-hydroylase (152). In concert with catecholamine synthesis, tyrosinase, a cuproenzyme, produces a second symptom of copper deficiency in that a drop in copper level drops the activity of tryosinase which is responsible for oxidation of tyrosine to produce DOPA which is further oxidized to a quinone, the precursor of melanin (152). Failure in melanin production leads to lack of pigmentation of wool and feathers. In hair there is an associated, yet unconnected, failure of the sulfhydryl groups to cross link reported to cause the kinky hair syndrome in Menkes' disease (33).

Other copper enzymes are known to exist that as yet do not have a recognized physiological function or are not associated with a specific pathology associated with copper deficiency. Some enzymes of this group are spermine oxidase, benzylamine oxidase, diamine oxidase, and urate oxidase (107).

Copper Deficiency and Toxicity

Copper is ubiquitous in nature and in most areas of the world it is generally assumed that diets contain copper in excess of minimum requirements for most mammals (141). There are however certain parts of the world where livestock suffer from too much or too little copper intake (152). Chronic copper poisoning under natural grazing conditions in multigastric mammals such as sheep and cattle have been observed (152). Acute copper poisoning in humans results in ulcerations of intestinal mucosa, hepatic necrosis, hemoglobinuria, nausea, vomiting, diarrhea, and jaundice (42). Cases of human poisoning are usually self-inflicted or due to acid contact with copper bearing plumbing (17). Frank copper deficiency in humans seems highly unlikely but cases of copper responsive anemia in infants (57) and in patients on total parenteral nutrition (89) have been reported in the literature.

Deficiency states of copper are manifested in various ways among animal species. Anemias of varying degree and type are found in most animal species (17). Incoordination of movement in neonates of many species are common and are probably produced by demyelination of nerves (103). Defects in connective tissue formation, in copper deficiency, produce abnormalities of the protein matrix of bone which produces skeletal malformations (83). Copper deficiency can produce elastin abnormalities in swine, rabbits and chicks that lead to failure of the aorta or other large vessels (83). Most animals other than pigs show alteration of pigmentation of hair or wool when copper is deficient (17).

Two states in man exist as a result of either copper deficiency, Menkes' disease, or copper toxicity, Wilson's disease (18). Menkes' disease represents an inability to mobilize and transport copper to target tissues whereas Wilson's disease represents a failure in metalloprotein biosynthesis (50). The two disease states produce the same pathological effects in humans as are commonly seen in animals in both the deficient state (Menkes' disease) and in the toxic state (Wilson's disease) (17).

Monoamine oxidase or lysyl oxidase functions in the oxidation of peptidyl lysine necessary for the formation of α-aminoadipic-δ-semi-aldhyde the precursor for cross linking compounds that occur in connective and elastic tissue (71). The enzyme is inactive without the presence of copper (71). Skeletal and cardiovascular defects are due to a drop in activity of lysyl oxidase (152).

Tyrosinase, a copper containing enzyme, catalyzes the first two steps in synthesis of melanin from tyrosine. Albinism results from a genetic absence of tyrosinase with achromotrichia and extreme sensitivity to light often observed (42).

Copper Intake

Copper nutrition in man was addressed by Cartwright and Wintrobe (26) who in 1964 stated that based on their research and the research of others (125) a copper intake of 1.3 and 2 mg per day appeared to maintain balance in preadolescent girls and adults respectively. Schroeder *et al.* (141) stated that copper is widely distributed in

foods and most diets provide the 2 mg/day sufficient to maintain balance. Since this 1966 work (141) there appears to have been a reduction in the copper content of the diet (91). Klevay (91) states that as of 1977 the geometric mean copper content of 20 analyzed diets was 0.82 mg copper per day, if one adds one standard deviation, 84 percent of U. S. diets contain less than 1.67 mg of copper per day. Guthrie et al. (59) found 131 of 164 diets contained less than 2 mg copper per day and 38 of the diets provided less than 1 mg per day. The possibility that diets may contain too little copper is currently being recognized in the scientific community.

Copper Absorption

The site of absorption of copper from the gastrointestinal tract varies with species (42). In the rat copper absorption is maximal from the stomach and duodenum (156). In dogs the small intestine is the major site of copper absorption (43). In hamsters the middle one-third of the small intestine is the site of maximum absorption (32). The small intestine in swine is the major absorptive area (13). In humans the stomach and upper small intestine (duodenum) is the site of maximum absorptive activity for copper (8,21). Oral administration of ^{64}Cu to the above species suggests either the stomach or the small intestine as the site of copper absorption (25).

The analysis of luminal contents for copper is not the best indication of the quantity of copper that is available for absorption (50). The form of copper ingested and the dietary components that accompany the copper play a major role in the amount of copper absorbed (17).

Copper is soluble at acidic pH, but at alkaline pH free copper is converted to Cu $(OH)_2$, an insoluble compound which is poorly absorbed unless the pH is again lowered (54). The presence of other transition elements such as calcium, silver, and zinc compete with copper for binding sites (17). The presence of molybdenum, sulfide, or sulfate radicals interfere with copper absorption in an antagonistic manner of unknown mechanisms (17). Co-ingested plant material diminishes copper absorption, perhaps by complexing copper to phytic acid or dietary fiber similar in nature to losses known to occur in zinc (136, 138).

Copper is absorbed from the gut by at least two mechanisms (17, 32). Active transport of a limited quantity of copper is facilitated by amino acids, these copper complexes are either preformed in the diet or arise from the first stages of protein digestion in the stomach (42). The second mechanism is the absorption by a copper binding ligand in the mucosa and later transport to the serosal side or excretion via the sloughing off of mucosal cells (32,43). The amino acid-copper complex is transported very rapidly while the movement of copper from mucosal ligands is somewhat slower and appears to be partially homeostatic in nature (43). One fraction of copper is contained within a protein which has the physical properties of superoxide dismutase (32,43). The second or larger fraction of copper in the cytosol is bound to a sulfhydryl rich metallothionein with a molecular weight of 10,000 (32,42,43). Both proteins have the ability to sequester copper from luminal contents (42,43). The saliva and

gastric juice both have the ability to complex copper, with saliva able to complex 209 µg copper/ml and gastric juice 294 µg/ml in humans (54). The binding of copper by low molecular weight (<5000) compounds in saliva and gastric juice is postulated by Gollan et al. to be protective in nature since the complexing of copper protects the copper from hydroxide precipitation at alkaline pH and from sulfide formation thereby preserving the copper for absorption (54). On the other hand the macromolecular binding substance in bile may inhibit copper absorption by virtue of its affinity for copper so that the net uptake of copper would represent a balance between opposing influences (54).

The identification of a copper-binding ligand in the particulate fraction of the cell and metallothionein in the cytosol by Evans and Johnson (43) led them to speculate that the synthesis of these two components may function in the regulation of the quantity of copper passed into the blood after the ingestion of copper as a form of homeostatic control. Tracer studies in mammals have shown that the movement of copper from the gut to the liver is as a copper-albumin, copper-histidine or as mixed complexes (50). Copper transport is unique in that several copper (II) chelates have been identified in the blood that serve only as transient forms for absorptive transport to the liver while ceruloplasmin is the principle copper-protein of vertebrate serum (50). Copper, upon reaching the liver, is stored as cupreins (hepatocupreins). Metabolism of copper in the liver mainly takes two forms: 1) synthesis of ceruloplasmin and 2) synthesis of

high molecular weight ligands to be routed to the bile for excretion (17,25,50,54,55). Evans and Johnson (43) have recently elicited the makeup of several important metallothioneins using anaerobic conditions of extraction which radically revised earlier work in sulfur amino acid composition. Metallothioneins extracted under anaerobic conditions contained approximately 12 times as much cysteine as the same compound analyzed under aerobic conditions. The presence of copper in the gut in the rat will induce the synthesis of both the copper proteins (43). A second function for the metallothionein may be as a block to protect absorption of toxic levels of copper as well as other trace elements of the first transition series of the periodic chart (42).

Total body copper in a 70 kg man ranges from 80 mg (25) to 120 mg (17). Liver and brain are similar in copper content with liver values being 8 mg (range 4 to 13 mg) and total brain copper 8 mg (range 7 to 10 mg) (25). Total serum copper is approximately 114 µg/100 ml (25) and total plasma copper 109 µg/100 ml (17) in normal nonpregnant adults. The copper content of other tissues such as muscle and bone is approximately 1 µg/gm (17,25). Copper distribution in blood plasma or serum varies with species, but ceruloplasmin is the major carrier protein with 60 to 99 percent of the copper in circulation attached to this metalloprotein (44). Normal human values for ceruloplasmin bound copper is 93 percent with 7 percent as "free" or albumin bound copper (25). Ceruloplasmin values for normal subjects range from 33 mg per 100 ml serum (25) to 38 mg/100 ml (96) in young women to 46 mg/100 ml

in oral contraceptive users (96). Ceruloplasmin values do not change significantly with age but women are significantly higher than men in ceruloplasmin values (162) (women 44±11 mg/100 ml, men 32±8 mg/100 ml).

When ^{64}Cu is administered either I.V. or orally there is a rapid rise in circulatory radioactivity followed by a precipitous drop which coincides with the uptake of ^{64}Cu by the liver with a second elevation of ^{64}copper in circulation taking place with the release of newly synthesized ceruloplasmin -^{64}Cu (25). Copper (^{64}Cu), either I.V. or orally, is first taken up by serum alblumin or the free sulfur amino acid cystine in the blood (25). A notable difference exists in that at least one mammalian species, the dog, does not bind copper with albumin in the serum, probably due to the lack of a specific first binding site as a result of histidine being substituted for cysteine (4). This lack of albumin binding of copper in dog serum is demonstrated by the extreme susceptibility of dogs to copper poisoning (56).

Tracer studies utilizing ^{64}Cu by Gollan and Deller (55) have shown in liver there are two major types of synthesis occurring: 1) the synthesis of ceruloplasmin for release into the blood and 2) synthesis of a macromolecular protein component that complexes copper for biliary excretion. Ceruloplasmin synthesis in the liver includes the addition of copper from the cytosol and once the ceruloplasmin is released into circulation the copper in ceruloplasmin is not exchangeable with non-ceruloplasmin copper in the blood (21). Owen (109) observed that after intravenous injection of ^{64}Cu, the isotope did not accumulate in extrahepatic tissues until after the emergence of

^{64}Cu-ceruloplasmin into circulation, suggesting that ceruloplasmin is the main copper donor for other than hepatic tissues. Copper in biliary excretion is mainly bound to protein of 50,000 to 200,000 in molecular weight. The copper in bile seems to arise from at least two sources: 1) copper that permeates the bile canaliculus and combines nonspecifically with proteins in the bile, and 2) protein bound copper that is deposited in the bile as the result of protein catabolism and pinocytosis by the hepatic lysomes (42). Isotope studies using ^{64}Cu showed that 2 to 6 percent of administered radiocopper was in the gallbladder after 4 hours (55). The rapid appearance of ^{64}Cu in the bile, before the synthesis of appreciable ceruloplasmin -^{64}Cu, and the difference in atomic weight of ceruloplasmin and macromolecular biliary protein makes ceruloplasmin a poor choice as the excretory protein for copper (54,55).

In humans biliary copper is complexed to a heat stable macromolecular protein with a molecular weight in excess of 50,000 (54,55). Below a pH of 4 large amounts of Cu can be dissociated from the high molecular weight complex, but if the pH is raised to 6, a condition similar to that which prevails in the upper G.I. tract of man, the copper is readily reabsorbed by this complex (55). In studies with rats and humans Gollan and Deller (55) found little enterohepatic circulation of *in vivo* ^{64}Cu labled bile. Biliary excretion of copper in humans accounts for approximately 80 percent of copper excretion (21,25). Cartwright and Wintrobe (25) administered radiocopper orally to patients; 72.4 percent was excreted in the stools and another 0.1 percent was excreted via the urine.

Oral Contraceptives

The development of oral contraceptive agents in pill form has had a major impact on life in North America and Western Europe. It is estimated that 50 million women are currently using the "pill" (133). The oral contraceptive agents are generally of two types, 1) an estrogen given in combination with a progestogen for 21 days, and 2) a sequential pill with estrogen given for days 1-14 and progestogen given days 15-20. A seven day interruption of therapy is made at which time menstruation occurs (105). The first oral contraceptive pills contained a daily dose of 80-100 µg of estrogen, most oral contraceptives now contain 30-50 µg of estrogen per pill (105).

Since the introduction of oral contraceptives into general use many side effects have been noted. Nelson (105) divides the side effects into two groups: 1) those primarily caused by estrogens and 2) those caused by progestogens. Symptoms associated with estrogen use include: gastrointestinal upsets, fluid retention, capillary engorgements, cystic changes in breasts, skin pigmentation, visual changes, hypertension, altered glucose tolerance, altered thyroid function tests, altered tryptophan load tests, and changes in serum lipid levels (12,105). Symptoms associated with progestogen include: oligomenorrhea, amenorrhea, hirsutism, breast regression, appetite increase, fatigue, and hair thinning (12,105). Korba and Heil (92) in an eight year study involving 6,806 women experienced a 4.5 percent drop out rate due to side effects. Maile and Kent (102) published a review of 100 laboratory tests with the tests altered by the use of

oral contraceptive agents. The test results changed by oral contraceptives are for the most part the normal tests that would be run to assess state of health and nutrition.

The normal menstrual cycle can be divided into four parts: 1) the follicular phase, 2) ovulatory phase, 3) luteal phase, and 4) the menstrual phase (110). During the follicular phase the levels of circulating estrogens slowly increase until ovulation occurs, after ovulation there is a gradual drop in estrogen and a marked increase in progestogen (110). This view of the changes in steroid chemistry is very limited in scope and does not truly reflect the intricate system of hormonal control over other tissues and glandular secretions.

Effects of Oral Contraceptives on Steroids and Lipids

The exogenous intake of steroid hormones as a means of fertility control basically suppresses natural hormonal variation (47). Fern et al. (47) found that oral contraceptive therapy suppressed the synthesis of androgenic steroids and their precursors. The use of oral contraceptives produces a pseudo-pregnant state in women thus the inference made by Coats et al. (29) that plasma prostaglandins should increase in patients using oral contraceptives should hold true.

The effect of oral contraceptive agents on circulating lipids has been heavily researched. Martin et al. (97) found that when subjects were placed on oral contraceptives (combined type) that cholesterol levels in the serum increased 13 percent and triglyceride levels increased 41 percent. Donde and Virkar (37) and deAlverez et al. (36) found increases in cholesterol, free fatty acids, triglycerides, and

phospholipids. Two groups of subjects were given either a combined oral contraceptive or a "mini" pill (progestogen only), there was the expected rise in cholesterol and triglyceride levels with the combined product, but the "mini" pill was found to elevate triglyceride and not cholesterol levels (144). Beck (9) states that the changes observed in blood lipids are favored by an increase in circulatory levels of insulin, growth hormone, and a blunting of the aminogenic effect of glucagon. The changes that occur in cholesterol metabolism are postulated to be a prime cause in the increase of surgically proven gallstones among oral contraceptive users, the normal rate of gallstone formation was given as 70/100,000 women whereas the gallstone rate in oral contraceptive users was given as 158/100,000 women (9).

Oral Contraceptives and Vitamins

Oral contraceptive agents are known to cause changes in vitamin levels and apparent activity of vitamins in the body (118). Larson-Cohn (94), Prasad et al. (118), and Ahmed and Bamji (1) all observed elevation of plasma vitamin A levels with oral contraceptive therapy. Some controversy exists as to the amount of effect of oral contraceptives on blood levels of vitamin C with both no change or reductions in vitamin C noted (94,118). Wynn et al. (161) showed that oral contraceptive users showed reduced brain amine synthesis caused by induction of the tryptophan-nicotinic acid ribonuclease pathway and functional pyridoxine phosphate coenzyme deficiency. Approximately half of Wynn's (161) subjects showed B_6 deficiency which positively responded to supplemental B_6 (Pyridoxine-HCl). This work was confirmed

in women by Ahmed and Bamji (2) but a similar study in rats did not show the same effects (1). Levels of folate, thiamin and riboflavin have all been reported as depressed to near deficient levels in women using oral contraceptive agents (2,118). The intake of at least 10 mg of pyridoxine, 3 mg of thiamin, and 2 mg of riboflavin a day is recommended by Ahmed and Bamji (2) to at least keep vitamin levels from falling below normal values. Prasad (118) attributed the high levels of vitamin A to estrogen induced hepatic synthesis of α-globulin, the vitamin A carrier protein. Based on current research there does seem to be reason for concern about vitamin nutriture in subjects taking oral contraceptive agents.

Effects of Oral Contraceptive Agents on Blood Levels of Copper, Zinc, Ceruloplasmin, and Albumin

Various researchers have found elevations in serum copper in patients taking oral contraceptives. Horwitt *et al.* (80) found serum copper to be elevated to 204 µg/100 ml compared to control values of 115 µg/100 ml. Johnson (87) found copper elevated to 233.7 µg/100 ml compared to control values of 116 µg/100 ml. Schenker *et al.* (139,140) found in two separate studies that values from the control group were 129 µg of copper per 100 ml whereas oral contraceptive users had copper values of 205 and 207 µg/100 ml. Others (23,60,122) have produced similar results with control subjects having copper levels of 133.8, 142, 121, and 146 µg of copper per 100 ml of serum while the oral contraceptive users in the same studies had serum copper levels of 234, 228, 258.4 and 221 µg/100 ml.

Schenker et al. (139) found no change in serum zinc values of oral contraceptive users compared to normal controls with both groups having serum zinc levels of 116 µg per 100 ml. Prasad et al. (122) in examining a group of women of "high" or "low" economic status found values of zinc in the control groups to be 118 µg/100 ml for the "high" group and 118.9 µg/100 ml for the "low" group. In the same study (122) the oral contraceptive users had serum zinc values of 113.8 µg/100 ml for the "high" group and 114.4 µg/100 ml in the "low" economic group. Briggs and Briggs (15) and Hahn et al. (60) found a significant lowering of serum zinc due to oral contraceptive therapy. Hahn et al. (60) found control subjects to have 109 µg of zinc per 100 ml of serum while contraceptive users had serum zinc levels of 80 µg for 100 ml. Briggs and Briggs (15) reported values of 99 µg/100 ml for control subjects and 83 µg/100 ml for oral contraceptive users. McBean et al. (98) found that use of oral contraceptives significantly reduced serum zinc in rats from control values of 179 µg/100 ml to 139 µg/100 ml, but the levels of estrogen fed greatly exceeded normal human therapy levels. Other trace mineral levels were studied by Hahn (60) who found that use of oral contraceptives raised the serum levels of iron and magnesium while reducing serum calcium.

Changes have been found to occur in various proteins in the blood due to oral contraceptive therapy (16,36,97,115). Martin et al. (97) found increases in serum proteins associated with the lipid fraction and decreases in serum albumin. deAlvarez (36) found similar changes in protein patterns with a positive dose-response relationship. Pilgeram et al. (115) found that estrogen in oral contraceptive pills

caused an enhanced conversion of fibrinogen to fibrin, the soluble fibrin content was elevated 97 percent for subjects who used oral contraceptives compared to control subjects. Briggs and Briggs (16) found a dose respondent effect with estrogen containing contraceptives administered orally which produced a drop in serum albumin, a haptoglobin, and orosmucoid while producing large increases in ceruloplasmin. The effects on serum protein are caused by estrogen, but some progestogens are metabolized to estrogen-like metabolites that may produce serum protein changes (115).

CHAPTER III

EXPERIMENTAL PROCEDURE

Experimental Design

This study was designed to test the hypothesis that utilization of dietary copper and zinc is different in young women using oral contraceptive agents when compared with a control group of young women not using oral contraceptive agents. The subjects were young women of childbearing age who either had never taken oral contraceptive agents (controls) or who had been on oral contraceptive therapy for a minimum of two menstrual cycles. Past research has shown variations in circulating levels of plasma copper and zinc with the use of oral contraceptive agents producing significant elevations in plasma copper and no effect or some lowering of plasma zinc (15,23,38,62). The variations in circulating levels of copper and zinc in plasma give rise to the question: Do oral contraceptive agents also change the metabolic handling of copper and zinc and is variation in blood levels of minerals indicative of changing utilization patterns under a normally altered physiologic state?

Subjects were recruited and, based on their history of use or non-use of oral contraceptives, placed into the two experimental groups. A description of the oral contraceptive agents used by subjects is found in Appendix I. The diets for both groups were identical for each of the experimental periods.

The study spanned 18 days divided into three six day periods. The first 6 day period was not used in calculations for mineral

balance but served as an adjustment period to stabilize the subjects in terms of mineral intake. Periods two and three were used in calculating mineral balances for copper and zinc. All urine and feces were collected from day 1 through day 18. Blood samples were drawn at the end of each experimental period for analysis of copper, zinc, and cholesterol. Total nitrogen balance data was collected. Daily urinary creatinines were determined as a check for total collection of urine by the subjects.

Experimental Subjects, Management, Diet and Supplement Description

Sixteen female subjects ranging in age from nineteen years to twenty-four years and five months, were recruited from the Montgomery County area of Virginia. Subjects in the control group were assigned numbers from one to ten and oral contraceptive users were assigned numbers from eleven to twenty. All sample materials collected from subjects were labeled with subject's number and initials at the time of collection.

The subjects in the study were instructed in sample collection and labeling. Each subject was given a canvas shoulder bag equipped with bottles for urine collection, plastic lined paper cartons for fecal collection and bottles for deionized water for drinking away from the dietary unit. All subjects were asked to report any illness and provide a record of medicine prescribed, dosage level and a sample for trace metal analysis.

The subjects were fed all meals in the departmental metabolic unit. Subjects were provided with all food and drink consumed in the

18 days of the balance study. Except for the dietary restriction and sample collection, no other changes in normal routine by the subjects were imposed.

The food for the subjects was prepared in the metabolic kitchen and served in an adjoining dining room. Food and drinks other than water were completely consumed by the subjects. The different daily menus were provided with all subjects consuming the same menu for a given day. The menus cycled A, B, and C every three days for the duration of the study. Food items were selected on the basis of normal food patterns for the age group with the content of zinc and copper being constraints in selecting mainly meats from the fish and poultry area (Appendix II).

The diet provided approximately 2,000 kilocalories and approximately 50 grams of protein per day. The diets were so selected as to contain levels of copper and zinc below the level of the projected intakes during the study. The diets were supplemented with solutions of copper sulfate and zinc sulfate added to juice at breakfast to provide approximately 2 mg of copper and 9 mg of zinc per day (Table 1). Subjects were provided with a vitamin supplement[1] at breakfast each day which contained all the recommended vitamins but by chemical analysis showed negligible content of both zinc and copper.

All water used in food preparation for cooking, reconstituting beverages, final rinsing of dishes, and drinking was supplied as distilled-in-glass water with no detectable copper or zinc contamination. Subjects were allowed to consume water ad libitum both at

---

1. ONE-A-DAY (Brand), Miles Laboratories, Inc., Elkhart, Indiana 46514.

TABLE 1

Copper, zinc, and nitrogen content of the experimental diet

Experimental Period	Copper mg/day	Zinc mg/day	Nitrogen g/day
I	1.99	8.98	8.18
II	2.02	9.15	8.10
III	2.11	9.10	8.01

the metabolic unit and from water supplied by the metabolic unit while pursuing normal daily activities.

Food composites were selected daily from trays prepared for subjects. Food from two sample trays for each meal were selected and placed in separate polyethylene jars until all three meals for a given day were in each container. The food composite containers were kept refrigerated between meals. At the end of each day the jars were emptied into five-quart Waring blenders, care to transfer all food was taken and the food containers were rinsed with distilled water and the rinsing water added to the composite in the blender. The food was brought up to a standardized weight and homogenzied to a free flowing liquid consistency. An aliquot of 150 grams of food slurry from each of the duplicate samples was placed in acid washed polyethylene containers, sealed, and frozen. This process was repeated daily for each of the six days of each experimental period with successive aliquots being placed in the same bottle for a total 900 gram composite with equal amounts from each day. The food sample when completed was thawed and thoroughly mixed, then quantitatively analyzed for copper, zinc, and nitrogen.

Fecal markers composed of 40 mg brilliant blue and 100 mg methylcellulose in a gelatin capsule were given to each subject prior to breakfast on days 1, 7, and 19. The presence of the marker allowed the separation of feces on a period basis. All feces collected prior to the passage of the first fecal marker were discarded. All feces for each period containing the marker at the beginning of the period, but excluding any marked feces at the end of each period, were

composited as feces belonging to that period. Daily fecal excretion of copper, zinc and nitrogen were based on analysis of the last two 6-day composites for each subject.

Urine and Fecal Collection

All urine for each subject was collected from day 1 through day 18, inclusive. A 24-hour urine collection began with the second voiding of the day and continued through the first voiding of the subsequent day. Subjects brought their urine collection bottles to all meals where they exchanged used containers for clean dry containers. The urine was placed in acid washed two liter pyrex bottles containing 10 ml of 6N hydrochloric acid. With the completion of each 24-hour urine collection the urine samples were diluted to volume, mixed, and two aliquots taken from each composite. A 100 ml aliquot was taken for daily nitrogen and creatinine analysis. A 150 ml aliquot was placed in an acid washed liter polyethylene bottle for each subject. Successive aliquots were added to the same liter bottle until an aliquot for each of the 6 days in a period had been taken. Urine samples were kept frozen until thawed for copper and zinc analysis. Data from the analysis for copper and zinc of food, urine, and feces from periods II and III were used in assessing mineral balance.

Blood Collection

Blood samples were taken at the end of each period of the study. Blood was drawn from the antecubital vein by means of a 20 ml eccentric

tip plastic syringe[1] equipped with a stainless steel needle with a plastic leur[2]. Blood samples were drawn by syringe over a period of 2 to 4 minutes with minimum vacuum pulled in the syringe to lessen hemolysis of red cells from hydrostatic shock. The blood was then transferred to two polypropylene centrifuge tubes numbered for each subject. Centrifuge tubes were prepared by first acid washing and drying the tubes, then approximately 2 gm of blood barrier beads[3] and a liquid solution of heparin were added to the tubes. The tubes were shaken to coat both the tube walls and the barrier beads, the tubes were then dried. The heparin added to the tubes was sufficient to produce a level of heparin ten times normal physiological concentration (1µg/ml). After transfer of blood to the prepared centrifuge tubes the tubes were covered with parafilm and inverted gently three times. Blood samples were centrifuged at 1500xG for 10 minutes. Blood plasma was immediately separated from red cells by means of an acid washed Mohr pipet. Plasma was stored frozen in polycarbonate plastic vials labeled with the subject's number and initials. Plasma was analyzed for copper, zinc, and cholesterol concentration.

Analytical Methods and Analysis Conditions

Nitrogen in food and fecal samples was analyzed using a modified Kjeldahl-Gunning-Arnold method (5) with selenium coated boiling chips

---

1. Polypac Syringes, Eccentric Tip, Becton-Dickenson Corporation, Rutherford, NJ 07070.

2. Needle #8956E56, 1½", 21 gauge, Arthur H. Thomas, Philadephia, PA 19105.

3. Blood barrier beads, Walter Sarstedt, Inc., Princeton, NJ 08540.

added to catalytically aid the sulfuric acid digestion of samples. Urinary nitrogen and creatinine were determined simultaneously with a Technicon Autoanalyzer II[1]. A modified Missouri-automated nitrogen procedure was utilized for nitrogen and a modified picric acid color reaction for creatinine. Nitrogen standards were known concentration solutions of ammonium sulfate. Creatinine standards were aqueous solutions of known concentration of creatinine.

Samples for determination of urinary zinc were pipeted from thawed mixed composites into acid washed 250 ml beakers. After the transfer of the 10 ml urine sample to the beaker 10 ml of concentrated nitric acid was added. The beaker was covered with an acid washed watch glass and placed on a 125°C hotplate. The samples were allowed to remain on the hotplate for approximately 1 hour after which the watch glasses were removed and the volume reduced to roughly 1 ml, at this time a fresh 5 ml of nitric acid was added followed by an addition of 2 ml of 30 percent hydrogen perioxide. The samples were allowed to react while covered with watch glasses for 30 minutes. (The last nitric acid hydrogen peroxide digestion step was repeated a total of three times.) The samples, following the completion of digestion of organic matter, were evaporated to dryness. The sample was quantitatively transferred to a volumetric flask with 1N hydrochloric acid and made to a final volume of 10 ml.

Blood plasma samples of 2 or 3 ml volume (depending on quantity of plasma available) were transferred to acid washed 150 ml beakers.

---

1. Technicon Auto-analyzer II, Technicon Instruments Corporation, Tarrytown, NY 10591.

The samples were covered with watch glasses and placed in a 90°C drying oven for 30 minutes. The sample beakers were then transferred to a 125°C hotplate with the addition of 10 ml of concentrated nitric acid. The samples were allowed to digest for 1 hour, the watch glasses were removed, and the volume reduced to roughly 1 ml. The beakers were partially recovered and fresh additions of 5 ml of concentrated nitric acid and 2 ml of 30 percent hydrogen peroxide were made and the mixture was allowed to react for 45 minutes. This last digestion step was repeated once, then the beakers were moved to a perchloric acid hood and placed on a 150°C hotplate. The final digestion mixture consisted of an addition of 2 ml of 70 percent perchloric acid and 5 ml of concentrated nitric acid. This mixture was allowed to react to the stage of dense white fume generation. The watch glasses were removed and the samples were allowed to evaporate to dryness on the hotplate. The samples were quantitatively eluted from the beakers with 1 N hydrochloric acid into acid washed pyrex culture tubes. The tubes were closed with a layer of parafilm, stored at ambient temperature until analysis for copper and zinc. Plasma samples of 3 ml were made to a final volume of 10 ml and 2 ml plasma samples were made to a final volume of 6 ml.

Fecal composites were thawed, mixed and 10 gm aliquots transferred to acid washed 400 ml beakers. The beakers were covered with acid washed watch glasses and placed on a 150°C hotplate and allowed to dry for 30 minutes. Three 10 ml additions of concentrated nitric acid with volume reductions between additions were made with 1 hour reaction times allowed for each new acid addition. Two additions of

5 ml of concentrated nitric acid and 2 ml of 30 percent hydrogen peroxide were made to each sample with 30 minutes allowed for each new acid-peroxide reaction to occur. The watch glasses were removed and the sample evaporated to approximately 1 ml. The samples were covered with watch glasses and transferred to the perchloric acid hood for final digestion. The samples were placed on a 150°C hotplate and 6 ml of concentrated nitric acid and 3 ml of 70 percent perchloric acid added to each sample. The final digestion reaction was allowed to progress to the stage of dense white fume generation at which point the watch glasses were removed and the sample allowed to evaporate to dryness. The samples were transferred with 1 N hydrochloric acid into acid washed 50 ml volumetric flasks and brought to volume. The flasks were stoppered and stored at ambient temperature until analyzed for copper and zinc.

Food composites were thawed, mixed and 10 gm aliquots placed in acid washed 400 ml beakers. The beakers were covered with watch glasses and placed in a 90°C drying oven and allowed to dry for 1 hour. The beakers were then transferred to a 150°C hotplate where four serial additions of 10 ml of concentrated nitric acid were made. After each addition of acid a 30 minute digestion was allowed before new acid was added. The watch glasses were removed and the samples were allowed to evaporate to approximately 2 ml in volume. An addition of 0.5 ml of 98 percent sulfuric acid was made to each sample and the samples again covered with watch glasses. The samples were allowed to char for 30 minutes. Three serial additions of 5 ml of concentrated nitric acid and 3 ml of 30 percent hydrogen peroxide were made. Each acid-

peroxide reaction was allowed to proceed for 30 minutes with serial additions being interrupted with removal of watch glasses, evaporation to 2 ml volume, and installation of new watch glasses. After the final digestion the watch glasses were removed and the samples evaporated to a volume of approximately 0.5 ml. The samples were then transferred to acid washed 50 ml volumetric flasks and stoppered until copper and zinc analysis.

The urine samples were thawed and mixed and 100 ml aliquots of urine were placed in 400 ml acid washed beakers. The pH of the urine was adjusted with 6 N hydrochloric acid until a pH of 2.3 to 2.5 was achieved. The urine was transferred to a 250 ml acid washed separatory funnel equipped with a teflon stopcock and stopper. To the urine was added 1 ml of a 2 percent aqueous solution of ammonium pyrrolidine dithiocarbamate (APDC). The separatory funnel was stoppered and shaken for 1 minute to chelate the copper with the APDC. The funnel was unstoppered and 10 ml of reagent grade methyl isobutyl ketone (MIBK) was added to each sample. The separatory funnels were stoppered and vigorously shaken for 2 minutes. Care was taken to relieve pressure build up in the shaking process by inverting the funnel at 15 second intervals during shaking and slowly opening the stopcock. The flasks were allowed to stand for 10 minutes for aqueous and organic layers to separate. The aqueous layer was discarded and the MIBK layer transferred to a pyrex tube and covered with parafilm. The MIBK extract was placed in a 0°C refrigerator for 1 hour to remove any residual water. Standards of known copper concentration were run by APDC

chelation and MIBK extraction to have standards in the same matrix and to negate the need to recalculate for percent recovery.

APDC was synthesized by a modification of the method of Malissa and Schoeffman as suggested by Slavin (145). One hundred ml of ethyl alcohol and 45 ml of pyrrolidine were added to a 300 ml Erlenmeyer flask. The flask was equipped with a reflux condenser and cooled with ice. When the solution had cooled to near 0°C three 10 ml additions of carbon disulfide were made through the condenser and swirled with each new addition. A white precipitate was formed. The precipitate was filtered and washed with pure ethyl alcohol.

Blood cholesterol values were determined as methyl-derivatives of cholesterol from the blood plasma. Cholesterol analysis was by gas-liquid chromatography. Cholesterol derivatives were in a N-hexane matrix. Analyses were performed in a service laboratory in the Department of Biochemistry.

Copper and zinc were analyzed with a Perkin Elmer model 305 atomic absorption spectrophotometer[1] equipped with a titinium 3 slot Boiling burner head. The fuel was acetylene and the oxidant was air. Samples were aspirated into the burner in a dilute hydrochloric acid matrix with the exception of urinary copper. Copper-APDC chelates were aspirated in a matrix of reagent grade MIBK. For copper analysis a hollow cathode intensitron copper lamp[1] was used as a light source with analysis carried out at a wavelength of 324.8 nm and a slit width of 4 mm; an oxidizing flame was used in all copper analysis.

---

1. Perkin-Elmer Atomic Absorption Spectrophotometer, Model 305, Perkin-Elmer Corporation, Norwalk, CT 06856.

Urine samples and standards were run with standard wavelength and slit settings for copper, but the flame was adjusted for a fuel flow that would just maintain a flame without pulling from the burner head. The gaskets in the burner nebulization chamber were changed to corkprene, a material compatible with ketone solvents. Recovery values for copper added to urine and chelated and extracted ranged from 89 to 101 percent. The average recovery value for the procedure was 94 percent. Zinc was analyzed with a hollow cathode intensitron zinc lamp[1] as a light source. Zinc analyses were run at a wavelength of 213.9 nm with an optical slit width of 4 mm; analyses were carried out with oxidizing flame conditions.

Standards for analysis of copper and zinc were diluted from stock, certified 1000 ppm standard solutions[2]. Acid washed class A pipets and volumetric flasks were used in all sampling and transfer operations. Pipets used in standard solution makeup were first filled with the standard and allowed to drain into a waste container. Pipets that showed any droplet formation on the inside of the bore were rejected for use in making standard solutions.

Data from the atomic absorption spectrophotometer and the autoanalyzer II were outputted as a tracing from linear electronic recorders. Steady state peak heights were used as indicative of sample amounts and were calculated by regression analysis based on standards assayed with each set of samples.

---

1. Perkin-Elmer Atomic Absorption Spectrophotometer, Model 305, Perkin-Elmer Corporation, Norwalk, CT 06856.

2. Copper and zinc standards, 1000 ppm, Harleco division of American Hospital Supply Corporation, Gibbstown, NJ 08027.

The values for hemoglobin and hematocrit were arrived at by normal clinical methods.

The type, grade and sources for lab supplies and reagents is found in Appendix III.

Statistical Testing

Data generated from the study were analyzed with the SAS 72 (7) procedure for analysis of variance and regression correlation to observe possible interactions among measured parameters. The experiment was a fixed-effects model utilizing one variable. Analysis of variance was used to test the effect of oral contraceptive agents on nitrogen balance, zinc balance, copper balance, urinary zinc excretion, urinary copper excretion, plasma copper levels, plasma zinc levels, and plasma cholesterol levels.

CHAPTER IV

RESULTS

Anthropometric and Biochemical Measurement on Subjects

The subjects' initial weights at the beginning of the study ranged from 45.9 kg to 73.6 kg. The mean weight for the non-oral contraceptive group was 57.2 kg. The mean weight for the oral contraceptive group was 59.5 kg. On the initial day of the study, hemoglobin and hematocrit values were determined as a measure of general health to detect iron deficiency anemia. Initial hemoglobin values ranged from 13.39 g/100 ml to 16.6 g/100 ml. The mean hemoglobin for the control subjects was 14.93 g/100 ml and the mean hemoglobin for the oral contraceptive group was 15.09 g/100 ml. Hematocrit values ranged from 40 to 54 percent by volume. The means of the hematocrit values were 45.7 percent by volume for controls and 44.7 percent by volume for oral contraceptive users. No statistical significance was detected between the two groups' hemoglobin or hematocrit values at the beginning of the study (Table 2).

The subjects' weights at the end of the study ranged from 44.2 kg to 70.2 kg. The mean weight of the control group at the end of the study was 55.7 kg and the mean weight of the oral contraceptive group was 58.2 kg. Both groups lost approximately 2 percent of body weight during the course of the experiment. Individual subjects' weights, hemoglobins, and hematocrits are shown in Table 2.

TABLE 2

Age, oral contraceptive agent, and initial and final weight[a], hemoglobin and hematocrit of subjects

Subject Number	Age yrs	Age mos	Initial Weight kg	Final Weight kg	Initial Blood Values Hemoglobin g/100 ml	Initial Blood Values Hematocrit Vol. %	Final Blood Values Hemoglobin g/100 ml	Final Blood Values Hematocrit Vol. %	Oral Contraceptive Agent
1	19	5	62.4	60.5	13.38	40.0	13.61	39.5	
2	21	0	56.8	55.8	15.96	--	14.75	41.0	
4	20	9	45.9	44.2	15.74	54.0	15.70	44.0	
5	24	5	49.7	49.0	15.43	44.0	15.94	44.5	
6	21	11	56.4	56.0	14.10	45.0	13.97	40.0	
9	22	10	55.9	55.5	16.53	50.0	14.94	44.0	
10	19	6	73.6	70.2	13.39	41.0	12.98	36.5	
Average	21	6	57.2	55.9	14.93	45.7	14.56	41.4	
SD	--	--	8.96	8.27	1.29	5.39	1.09	2.96	
11	19	0	63.6	59.5	15.82	46.0	15.74	43.5	Ortho-Novum 1/50
12	21	5	55.6	54.5	14.44	45.5	13.56	39.5	Norinyl 1/50
13	20	8	65.9	65.9	15.06	47.0	14.35	42.5	Ovulen 21
15	19	6	60.7	60.0	14.32	44.0	14.76	42.0	Norinyl 1/50
16	21	4	56.0	54.5	15.08	43.0	14.55	42.0	Ortho-Novum 1/50
17	23	9	56.8	55.5	13.94	40.0	14.78	43.0	Zorane 1/50
18	22	8	55.0	54.3	14.00	41.0	13.21	38.0	Demulen
19	21	0	60.9	58.9	16.60	48.5	16.60	49.0	Low Ovral
20	21	0	60.9	60.7	16.59	47.5	16.44	46.5	Loestrin 1.5/50
Average	21	2	59.5	58.2	15.09	44.7	14.89	42.9	
SD	--	--	3.84	3.89	1.04	2.94	1.18	3.31	

[a]Subjects weighed fully clothed, without shoes.

Nitrogen Excretion

The urinary loss of nitrogen was determined for all three experimental periods, but the first period was disregarded as an adjustment period. The urinary nitrogen loss for period II ranged from 5.77 g/day to 7.76 g/day. The mean daily nitrogen loss for period II was 6.62 g/day for controls and 6.72 g/day for oral contraceptive users. Urinary nitrogen loss for period III ranged from 5.33 g/day to 8.98 g/day. The mean urinary nitrogen loss for controls for period III was 6.59 g/day and for oral contraceptive users was 6.62 g/day. When period II and period III were examined as a single group of data the mean urinary nitrogen loss was 6.61 g/day for controls and 6.67 g/day for the oral contraceptive group. There was no statistically detectible difference in urinary nitrogen excretion. Urinary nitrogen values are shown in Table 3.

The fecal loss of nitrogen was analyzed for all three experimental periods, but only fecal losses for periods II and III were used in calculating balances for nitrogen. Fecal nitrogen losses for period II ranged from 0.63 g/day to 1.45 g/day. The mean fecal nitrogen excretion for period II was 1.12 g/day for controls and 0.98 g/day for oral contraceptive users. The mean fecal nitrogen losses for period III ranged from 0.74 g/day to 1.51 g/day. The mean fecal nitrogen loss for period III was 1.11 g/day for controls and 1.13 g/day for oral contraceptive users. When periods II and III are treated as a single period the mean fecal nitrogen loss was 1.12 g/day for controls and 1.05 g/day for the oral contraceptive group. Mean daily fecal nitrogen excretion data are shown in Table 4. Testing the means of the

TABLE 3

Urinary excretion of nitrogen and creatinine

Subject Number[a]	Period I		Period II		Period III	
	Nitrogen g/day	Creat. g/day	Nitrogen g/day	Creat. g/day	Nitrogen g/day	Creat. g/day
1	8.48	1.19	7.76	1.14	8.98	1.24
2	7.75	1.03	5.80	1.07	5.40	1.07
4	6.21	0.96	6.31	0.97	6.11	1.00
5	6.49	0.93	6.47	0.97	6.43	0.94
6	7.05	1.06	6.28	1.05	6.00	1.02
9	8.37	1.13	6.98	1.11	6.03	1.12
10	7.61	1.22	6.75	1.30	7.17	1.31
Average	7.42	1.07[b]	6.62	1.09[c]	6.59	1.10[d]
SD	0.88	0.11	0.63	0.11	0.18	0.13
11	7.60	1.20	7.47	1.18	7.52	1.18
12	6.23	1.09	5.77	1.07	6.11	1.12
13	7.85	1.12	7.24	1.36	6.13	1.21
15	6.68	1.25	6.76	1.22	7.54	1.21
16	7.42	1.22	7.40	1.25	6.48	1.25
17	6.62	1.18	6.11	1.14	6.62	1.18
18	7.63	1.26	7.38	1.28	7.40	1.37
19	6.75	1.22	5.98	1.27	5.33	1.23
20	7.06	1.19	6.38	1.17	6.45	1.18
Average	7.09	1.19[b]	6.72	1.22[c]	6.62	1.22[d]
SD	0.56	0.06	0.68	0.09	0.75	0.07

[a] 1-10, control subjects; 11-20, subjects on oral contraceptive agent therapy.

[b,c,d] significantly different ($p < 0.05$).

TABLE 4

Fecal excretion of copper, zinc and nitrogen

Subject Number[a]	Period I			Period II			Period III		
	Copper mg/day	Zinc mg/day	Nitrogen g/day	Copper mg/day	Zinc mg/day	Nitrogen g/day	Copper mg/day	Zinc mg/day	Nitrogen g/day
1	1.77	9.76	0.67	2.27	11.17	0.86	2.34	10.95	0.90
2	2.12	10.00	1.12	2.28	9.33	1.08	2.19	8.48	0.96
4	1.66	7.12	1.17	2.07	8.16	1.25	2.20	8.24	1.38
5	2.26	10.19	1.33	2.18	9.17	1.30	2.09	8.61	1.27
6	1.69	8.26	0.94	2.38	10.17	1.45	2.02	7.90	1.04
9	1.48	8.39	0.67	2.53	12.42	1.16	1.88	8.28	0.98
10	2.27	9.08	1.03	1.47	5.98	0.74	2.81	11.81	1.27
Average	1.89	8.97	0.99	2.17	9.48	1.12	2.22	9.18	1.11
SD	0.32	1.11	0.25	0.34	2.08	0.25	0.30	1.54	0.19
11	1.58	9.42	0.98	2.38	10.74	0.98	2.80	11.90	1.37
12	1.31	5.09	0.61	2.02	7.58	1.25	2.44	9.30	1.51
13	1.60	7.14	1.10	1.76	7.04	0.99	2.16	9.47	1.04
15	1.32	8.06	0.75	2.41	10.02	1.11	--	--	--
16	2.44	12.78	1.21	2.06	8.38	0.95	2.21	10.52	1.16
17	1.85	12.02	0.87	1.45	6.40	0.63	2.46	10.32	1.00
18	1.40	6.03	0.67	2.15	9.01	1.74	2.15	9.01	0.74
19	1.89	10.79	1.40	2.11	9.37	1.23	1.96	7.70	1.24
20	0.85	3.82	0.42	2.15	9.07	0.93	1.98	7.98	0.85
Average	1.58	8.35	0.89	2.05	8.62	0.98	2.27	9.53	1.13
SD	0.45	3.13	0.31	0.30	1.41	0.20	0.28	1.38	0.25

[a] 1-10, control subjects; 11-20, subjects on oral contraceptive agent therapy.

fecal nitrogen failed to show any statistical difference between groups.

Nitrogen Balance

Nitrogen balance is widely used to evaluate nitrogen requirements and utilization. Nitrogen balance is maintained in adults when there are adequate supplies of essential amino acids, total nitrogen and calories being consumed for replacement of nitrogen losses through the kidneys, intestinal losses, and integumental losses. Nitrogen balance may be shown in equation form to be: $B = I - (U + F + S)$, where B is nitrogen balance, I is nitrogen intake, U is urinary nitrogen, F is fecal nitrogen, and S is sweat nitrogen. The loss of sweat nitrogen is not included in this study, but past research shows losses of the order of 0.15 to 0.5 g/day with the higher figures representing subjects consuming high protein diets while living in a hot environment.

Individual mean daily nitrogen balances have been calculated for all subjects in all three experimental periods. Nitrogen intake for period II averaged 8.09 g/day. Mean daily nitrogen balance ranged from -0.525 g/day to 1.22 g/day during period II. The mean daily nitrogen balance was 0.345 g/day for the controls and 0.416 g/day for oral contraceptive users in period II. Nitrogen intakes for period III averaged 8.01 g/day. Mean daily nitrogen balances ranged from -0.880 g/day to 1.65 g/day during period III. The mean daily nitrogen balance was 0.548 g/day for controls and 0.391 g/day for oral contraceptive users. No difference could be detected statistically between the two groups. Period nitrogen balance data are shown in Table 5.

TABLE 5

Copper, zinc, and nitrogen balance[a] data

Subject Number[b]	Period I			Period II			Period III		
	Copper mg/day	Zinc mg/day	Nitrogen g/day	Copper mg/day	Zinc mg/day	Nitrogen g/day	Copper mg/day	Zinc mg/day	Nitrogen g/day
1	0.214	-1.334	-0.975	-0.261	-2.435	-0.525	-0.280	-2.390	-0.187
2	-0.136	-1.317	-0.695	-0.276	-0.369	1.220	-0.131	0.325	1.650
4	0.322	1.616	0.795	-0.061	0.715	0.535	-0.140	0.508	0.520
5	-0.267	-1.498	0.355	-0.170	-0.310	0.325	-0.029	0.071	0.310
6	0.297	0.495	0.185	-0.375	-1.322	0.365	0.043	0.862	0.970
9	0.508	-0.049	-0.865	-0.529	-3.669	-0.045	0.173	0.339	1.000
10	-0.287	-0.441	-0.465	0.532	2.838	0.605	-0.752	-3.111	-0.430
Average	0.092	-0.361	-0.238	-0.163	-0.650	0.345	-0.159	-0.484	0.548
SD	0.318	1.147	0.628	0.340	2.120	0.544	0.299	1.579	0.724
11	0.406	-0.634	-0.405	-0.374	-1.764	-0.355	-0.740	-3.083	-0.880
12	0.678	3.593	1.335	0.020	1.283	1.075	-0.384	-0.540	0.390
13	0.375	1.300	-0.775	0.248	1.647	-0.135	-0.106	-0.864	0.835
15	0.664	0.649	0.745	-0.404	-1.203	0.225	---	---	---
16	-0.460	-4.114	-0.455	-0.061	0.490	-0.255	-0.151	-1.731	0.370
17	0.137	-3.151	0.685	0.548	2.636	1.355	-0.401	-1.496	0.390
18	0.583	2.516	-0.125	-0.148	-0.232	-0.025	-0.095	-0.475	-0.130
19	0.095	-2.314	0.025	-0.106	-0.715	0.885	0.094	0.736	1.440
20	1.134	5.007	0.675	-0.141	-0.085	0.785	0.076	0.788	0.710
Average	0.401	0.316	0.180	-0.051	0.228	0.416	-0.213	-0.833	0.391
SD	0.450	3.125	0.701	0.295	1.423	0.678	0.280	1.288	0.685

[a]-denotes negative balance.

[b]1-10, control subjects; 11-20, subjects on oral contraceptive agent therapy.

Creatinine Excretion

The excretion of creatinine via the urine was used as a check for completeness of collection of urine. The creatinine content of each subject's urine was measured daily. Only creatinine excretion from period II and period III were considered with period I allocated to an adjustment period.

The mean daily creatinine excretion for period II ranged from 0.97 g/day to 1.36 g/day. The mean creatinine excretion for period II was 1.09 g/day for controls and 1.22 g/day for oral contraceptive users. Creatinine excretion for period III ranged from 0.94 g/day to 1.37 g/day. The mean creatinine excretion for period III was 1.10 g/day for controls and 1.22 g/day for oral contraceptive users. When period II and period III are combined into a single period the mean daily creatinine excretion was 1.09 g/day for controls and 1.22 g/day for oral contraceptive users. The means for creatinine excretion for the two groups were statistically significant at the 0.05 level. Mean daily creatinine excretion is shown in Table 3.

Zinc Excretion

Means of urinary zinc excretion were measured for all subjects for all three periods. The mean excretion of zinc during period II ranged from 0.11 mg/day to 0.49 mg/day. The mean daily urinary excretion of zinc for period II was 0.31 mg/day for controls and 0.30 mg/day for oral contraceptive users. The mean daily zinc excretion for period III ranged from 0.17 mg/day to 0.56 mg/day. The mean daily urinary excretion of zinc was 0.30 mg/day for controls and 0.30 mg/day

for oral contraceptive users. When period II and period III are considered as a single experimental period the mean urinary zinc excretion for the control group is 0.31 mg/day versus 0.30 mg/day for oral contraceptive users. No significant difference could be detected statistically between the two groups. Urinary zinc output means are found in Table 6.

Fecal zinc analyses were measured using a 6-day pooled sample for all three experimental periods. Mean daily fecal zinc for period II ranged from 5.98 mg/day to 12.42 mg/day. The mean fecal zinc excretion for the control group in period II was 9.48 mg/day and the mean for oral contraceptive users was 8.62 mg/day. Mean daily fecal zinc excretion for period III ranged from 7.7 mg/day to 11.90 mg/day. The mean fecal zincs for period III were 9.18 mg/day for controls and 9.53 mg/day for oral contraceptive users. The variability of fecal marker detection makes the combination of period II and period III manditory for reasonable results. When period II and period III are considered as a single period the mean fecal zinc excretion was 9.33 mg/day for controls and 9.05 mg/day for oral contraceptive users. No statistical difference was detected in the means of the two groups. Fecal excretion of zinc is shown in Table 4.

Zinc Balance

Dietary intake of zinc for the three periods was: 8.98 mg/day for period I, 9.15 mg/day for period II, and 9.10 mg/day for period III. The RDA for zinc for this sex-age group is 15 mg/day but current research indicates that dietary intakes in the general population are

TABLE 6

Urinary copper and zinc excretion

Subject Number[a]	Period I		Period II		Period III	
	Urinary Zinc mg/day	Urinary Copper µg/day	Urinary Zinc mg/day	Urinary Copper µg/day	Urinary Zinc mg/day	Urinary Copper µg/day
1	0.55	11.5	0.42	10.6	0.44	11.3
2	0.30	12.5	0.18	12.7	0.19	13.1
4	0.24	11.0	0.28	12.3	0.25	10.5
5	0.29	10.9	0.29	9.7	0.31	9.5
6	0.22	10.3	0.30	10.2	0.24	12.4
9	0.64	13.8	0.39	13.2	0.38	12.5
10	0.34	13.1	0.33	13.2	0.30	12.3
Average	0.37	11.9[b]	0.31	11.7[c]	0.30	11.7[d]
SD	0.162	1.29	0.078	1.50	0.086	1.29
11	0.19	13.3	0.17	11.2	0.18	12.0
12	0.30	14.8	0.29	13.5	0.24	12.6
13	0.55	19.0	0.46	12.6	0.39	14.6
15	0.27	14.2	0.34	14.8	0.30	12.6
16	0.32	14.0	0.28	14.0	0.21	11.9
17	0.11	15.7	0.11	17.7	0.17	15.7
18	0.44	13.3	0.37	14.9	0.46	15.0
19	0.51	15.3	0.49	14.4	0.56	14.5
20	0.16	14.0	0.16	10.8	0.23	12.9
Average	0.32	14.9[b]	0.30	13.8[c]	0.30	13.5[d]
SD	0.153	1.77	0.131	2.09	0.137	1.41

[a] 1-10, control subjects; 11-20, subjects on oral contraceptive therapy.

[b,c,d] significantly different ($P < 0.01$).

around 9 mg/day (59,78). Mean daily zinc balances were calculated for all subjects using the same equation as for nitrogen balance. Skin loss zinc was omitted from the balance, but one researcher has shown zinc loss through skin in young women to be approximately 0.67 mg/day (78).

Mean daily zinc balances were calculated for all subjects during period II and period III. Mean zinc balance ranged from -3.67 mg/day to 2.84 mg/day during period II. The mean balances for period II were -0.650 mg/day for controls and 0.228 for the oral contraceptive group. Mean zinc balances ranged from -3.11 mg/day to 0.86 mg/day during period III. The mean value for the controls was -0.48 mg/day versus -0.83 mg/day for oral contraceptive users. If period II and period III are combined the means for the combined period were -0.57 mg/day for the control group and -0.27 mg/day for the oral contraceptive group. There was no statistical difference between the two groups of subjects. Zinc balance data are shown in Table 5.

Copper Excretion

Copper is primarily excreted through the bile, but a small amount does exit the body via the urine (25,55). Weinmann et al. (160) have postulated a difference in urinary metal excretion under the influence of oral contraceptives.

Mean daily urinary copper excretion was analyzed for all three experimental periods. Urinary copper excretion ranged from 9.7 µg/day to 17.7 µg/day for period II. The mean urine copper for the control group was 11.7 µg/day versus 13.8 µg/day for the oral contraceptive group during period II. Urinary copper ranged from 9.5 µg/day to

15.7 µg/day during period III. The mean urinary copper for period III was 11.7 for the control group and 13.5 for the oral contraceptive group.

Since urinary copper is not a direct function of dietary intake all three experimental periods were examined together for treatment effects. The means for all three periods are 11.7 µg/day for controls and 14.0 µg/day for oral contraceptive users. There was a significant difference at the 0.01 level for urinary copper excretion between groups (Table 6).

Fecal excretion values were calculated based on 6-day composites of feces and mean daily fecal copper excretion was calculated. The range of fecal copper values for period II was from 1.45 mg/day to 2.53 mg/day. Mean fecal excretion of copper for period II was 2.17 mg/day for controls and 2.05 mg/day for oral contraceptive users. The range of fecal copper for period III was from 1.88 mg/day to 2.80 mg/day. The means for period III for fecal copper were 2.22 mg/day for controls and 2.27 mg/day for oral contraceptive users. When experimental period II and experimental period III were combined into a single period the mean fecal copper excretion was 2.19 mg/day for controls and 2.15 mg/day for oral contraceptive users. No significant differences were statistically evident between the two groups in copper excretion (Table 4).

Copper Balance

The experimental diet provided approximately 2 mg/day of copper. The mean daily copper content of the diets was: 1.99 mg/day for period I, 2.02 mg/day for period II, and 2.11 mg/day for period III.

Currently there does not exist an RDA for copper (104), but various researchers have suggested than an intake (dependent on age-sex) between 1.5 and 2.0 mg/day should meet all body needs (125,141). Copper balance was calculated following the same equation as for nitrogen balance. No studies of a reliable nature for skin losses for copper have appeared, hence the term S is deleted from the equation.

Mean copper balances were calculated for all subjects for experimental periods II and III. Mean copper balances for period II ranged from -0.53 mg/day to 0.53 mg/day. Mean values for the two groups for period II were -0.16 mg/day for controls and -0.05 mg/day for oral contraceptive users. The range of copper balance for period III was from -0.75 mg/day to 0.17 mg/day. The means for the two groups were -0.16 for controls and -0.21 mg/day for oral contraceptive users. The combination of period II and period III into a single period yielded means of -0.16 mg/day for controls and -0.13 mg/day for oral contraceptive users. No statistically significant difference could be detected between the copper balance of the two groups (Table 5).

Plasma Copper, Zinc, and Cholesterol

Blood plasma was analyzed four times during the experiment. Samples were taken for periods I, II, III, and a post period sample taken 6 days after the last balance period. The last blood sample was taken after a 6-day period during which the subjects consumed a self selected diet and were given a 25 mg/day zinc supplement.

Blood plasma zinc levels for each period were in the following ranges: period I, 95.2 µg/100 ml to 185.7 µg/100 ml; period II, 100.8 µg/100 ml to 182 µg/100 ml; period III, 111.7 µg/100 ml to 168.9 µg/100 ml; post period IV, 88.8 µg/100 ml to 146.5 µg/100 ml. The means of period I zinc were 130.5 µg/100 ml for controls and 126.3 µg/100 ml for oral contraceptive users. Period III means were 151.8 µg/100 ml for controls and 130.1 µg/100 ml for oral contraceptive subjects. The post period, period IV values were 113.7 µg/100 ml for controls and 108.6 µg/100 ml for oral contraceptive users. When all four periods were combined the plasma zinc was 134.9 µg/100 ml for control subjects versus 125.3 µg/100 ml for users of oral contraceptives. No statistical significance between groups for plasma zinc values were found (Table 7).

Plasma copper values were measured for all four periods, including the post period. The plasma copper values for the periods were: period I, 86.3 µg/100 ml for controls and 213.2 µg/100 ml for oral contraceptive users. Period II means were 121.3 µg/100 ml for controls and 210.2 µg/100 ml for oral contraceptive agent users. Plasma copper for period III was 131.0 µg/100 ml for controls and 200.6 µg/100 ml for the oral contraceptive users. The post period IV means for copper were 115.4 µg/100 ml for controls and 196.3 µg/100 ml for oral contraceptive users. With all four periods combined into a single period the means were 121.0 µg/100 ml for copper in controls versus 198.5 µg/100 ml in subjects using oral contraceptives. The levels of copper in plasma were significantly different statistically at the 0.01 percent level (Table 7).

TABLE 7

Blood levels of zinc, copper, and cholesterol

Subject Number[a]	Blood I			Blood II			Blood III			Blood IV		
	Zinc µg/100ml	Copper µg/100ml	Chol. mg/100ml	Zinc µg/100ml	Copper µg/100ml	Chol. mg/100ml	Zinc µg/100ml	Copper µg/100ml	Chol. mg/100ml	Zinc µg/100ml	Copper µg/100ml	Chol. mg/100ml
1	185.7	127.8	163.1	122.8	118.8	162.1	159.3	125.7	108.5	103.2	107.5	125.0
2	180.2	130.7	174.7	133.2	120.8	219.0	128.3	115.0	124.4	124.7	131.2	228.3
4	95.2	86.3	125.8	132.5	106.0	103.5	---	---	138.6	123.3	105.8	120.5
5	109.7	122.2	159.8	145.0	118.0	131.6	144.8	136.1	144.7	105.5	125.5	157.1
6	97.3	104.0	84.9	152.0	148.3	117.4	165.2	134.9	74.3	---	---	---
9	129.0	101.7	135.1	105.5	110.2	81.4	144.0	130.3	78.9	109.0	110.0	113.2
10	116.7	119.2	180.8	120.7	127.3	136.6	168.9	144.0	95.3	116.3	112.5	101.6
Average	130.5	113.1[b]	146.3[f]	130.2	121.3[c]	135.9[g]	151.8	131.0[d]	109.2[h]	113.7	115.4[e]	140.9[i]
SD	37.6	16.2	33.6	15.6	13.8	44.7	15.2	9.9	27.9	9.2	10.4	46.7
11	143.7	205.5	166.3	139.9	197.8	137.3	151.8	181.6	149.4	107.3	196.0	186.0
12	104.2	212.2	142.8	113.7	234.5	174.2	128.3	225.7	119.8	96.0	219.5	161.5
13	102.8	214.2	231.4	167.0	244.3	264.8	125.7	220.8	236.0	88.8	214.8	245.4
15	107.7	212.7	252.7	116.8	184.2	186.3	129.0	185.2	189.5	120.0	204.5	185.0
16	106.3	183.2	205.3	118.7	192.7	178.9	111.7	190.3	151.7	105.3	190.7	207.6
17	123.2	227.0	206.3	182.0	254.0	202.1	125.7	206.8	169.9	108.2	193.5	223.4
18	131.8	208.8	233.0	135.3	185.0	198.1	127.0	192.3	179.3	97.0	200.3	112.3
19	128.3	206.0	164.2	100.8	203.7	194.8	139.8	193.1	229.9	146.5	175.3	219.6
20	188.5	213.2	157.5	151.3	196.0	130.7	132.0	209.7	160.4	108.5	171.8	228.3
Average	126.3	209.2[b]	195.5[f]	136.2	210.2[c]	185.2[g]	130.1	200.6[d]	176.2[h]	108.6	196.3[e]	196.6[i]
SD	27.4	11.6	39.1	26.8	26.7	39.2	11.0	15.8	37.8	16.8	16.0	40.8

[a] 1-10, control subjects; 11-20, subjects on oral contraceptive therapy.

[b,c,d,e] significantly different ($P < 0.01$).

[f,g,h,i] significantly different ($P < 0.01$).

Plasma cholesterol was measured for the same samples as zinc and copper. Plasma cholesterol ranges for the four periods were: period I, 84.9 mg/100 ml to 227.0 mg/100 ml; period II, 81.4 mg/100 ml to 264.8 mg/100 ml; period III, 74.3 mg/100 ml to 236.0 mg/100 ml; period IV, 101.3 mg/100 ml to 245 mg/100 ml. When all four periods were combined into a single period the means for cholesterol were 132.8 mg/100 ml for controls and 185.6 mg/100 ml for oral contraceptive users. There was a statistically significant difference in the level of plasma cholesterol between the two groups, the level of significance was the 0.01 percent level (Table 7).

CHAPTER V

DISCUSSION AND CONCLUSIONS

Nitrogen Balance

None of the three parameters measured concerning nitrogen; urinary nitrogen excretion, fecal nitrogen excretion, and total nitrogen balance showed any statistically significant differences between the control group and the group on oral contraceptive therapy. Both groups were in positive nitrogen balance for the final two periods. During period II, six subjects were in negative balance while the remainder were in positive balance. During period III only four subjects were in negative balance while the remaining subjects were in positive balance. Only three of sixteen subjects remained in negative balance for both periods or in total negative balance for the two periods on a combined basis.

Average weight changes for the two balance periods dropped 1.32 kg per subject. This represents a 2 percent weight change and was attributed, for the most part, to changes in clothing and activity of the subjects with the winter to spring climate change.

The results of this study strongly suggest that there is no apparent difference in nitrogen utilization between oral contraceptive users and normal subjects not taking oral contraceptive agents. Kudzma *et al.* (93) also found oral contraceptives to have no effect on nitrogen balance in young women.

Creatinine Excretion

The creatinine excretion via the kidneys is usually a fair indication of lean body mass. The creatinine excretion was significantly different ($P<0.05$) for the two groups with the oral contraceptive groups excreting more creatinine. This might be explained by results found in a study dealing with body composition where there was an apparent loss of fat and gain in non-fat body mass postulated to be due to the anabolic effect of estrogen (93). Weinmann et al. (160) detected a significant ($P<0.01$) difference in creatinine excretion between oral contraceptive users and non-users.

Zinc Balance

Of the three parameters of zinc measured; urinary zinc, fecal zinc, and zinc balance none of the three were statistically different. The subjects' average daily zinc balances were negative for both groups. Therefore it appears that a dietary intake of approximately 9 mg/day is not sufficient to meet the needs of healthy young women. The zinc balances were negative without including the skin loss of zinc which Hess et al. (78) estimated at 0.67 mg/day. Menstrual blood loss was not accounted for, but work by Nilsson (106) places menstrual zinc loss at 0.008 mg/day while Hess et al. (78) estimates menstrual zinc loss to be 0.005 mg/day. It would appear that an intake of the RDA level of zinc, 15 mg/day, would be sufficient to meet the needs of the age-sex group except that Margen and King (96) fed levels of zinc approaching the RDA for zinc and still had negative balances. Margen and King fed 14.8 mg/day of zinc and found fecal excretion alone to be

in excess of intake without consideration of urinary or skin loss zinc (96). When this study in concert with the work of others is looked at in light of zinc dietary levels that have in current years apparently decreased to around 9 mg/day of zinc (59,78), the question arises not of overt zinc deficiency but of suboptimum levels affecting the population.

Copper Balance

The intake of oral contraceptive agents did not significantly alter the metabolic balance or fecal excretion of copper. Urinary copper excretion was significantly elevated ($P<0.01$) for subjects on oral contraceptive therapy.

Copper balance was negative for the mean of both experimental groups. There does not exist as yet an RDA for copper, but the current thinking is that 2 mg/day of copper should meet most metabolic needs (104). Schroeder (141) in looking at copper nutriture in 1966 expressed the opinion that most normal diets would provide about 2 mg/day of copper, but recent work by Guthrie *et al.* (59) found that average diets in New Zealand averaged 1.5 mg/day of copper with 38 of 164 diets containing less than 1 mg/day of copper. Klevay (91) in examining diets in the U. S. found an average copper content of 1.05 mg/day with only 2 of 20 diets containing 2 mg/day of copper and 13 of 20 diets below 1 mg/day. It would appear that copper nutriture is questionable and a suboptimal nutritional state for copper probably exists.

The analysis of urinary copper was a series of trial and error attempts in this study. Most reported research on urinary copper is

either reported for straight urine or urine that has been diluted. Copper values reported by Henkin (74) indicated copper in the range of 14 µg/100 ml of urine, which with the average daily output of urine being approximately 1000 ml would indicate a urinary copper excretion exceeding 0.1 mg/24 hours. This type of analysis is at variance with other studies of copper excretion that show about 1 percent or less of ^{64}Cu eliminated via the urine (25). With dietary intakes of 2 mg/day of copper the urinary copper should be in the 20 µg/day range or less.

The first attempt at copper analysis by the method in the atomic absorption spectrophotometer manual (3) produced values in the 100 µg/day range with poor duplication due to sodium interference. The second attempt was with the graphite furnace equipped atomic absorption spectrophotometer utilizing a modification of the procedure of Ediger et al. (39) for copper in saline water. The second attempt produced reasonable values in the 30 µg/day range but 20 percent or more variation between duplicates. The third and successful attempt was a modification of Slavin's (145) technique involving extraction of divalent ions complexed to an ammonium pyrrolidine dithiocarbamate chelator and extracted into a ketone solvent for analysis. This method yielded duplicates within 2 percent of each other and values in the range of 9.5 to 19 µg/day. Recovery trials for accuracy of the method consistently yield above 90 percent recovery of added copper with an average recovery of 94 percent.

The values for urinary copper were significantly ($P<0.01$) different between the groups, with the oral contraceptive group excreting more copper. This difference was probably caused by the greatly

increased plasma copper levels associated with oral contraceptive therapy. Copper is normally excreted via the bile system with 96 percent (38) or more of plasma copper tied to ceruloplasmin which is not degraded in the kidneys (21,25,55) when present at normal levels. Two possible causes for increased urinary copper excretion exist; the degradation of some ceruloplasmin by the kidneys, or an increase in copper complexed to amino acids that can be degraded in the kidney with the loss of copper via the urine. The first of the two hypotheses seems the more likely.

Plasma Copper, Zinc, and Cholesterol

The use of subjects who are "free living" and not housed in one place presented certain problems in the study. The scheduling of time periods to draw blood samples presented a problem so that the blood samples were not fasting samples, but samples taken at least 2-1/2 hours after a meal. The time of sampling was between 10:30 A.M. and 12:30 P.M. This time was chosen based on the circadian variation postulated for plasma copper and zinc. Work by Lifschitz *et al.* (95) showed that serum copper and zinc are at fairly stable concentrations during the time period selected for blood sampling and both within 5 percent of the mean daily value.

The plasma copper for the oral contraceptive group is significantly ($P<0.01$) elevated over the control group. Plasma copper level for the oral contraceptive group on a total basis was 198.5 µg/100 ml while the control level was 121 µg/100 ml. These values are comparable with other researchers (23,60,80,87,122,139,140) who found copper values

in the range of 204 to 258 µg/100 ml for oral contraceptive users and control values in the range of 115 to 142 µg/100 ml for plasma copper. The range of values in the literature and in this study were probably due to the differing potentcies and dose levels of the individual steroids that make up the various oral contraceptive agents currently on the market (12). The actual change in copper level in plasma is attributed to the action of estrogen on the liver; estrogen induces the synthesis of ceruloplasmin, the carrier protein for copper in the plasma (14,23,80). The synthesis of ceruloplasmin occurs in the liver and copper is complexed to the protein before the release of the protein into the circulation (50). The status of liver copper reserves in women on oral contraceptive agents is as yet unknown.

Plasma zinc values were similar for oral contraceptive users and non-users. Plasma zinc concentrations on a total average basis for the experiment were 134.9 µg/100 ml for controls and 125.3 µg/100 ml for oral contraceptive users. Schenker et al. (139) showed no significant difference in serum zinc levels. Published values for blood zinc are most usually values for serum zinc. Various researchers have shown serum zinc to range from 95 to 179 µg/100 ml of serum while levels for women on oral contraceptive therapy range from 80 to 139 µg/100 ml (15,51,60,62,65,66,98,122,139). Foley (48) postulates a difference between serum and plasma zinc values with serum zinc 16 percent larger. In the current study a new blood handling technique was developed with special procedures to minimize hemolysis and to keep centrifugation speeds to a minimum. Values from this study indicate good precision and furthermore it is postulated that more serum proteins with their

attached zinc are retained in the plasma thus explaining higher values for plasma zinc in this experiment. Most other researchers (64,74) are letting blood clot, centrifuging, and precipitating the protein from the serum as a part of the preparation of samples for zinc analysis, possibly discarding a portion of the zinc.

Plasma cholesterol level has been reported to increase in oral contraceptive therapy (36,102,144). The diet in this study was calculated to contain 181 mg/day of cholesterol (45). The cholesterol levels of the two groups were significantly ($P<0.01$) different. The average levels for all periods treated as a single period were 132.8 mg/100 ml for controls and 186.6 mg/100 ml for oral contraceptive users. The result for oral contraceptive users reported in a study by Donde and Virkir (38) was 191 mg/100 ml. The estrogen content of the oral contraceptive preparation has been shown to be the agent responsible for the elevated cholesterol values (36).

Statistical Correlation

Statistical correlation showed that a relationship exists between two parameters. This correlation does not indicate a cause-effect relationship. Table 8 shows some of the parameters that were correlated, but the trivial cases where the positive correlation is also the statistically measured variable interaction are eliminated.

Recommendations for Future Study

The experiment as designed yielded a great deal of information, but several other facets could have been added that would have increased

TABLE 8

Statistical correlation of experimental parameters

Correlated Variables	r Values
Plasma copper/plasma cholesterol	$r = 0.6203$ $p < 0.0001$
Copper balance/zinc balance	$r = 0.8987$ $p < 0.0001$
Copper balance/nitrogen balance	$r = 0.5482$ $p < 0.001$
Zinc balance/nitrogen balance	$r = 0.6104$ $p < 0.0003$

the value of the study. It is recommended that an exact record of menstrual cycle timing and dates of menstruation be kept. That any future study would include a balance period that extended over a full menstrual cycle since previous work has shown differences in physical assessments such as blood levels of zinc and copper, nitrogen utilization, and rhythmic changes in urinary output of some trace minerals (78,160). That the standardization of conditions surrounding the sampling blood for trace minerals be standardized not just in terms of time of day and length of time since consuming food, but the physical activity and stress levels immediately before the drawing of blood samples. Persigehl et al. (114) recently published a study of the effects of diet, exercise, repose, and dietary components and their effects on serum zinc values, the range of perturbations of serum zinc ranged from 8 to 55 percent.

CHAPTER VI

SUMMARY

An 18-day balance study was conducted in the spring of 1976 in which the effects of oral contraceptive agents on copper and zinc balance in young women were studied. The experimental subjects were divided into two groups; one group (controls) of seven young women who had never taken oral contraceptives, and nine young women taking combined oral contraceptive agents. The mean age and weight for the two groups were essentially equal.

The subjects were fed identical diets that provided approximately 9 mg of zinc, 2 mg of copper, 50 gm of protein, and 2000 kilocalories per day. In addition to the diet the subjects were given a multivitamin capsule with breakfast each day. This dietary regimen was carried out using three menus, which cycled daily to provide some variety in diet for the subjects. All portions of food were prepared in a metabolic kitchen. All food was weighed and subjects consumed all food served. The experiment was divided into three 6-day periods, the first of which served as an adjustment period and the latter two as balance periods.

All urine and fecal material were collected for the entire 18 days of the experiment. Duplicate food samples were collected each day for the entire experiment. Nitrogen, creatinine, copper and zinc levels were determined in urine. Homogenized composites of fecal material and of food samples were prepared and analyzed for nitrogen, copper, and zinc. Fecal and food nitrogen was assayed by the Kjeldahl

method (5). Urine nitrogen and creatinine were measured daily on a Technicon Auto-analyzer II utilizing standard procedures. Analysis of food and feces for copper and zinc and urine zinc included wet-ashing of samples subsequently assayed with a Perkin-Elmer atomic absorption spectrophotometer. Urinary copper was treated to form an organic chelate, extracted into a ketone solvent, and subsequently analyzed by flame atomic absorption.

Blood samples were drawn from the subjects in each experimental period and after a 6-day post period. The subjects consumed a 25 mg/day supplement of zinc in addition to a self selected diet during the post period. Plasma levels of copper and zinc were determined on wet-ashed samples measured with the atomic absorption spectrophotometer. Plasma cholesterol samples were made into methyl-cholesterol derivatives and measured by gas-liquid chromatography.

The balance data did not show a significant difference between controls and subjects on oral contraceptive therapy for copper, zinc, or nitrogen. Nitrogen balance means for the two groups were positive. Copper and zinc balances both indicated a state of negative balance on a copper intake of 2 mg/day and a zinc intake of 9 mg/day. Mean copper balance was -0.16 mg/day for the control group and -0.13 mg/day for oral contraceptive users. Mean zinc balance was -0.57 mg/day for the control group and -0.27 mg/day for oral contraceptive users. Neither copper nor zinc balance data was corrected for integumental or sweat loss. Data for copper and zinc balance indicate a need for more than

2 mg/day of copper and 9 mg/day of zinc for optimum status in this sex-age group.

Analysis of blood plasma detected significantly ($P<0.01$) higher levels of copper and cholesterol in oral contraceptive users. Plasma zinc was not significantly different between groups. Plasma copper means were 198.5 µg/100 ml for oral contraceptive users and 121.0 µg/100 ml for controls. Plasma cholesterol means were 132 mg/100 ml for controls and 185 mg/100 ml for the oral contraceptive group. Mean plasma zinc values were 134 µg/100 ml for controls and 125 µg/100 ml for oral contraceptive users.

The results indicate that if the RDA of 15 mg/day of zinc were met that zinc balance would probably be achieved, but the recommended 2 mg/day of copper may result in a condition of suboptimum copper status in this sex-age group.

LITERATURE CITED

1. Ahmed, Faizy and Bamji, M. S. (1976) Biochemical basis for the riboflavin defect associated with the use of oral contraceptives. A study in female rats. Contraception 14, 297-307.

2. Ahmed, Faizy and Bamji, M. S. (1976) Vitamin supplements to women using oral contraceptives. Contraception 14, 309-319.

3. Analytical Methods for Atomic Absorption Spectrophotometry (1975). Revised edition, Perkin-Elmer Corporation. Norwalk, CN.

4. Appleton, D. W. and Sarkar, B. (1971) The absence of a specific copper (II)-binding site in dog albumin. J. Biol. Chem. 246, 5040-5046.

5. Association of Official Agricultural Chemists. (1950) Official Methods of Analysis. 7th ed., Washington, D. C.

6. Barcia, P. J. (1970) Lack of acceleration of healing with zinc sulfate. Ann. Surg. 172, 1048-1050.

7. Barr, A. J. and Goodnight, J. H. SAS 72: Institute of Statistics, N. C. State University.

8. Bearn, H. G. and Kunkel, H. G. (1955) Metabolic studies in Wilson's disease using ^{64}Cu. J. Lab. Clin. Med. 45, 623-631.

9. Beck, Paul (1975) Alterations of lipid metabolism by contraceptive steroids. J. Steroid Biochem. 6, 957-959.

10. Becking, George C. (1976) Trace elements in drug metabolism. Med. Clin. North Amer. 60, 813-830.

11. Beisel, William R. (1976) Trace elements in infectious processes. Med. Clin. North Amer. 60, 831-849.

12. Berger, G. S. and Talwar, P. P. (1978) Oral contraceptive potencies and side effects. Obstet. Gynecol. 51, 545-547.

13. Bowland, J. P., Braude, R., Chamberlain, A. G., Glascock, R. F., and Mitchell, K. G. (1961) The absorption, distribution, and excretion of labeled copper in young pigs given different quantities, as sulfate or sulfide, orally or intravenously. Brit. J. Nutr. 15, 59-72.

14. Briggs, Michael, Austin, J., and Staniford, Maxine (1970) Oral contraceptives and copper metabolism. Nature, 225, 81.

15. Briggs, M. H., and Briggs, Maxine (1971) Effect of steroid pharmaceuticals on plasma zinc. Nature, 232, 480.

16. Briggs, Michael and Briggs, Maxine (1973) Effects of some contraceptive steroids on serum proteins of women. Biochem. Pharmacol. 22, 2277-2281.

17. Burch, R. E., Hahn, H. K. J., and Sullivan, J. F. (1975) Newer aspects of the roles of zinc, manganese, and copper in human nutrition. Clin. Chem. 21, 501-520.

18. Burch, R. E. and Sullivan, J. F. (1976) Diagnosis of zinc, copper, and manganese abnormalities in man. Med. Clin. North Amer. 60, 655-660.

19. Burch, R. E. and Sullivan, J. F. (1976) Clinical and nutritional aspects of zinc deficiency and excess. Med. Clin. North Amer. 60, 675-685.

20. Burns, R. R. and Fell, G. S. (1976) Estimation and Interpretation on plasma zinc fractions. Scott. Med. J. 21, 153-154.

21. Bush, J. A., Mahone, D. A., Markowitz, H., Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M. (1975) Studies in copper metabolism XVI. Radioactive copper studies in normal subjects and in patients with hepatolenticular degeneration. J. Clin. Investigation 34, 1766-1778.

22. Carrico, R. J. and Deutsch, H. F. (1970) The presence of zinc in human cytocuprein and some properties of the protein. J. Biol. Chem. 245, 723-727.

23. Carruthers, M. E., Hobbs, C. B., and Warren, R. L. (1966) Raised serum copper and caeruloplasmin levels in subjects taking oral contraceptives. J. Clin. Path. 19, 498-500.

24. Carter, J. P., Grivetti, L. E., Davis, J. T., Nasiff, S., Mansour, A., Mousa, W. A., Atta, A., Patwardham, V. N., Moneim, M. A., Abdou, I. A., and Darby, W. J. (1969) Growth and sexual development of adolescent Egyptian village boys. Effect of zinc, iron, and placebo supplementation. Amer J. Clin. Nutr. 22, 59-78.

25. Cartwright, G. E. and Wintrobe, M. M. (1964) Copper metabolism in normal subjects. Amer. J. Clin. Nutr. 14, 224-232.

26. Cartwright, G. E. and Wintrobe, M. M. (1964) The question of copper deficiency in man. Amer. J. Clin. Nutr. 15, 94-110.

27. Chvapil, M., Elias, S. L., Ryan, J. N., and Zukoski, C. F. (1972) Pathophysiology of zinc. In: Neurobiology of the Trace Metals Zinc and Copper. (Pfeiffer, C. C. ed.), p. 105, Academic Press, New York.

28. Chvapil, M., Peng, Y. M., and Aronson, A. L. (1974) Effect of zinc on lipid peroxidation and metal content in some tissues of rats. J. Nutr. 104, 434-443.

29. Coats, P., Walker, E., Youssefnejadian, E., and Craft, I. L. (1977) Variations in plasma steroid and prostaglandin concentrations during human pregnancy. Acta Obstst. Gynecol. Scand. 56, 453-457.

30. Cotton, F. A., and Wilkinson, G. (1972) Advanced Inorganic Chemistry. 3rd ed. p. 515, Interscience, New York.

31. Cousins, R. J. (1978) Synthesis and degradation of hepatic zinc-thionein and its role in zinc metabolism. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 59-63, Technisch Universität München G.D.R.

32. Crampton, R. F., Matthews, D. M., and Poisner, R. (1965) Observations on the mechanisms of absorption of copper by the small intestine. J. Physiol. 178, 111-126.

33. Danks, D. M., Campbell, P. E., and Steven, B. J. (1972) Menkes' kinky hair-syndrome. An inherited defect in copper absorption with wide spread effects. Pediatrics 50, 188-201.

34. Davies, N. T., and Wahle, K. W. J. (1978) A role for copper in mixed function oxidases. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.). pp. 23-27, Technisch Universität München, G.D.R.

35. Dawson, E. B., Frey, M. J., Monistere, N., and McGanity, W. J. (1973) Essential metals in oral contraceptive tablets. Amer. J. Obstet. Gynecol. 116, 412-414.

36. deAlvarez, R. R., Jahed, F. H., and Spitalny, K. J. (1973) The influence of oral contraceptive steroids of serum lipids. Amer J. Obstet. Gynecol. 116, 727-749.

37. Donde, U. M. and Virkir, K. (1975) Effect of contraceptive steroids on serum lipids. Amer. J. Obstet. Gynecol. 123, 736-741.

38. Donde, U. M. and Virkir, K. (1976) Effect of oral (combination and progestogen alone) and injectable contraceptives on serum copper levels. Indian J. Med. Res. 64, 787-791.

39. Ediger, R. D., Peterson, G. E., and Kerber, J. D. (1974) Application of the graphite furnace to saline water analysis. Atomic Absorption Newsletter, 13, 61-64.

40. Edwards, H. M., Jr. (1959) The availability to chicks of zinc in various compounds and ores. J. Nutr. 69, 306-308.

41. Elvehjem, C. A. (1935) The biological significance of copper and its relation to iron metabolism. Physiol. Rev. 15, 471-507.

42. Evans, G. W. (1973) Copper homeostasis in the mammalian system. Physiol. Rev. 53, 535-570.

43. Evans, G. W. and Johnson, P. E. (1978) Copper- and Zinc-binding ligands in the intestinal mucosa. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 98-105. Technisch Universität München, G.D.R.

44. Evans, G. W. and Wiederanders, R. E. (1969) Blood copper variation among species. Amer. J. Physiol. 213, 1183-1185.

45. Feeley, R. M., Criner, P. E., and Watt, B. K. (1972) Cholesterol content of foods. J. Amer. Diet. Assn. 61, 134-141.

46. Fell, G. S., Fleck, A., Cuthbertson, D. P., Queen, K., Morrison, C., Bessent, R. G., and Husain, S. L. (1973) Urinary zinc levels as in indication of muscle catabolism. Lancet, 280-282.

47. Fern, M., Rose, D. P., and Fern, E. B. (1978) Effect of oral contraceptives on plasma androgenic steroids and their precursors. Obstet. Gynecol. 51, 541-544.

48. Foley, B., Johnson, S. A., Hackley, B., Smith, J. C., and Halsted, J. A. (1968) Zinc content of human platelets. Proc. Soc. Exper. Biol. Med. 128, 265-269.

49. Frieden, E. (1978) Ceruloplasmin, a multifunctional metalloprotein. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 36-39. Technisch Universität München, G.D.R.

50. Frieden, E. (1978) Modes of metal metabolism. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 8-14. Technisch Universität München, G.D.R.

51. Funckes, C. G., Chvapil, M., Carrol, R. W., and Bressler, R. (1976) Effect of a long-acting contraceptive drug, norethindrone enanthate, on serum zinc and copper in human volunteers. Contraception 14, 291-295.

52. Giroux, E., Durieux, M., and Schechter, P. J. (1976) A study of zinc distribution in human serum. Bioinorganic Chem. 5, 211-218.

53. Giroux, E., Schechter, P. J., and Schoun, J. (1976) Diminished albumin binding of zinc in serum of pregnant women. Clin. Sci. Mole. Med. 51, 545-549.

54. Gollan, J. L., Davis, P. S., and Deller, D. J. (1971) Binding of copper by human alimentary secretions. Amer. J. Clin. Nutr. 24, 1025-1027.

55. Gollan, J. L. and Deller, D. J. (1973) Studies on the nature and excretion of biliary copper in man. Clin. Sci. 44, 9-15.

56. Goresky, C. A., Holmes, T. H., and Sasskortsak, A. (1968) The initial uptake of copper by the liver in the dog. Can. J. Phsio. Pharmacol. 46, 771-784.

57. Graham, G. C. and Cordano, A. (1969) Copper depletion and deficiency in the malnourished infant. Johns Hopkins Med. J. 124, 139-141.

58. Greger, J. L. and Buckley, S. B. (1977) Menstrual loss of zinc, copper, magnesium and iron by adolescent girls. Nutr. Reports Internatl. 16, 639-647.

59. Guthrie, B. E., McKenzie, J. M., and Casey, C. C. (1978) Copper status of New Zealanders. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 304-306. Technisch Universität München, G.D.R.

60. Hahn, N., Paschen, K., and Haller, J. (1972) Behaviour of copper, iron, magnesium, calcium and zinc in women during normal menstrual cycles, while taking ovulation inhibitors and during pregnancy. Archiv. für Gynäkologie 213, 176-186.

61. Halsted, J. A. (1970) Human zinc deficiency. Trans. Amer. Clin. Climat. Assn. 82, 170-176.

62. Halsted, J. A., Hackey, B. M., and Smith, J. C. (1968) Plasma-zinc and copper in pregnancy and after oral contraceptives. Lancet, 278-279.

63. Halsted, J. A. and Prasad, A. S. (1960) Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism, and geophagia. Trans. Amer. Clin. Climat. Assn. 72, 130-149.

64. Halsted, J. A., Ronaghy, H. A., Abadi, P., Haghshenars, M., Amirhakem, G. H., Barakat, R. M., and Reinhold, J. G. (1972) Zinc deficiency in man: The Shiraz experiment. Amer. J. Med. 53, 277-284.

65. Halsted, J. A. and Smith, J. C. (1970) Plasma-zinc in health and disease. Lancet, 322-324.

66. Halsted, J. A., Smith, J. C., Hackley, B. M., and McBean, L. (1969) Plasma zinc and copper levels. Amer. J. Obstet. Gynecol. 105, 645-646.

67. Halsted, J. A., Smith, J. C., and Irwin, M. I. (1974) A conspectus of research on zinc requirements of man. J. Nutr. 104, 345-378.

68. Hambidge, K. M. (1978) Zinc and chromium in human nutrition. J. Human Nutr. 32, 99-110.

69. Hambidge, K. M., Hambidge, C., Jacobs, M., and Baum, J. N. (1972) Low levels of zinc in hair, anorexia, poor growth, and hypogeusia in children. Pediat. Res. 6, 868-874.

70. Hambidge, K. M., Walravens, P. A., Nelder, K. H., and Daugherty, N. A. (1978) Zinc, copper and fatty-acids in acrodermatitus enteropathica. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 413-417. Technisch Universität München, G.D.R.

71. Harris, E. D. and Rayton, J. K. (1978) Regulatory aspects of copper metabolism. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 19-22. Technisch Universität München, G.D.R.

72. Hart, E. B., Steenbock, H., and Waddell, J. (1928) Iron in nutrition VII. Copper as a supplement to iron for hemoglobin building in the rat. J. Biol. Chem. 77, 797-812.

73. Hathcock, J. and Abraham, C. (1978) Effect of protein, amino acids, phytate, and cadmium on zinc absorption in rats. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 126-128. Technisch Universität München, G.D.R.

74. Henkin, R. I. (1971) Newer aspects of copper and zinc metabolism. In: Newer Trace Elements in Nutrition. (Mertz, W. and Cornatzer, W. E., ed.) pp. 255-309. Marcel Dekker, New York.

75. Henkin, R. I. (1978) Zinc dependent control of food intake, taste and smell function. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 190-198. Technisch Universität München.

76. Henkin, R. I. (1976) Trace metals in endocrinology. Med. Clin. of North Amer. 60, 770-798.

77. Henkin, R. I., Schechter, P. J., Hoye, R., and Mattern, C. F. T. (1971) Idiopathic hypogeusia with dysgeusia, hyposmia, and dysosmia. J. Amer. Med. Assn. 217, 434-440.

78. Hess, F. M., King, J. C., and Margen, S. (1977) Zinc excretion in young women on low zinc intakes and oral contraceptive agents. J. Nutr. 10, 1610-1620.

79. Hoekstra, W. G., Bremner, I., and Davies, N. T. (1978) Effect of zinc status of rats on the synthesis and degradation of copper-induced thioneins. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 52-56. Technisch Universität München, G.D.R.

80. Horwitt, M. K., Harvey, C. C., and Dahm, C. H. (1975) Relationship between levels of blood lipids, vitamins C, A, and E, serum copper compounds, and urinary excretions of tryptophan metabolites in women taking oral contraceptive therapy. Amer. J. Clin. Nutr. 28, 403-412.

81. House, W. A., Welch, R. M., and Van Campen, D. R. (1978) Zinc kinetics and metabolism in rats fed diets with or without phytic acid. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 171-174. Technisch Universität München, G.D.R.

82. Hsu, J. M., and Anthony, W. L. (1971) Impairment of cystine ^{35}S incorporation into rat skin protein by zinc-deficient rats. J. Nutr. 101, 445-452.

83. Hurley, L. S. (1976) Trace elements and teratogenesis. Med. Clin. North Amer. 60, 771-778.

84. Hurley, L. S., Duncan, J. R., Sloan, M. V., and Eckhert, C. D. (1977) Development of zinc binding ligands during the postnatal period. Fed. Proc. 36, 1138.

85. Hurley, L. S. and Swenerton, H. (1966) Congenital malformations resulting from zinc deficiency in rats. Proc. Soc. Expl. Biol. Med. 123, 692-696.

86. Jameson, Sten (1977) Effects of zinc deficiency in human reproduction. Acta Medica Scandinavica. supplementum 593, 77-89.

87. Johnson, N. C. (1961) Study of copper and zinc metabolism during pregnancy. Proc. Soc. Expl. Biol. Med. 108, 518-519.

88. Jones, K. L., Smith, D. W., Ulleland, C. N., and Streissguth, P. (1973) Pattern of malformation in offspring of chronic alcoholic mothers. Lancet i, 1267.

89. Karpel, J. T. and Peden, V. A. (1972) Copper deficiency in long term parenteral nutrition. J. Pediat. 80, 32.

90. Keilin, D. and Mann, T. (1940) Carbonic anhydrase, purification and nature of the enzyme. Biochem. J. 34, 1163-1172.

91. Klevay, L. M. (1978) Dietary copper and the copper requirement of man. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 307-311. Technisch Universität München, G.D.R.

92. Korba, V. D. and Heil, C. G. (1975) Eight years of fertility control with norgestrel-ethinyl estradiol (Ovral): an updated clinical review. Fert. Ster. 26, 973-981.

93. Kudzma, D. J., Bradley, E. M., and Goldzieher, J. W. (1972) A metabolic balance study of the effects of an oral steroid contraceptive on weight and body composition. Contraception 5, 31-37.

94. Larsson-Cohn, Ulf (1974) Oral contraceptives and vitamins: A review. Amer. J. Obstet. Gynecol. 121, 84-90.

95. Lifschitz, M. D. and Henkin, R. I. (1971) Circadian variation in copper and zinc in man. J. Appl. Physiol. 31, 88-92.

96. Margen, S. and King, J. C. (1975) Effect of oral contraceptive agents on the metabolism of some trace minerals. Amer. J. Clin. Nutr. 28, 392-402.

97. Martin, J. V., Martin, P. J., and Goldberg, D. M. (1976) Enzyme induction as a possible cause of increased serum-triglycerides after oral contraceptives. Lancet, 1107-1108.

98. McBean, L. D., Smith, J. C., and Halsted, J. A. (1971) Effect of oral contraceptive hormones on zinc metabolism in the rat. Proc. Soc. Expl. Biol. Med. 137, 543-547.

99. McClain, P. E., Wiley, E. R., Beecher, G. R., Anthony, W. L., and Hsu, J. M. (1973) Influence of zinc deficiency on synthesis and crosslinking of rat skin collagen. Biochimica Biophysica Acta. 304, 457-465.

100. Methfessel, A. H. and Spencer, H. (1973) Zinc metabolism in the rat. I. Intestinal absorption of zinc. J. Appl. Physiol. 34, 58-62.

101. McMahon, D. (1974) Chemical messengers in development: a hypothesis. Science 185, 1012-1020.

102. Miale, J. B. and Kent, J. W. (1974) The effects of oral contraceptives on the results of laboratory tests. Amer. J. Obstet. Gynecol. 120, 264-272.

103. Morgan, K. T. (1971) Chronic copper toxicity of sheep: An ultrastructural study of spongiform leucoencepathy. Res. Vet. Sci. 15, 83-95.

104. National Academy of Sciences. (1971) Recommended Dietary Allowances, Food and Nutrition Board, National Research Council, Washington, D. C.

105. Nelson, J. H. (1973) Selecting the optimum oral contraceptive. J. Reprod. Med. 11, 135-141.

106. Nilsson, C. G. (1977) Comparative quantities of menstrual blood loss with a d-norgestrel-releasing IUD and a nova-T-copper device. Contraception 15, 379-387.

107. O'Dell, B. L. (1976) Biochemistry of copper. Med. Clin. North Amer. 60, 687-703.

108. O'Dell, B. L. and Savage, J. E. (1957) Potassium, zinc, and distillers dried solids as supplements to a purified diet. Poultry. Sci. 136, 459-460.

109. Owen, C. A., Jr. (1965) Metabolism of radiocopper (^{64}Cu) in the rat. Amer. J. Physiol. 209, 900-904.

110. Owen, J. A. (1975) Physiology of the menstrual cycle. Amer. J. Clin. Nutr. 28, 333-338.

111. Pallauf, Jr. (1978) Effect of zinc deficiency on digestibility and utilization of nutrients. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 218-221. Technisch Universität München, G.D.R.

112. Parisi, A. F. and Vallee, B. L. (1969) Zinc metalloenzymes: characteristics and significance in biology and medicine. Amer. J. Clin. Nutr. 22, 1222-1239.

113. Pearson, W. N., Schwink, T., and Reich, M. (1966) In vitro studies of zinc absorption in the rat. In: Zinc Metabolism. (Prasad, A. S., ed.) pp. 239-249. Wiley, New York.

114. Persigehl, M., Hock, A., Kasperek, K., and Land, E. (1974) Changes in serum zinc concentration in different metabolic states. Zeitschrift fur Klinische Chemie und Klinisch Biochemie, 12, 171-175.

115. Pilgeram, L. O., Ellison, J., and Von Dem Bussche, G. (1974) Oral contraceptives and increased formation of soluble fibrin. Brit. Med. J. 3, 556-558.

116. Pories, W. J., Henzil, J. H., Rob, C. G., and Strain, W. H. (1967) Acceleration of healing with zinc sulfate. Ann. Surg. 165, 432-436.

117. Prasad, A. S., Halsted, J. A., and Nadimi, M. (1961) Syndrome of iron deficiency anemia, hepatosplenomegaly, hypogonadism, dwarfism, and geophagia. Amer. J. Med. 31, 532-546.

118. Prasad, A. S., Lei, M. D., Oberleas, D., Moghissi, K. S., and Stryker, J. C. (1975) Effect of oral contraceptive agents on nutrients: II. Vitamins. Amer. J. Clin. Nutr. 28, 385-391.

119. Prasad, A. S., Miale, A., Farid, Z., Sansted, H. H., and Schulert, A. R. (1963) Zinc metabolism in patients with the syndrome of iron deficiency anemia, hepatosplenomegaly, dwarfism, and hypogonadism. J. Lab. Clin. Med. 61, 537-549.

120. Prasad, A. S. and Oberleas, D. (1971) Changes in activities of zinc-dependent enzymes in zinc-deficient tissues of rats. J. Appl. Physiol. 31, 842-846.

121. Prasad, A. S. and Oberleas, D. (1970) Binding of zinc to amino acids and serum proteins in vitro. J. Lab. Clin. Med. 76, 416-425.

122. Prasad, A. S., Oberleas, D., Lei, K. Y., Moghissi, M. D., and Stryker, J. C. (1975) Effect of oral contraceptive agents on nutrients: I. Minerals. Amer. J. Clin. Nutr. 28, 377-384.

123. Prasad, A. S., Rabbani, P., Abbasi, A., Bowersox, E., and Fox, M. R. S. (1978) Experimental production of zinc deficiency in man. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 280-285. Technisch Universität München, G.D.R.

124. Price, C. A. and Vallee, B. L. (1962) Euglena gracilis, a test organism for study of zinc. Plant Physiol. 37, 428.

125. Price, N. O., Bunce, G. E., and Engel, R. W. (1970) Copper, manganese, and zinc balance in preadolescent girls. Amer. J. Clin. Nutr. 23, 258-260.

126. Quarterman, J., Mills, C. F., and Humphries, W. R. (1966) The reduced secretion and sensitivity to insulin in zinc deficient rats. Biochem. Biophys. Res. Commun. 25, 354-358.

127. Richards, M. P. and Cousins, R. J. (1977) Isolation of intestinal zinc-thionein: proposed function in zinc absorption. Fed. Proc. 36, 1106.

128. Rienhold, J. G., Nasr, K., Lahimgarzadeh, A., and Hedayati, H. (1973) Effects of purified phytate and phytate-rich bread upon metabolism of zinc, calcium, phosphorus, and nitrogen in man. Lancet, 283-284.

129. Riordan, James F. (1976) Biochemistry of zinc. Med. Clin. North Amer. 60, 661-674.

130. Roeser, H. P., Lee, G. R., Nacht, S., and Cartwright, G. E. (1970) The role of ceruloplasmin in iron metabolism. J. Clin. Investigation. 49, 2408-2417.

131. Roman, W. (1969) Zinc in porphyria. Amer. J. Clin. Nutr. 22, 1290-1303.

132. Ronaghy, H., Fox, M. R. S., Garn, S. M., Israel, H., Harp, A., Moc, P. G., and Halsted, J. A. (1969) Controlled zinc supplementation for malnourished school boys: a pilot experiment. Amer. J. Clin. Nutr. 22, 1279-1289.

133. Rose, David P. (1977) The pill and nutrition: a most intricate relationship. Professional Nutritionist. 9, 1-5.

134. Sandstrom, B., Arvidsson, B., Bjorn-Rsamussen, E., and Cederblad, A. (1978) Zinc absorption from bread meals. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 129-131. Technische Universität München, G.D.R.

135. Sansted, H. H., Prasad, A. S., Schulert, A. R., Farid, Z., Miale, A., Jr., Bassilly, S., and Darby, W. J. (1967) Human zinc deficiency, endocrine manifestations and response to treatment. Amer. J. Clin. Nutr. 20, 422-442.

136. Sansted, H. H., Shukry, A. S., Prasad, A. S., Gabr, M. K., El Hifney, A., Mokhtar, N., and Darby, W. J. (1965) Kwashiorkor in Egypt. I. clinical and biochemical studies with special reference to plasma zinc and serum lactic dehydrogenase. Amer. J. Clin. Nutr. 17, 15-26.

137. Schecter, P. J., Giroux, E., and Sjoerdsma, A. (1978) Zinc distribution in human serum, with data on alterations in pregnancy and hepatic cirrhosis. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 343-345. Technisch Universität München, G.D.R.

138. Schelling, J. L., Muller-Hess, S., and Thonney (1973) Effect of food on zinc absorption. Lancet i, 263.

139. Schenker, J. G., Hellerstein, S., Jungreis, E., and Polishuk, W. Z. (1971) Serum copper and zinc levels in patients taking oral contraceptives. Fert. Ster. 22, 229-234.

140. Schenker, J. G., Jungreis, E., and Polishuk, W. Z. (1971) Oral contraceptives and serum copper concentration. Obstet. Gynecol. 37, 233-237.

141. Schroeder, H. A., Nason, A. P., Tipton, I. H., and Balassa, T. J. (1966) Essential trace elements in man: copper. J. Chron. Disease. 19, 1001-103.

142. Schwarz, F. J. and Kirchgessner, M. (1978) Studies on the regulation of the intestinal absorption of zinc. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 110-115. Technisch Universität München, G.D.R.

143. Sever, L. E. and Emanuel, I. (1973) Is there a connection between maternal zinc deficiency and congenital malformations of the central nervous system in man? Teratology 1, 117-121.

144. Shahani, S. M. and Patel, K. L. (1974) Effects of different oral contraceptives (combined and mini pill) on lipid metabolism. Contraception 10, 263-272.

145. Slavin, Walter. (1968) Atomic Absorption Spectroscopy. pp. 75-77, Interscience, New York.

146. Smit, Z. M. and Pretorius, P. J. (1964) Studies in metabolism of zinc. part 2. serum zinc levels and urinary zinc excretion in South African Bantu kwashiorkor patients. J. Trop. Pediat. 9, 105-112.

147. Somers, M. and Underwood, E. J. (1969) Studies of zinc nutrition in sheep II. The influence of zinc deficiency in ram lambs upon digestibility of dry matter and utilization of the N and S of the diet. Austra. J. Agric. Res. 20, 899-903.

148. Steele, T. H. (1967) Dissociation of zinc excretion from other cations in man. J. Lab. Clin. Med. 81, 205-213.

149. Todd, W. R., Elvehjem, C. A., and Hart, E. B. (1934) Zinc in the nutrition of the rat. Amer. J. Physiol. 107, 146-156.

150. Topham, R. W. and Frieden, E. (1970) Identification and purification of a nonceruloplasmin ferroxidase of human serum. J. Biol. Chem. 245, 6698-6705.

151. Tucker, H. F. and Salmon, W. D. (1955) Parakeratosis or zinc deficiency disease in the pig. Proc. Soc. Exp. Biol. Med. 88, 613-616.

152. Underwood, E. J. (1971) Trace Elements in Human and Animal Nutrition. Ed. 2, pp. 57-115, Academic Press, New York.

153. Vallee, B. L. (1973) Cobalt substituted zinc metalloenzymes. In: Metal Ions in Biological Systems. (Dhar, S. K., ed.) p. 1, Plenium Press, New York.

154. Vallee, B. L. and Wacker, W. E. C. (1976) Metalloproteins. In: Handbook of Biochemistry, Vol. II, 3rd ed. (Fasman, G. D., ed.) pp. 276-292, CRC Press, Cleveland.

155. Vallee, B. L. and Williams, R. J. P. (1968) Metalloenzymes: The entatic nature of their active sites. Proc. Acad. Sci. U. S. 59, 498.

156. Van Campen, D. R. and Mitchell, E. A. (1965) Absorption of ^{64}Cu, ^{65}Zn, ^{99}Mo, and ^{56}Fe from ligated segments of the rat gastrointestinal tract. J. Nutr. 86, 120-124.

157. van Riz, A. M. and McKinzie, J. M. (1978) Hyperzincuria and zinc deficiency in total parenteral nutrition. In: Trace Element Metabolism in Man and Animals-3. (Kirchgessner, M., ed.) pp. 288-291. Technische Universität München, G. D. R.

158. Wegger, I. and Palludan, B. (1978) Zinc metabolism in swine with special emphasis on reproduction. In: Trace Element Metabolism in Man and Animal-3. (Kirchgessner, M., ed.) pp. 428-435. Technische Universität München, G. D. R.

159. Weigand, E. and Kirchgessner, M. (1978) Homeostatic adaptation of zinc absorption and endogenous zinc excretion over a wide range of dietary zinc supply. In: Trace Element Metabolism in Man and Animals-3. pp. 106-109.

160. Weinmann, S., Dawson, E. B., Smolensky, M. H., and Weisberg, R. F. (1976) Effect of oral contraceptives on rhythmic urinary metal excretion. Amer. J. Obstet. Gynecol. 124, 719-722.

161. Wynn, V., Adams, P. W., Folkard, J., and Seed, M. (1975) Tryptophan, depression and steroidal contraception. J. Steroid Biochem. 6, 965-970.

162. Yunice, A. A., Lindeman, R. D., Czerwinski, A. W., and Clark, M. (1974) Influence of age and sex on serum copper and ceruloplasmin levels. J. Gerontology. 29, 277-281.

APPENDIX I

Oral Contraceptive Agents Used by Subjects

O.C.A.	No. Sub. on O.C.A.	Progestogen	mg	Estrogen	mg
Ortho-novum 1/50	2	Norethindrone	1.0	Mestranol	.05
Norinyl 1/50	2	Norethindrone	1.0	Mestranol	.05
Ovulin 21	1	Ethynodiol Diacetate	1.0	Mestranol	.10
Zorane 1/50	1	Norethindrone	1.0	Ethinylestradiol	.05
Demulen	1	Ethynodiol Diacetate	1.0	Ethinylestradiol	.05
Low-Overal	1	Norgestrel	0.3	Ethinylestradiol	.03
Loestrin 1.5/30	1	Norethindrone	1.0	Ethinylestradiol	.02

APPENDIX II

Menus Served in Study

Menu A[a]

	Food Item	Amount, gm
Breakfast:	Shredded wheat	28
	Whole milk	122
	Sugar	10
	Orange juice	248
	Coffee *or*	3
	Tea	6
Lunch:	Bologna	28
	White bread	56
	Mayonnaise	28
	Lettuce	50
	Carbonated beverage (gingerale)	366
	Apple (raw)	180
	Potato chips	20
	Fondant mints	28
Dinner:	Chicken breast	60
	Baby peas	80
	Carrots (cooked)	155
	Cabbage, shredded	90
	Salad dressing (creamy)	30
	Roll	28
	Margarine	14
	Cake (white)	86
	Pretzel sticks	6
	Sugar	5

[a] Deionized water available *ad libitum* with all meals.

Menu B[a]

	Food Item	Amount, gm
Breakfast:	Sausage links	28
	Egg (hard cooked)	50
	Biscuit	28
	Margarine	14
	Jelly (grape)	14
	Applesauce	122
	Cran-apple juice	164
	Sugar	5
	Coffee or	3
	Tea	6
Lunch:	Tomato soup	245
	Saltines	11
	Peanut butter	24
	Applesauce	122
	Carbonated beverage (cola)	185
	Whole milk	122
	Non-fat dry milk (reconstituted)	122
Dinner:	White fish	85
	Green beans	125
	Baked potato	130
	Roll	28
	Margarine	28
	Lettuce	50
	Dressing (French)	32
	Peaches	110
	Graham crackers	28
	Sugar	5
	Orange	180

[a] Deionized water available ad libitum with all meals.

Menu C[a]

	Food Item	Amount, gm
Breakfast:	Puffed wheat	15
	Bananna	119
	Orange juice	248
	Whole milk	122
	Bread (toasted)	28
	Margarine	14
	Jelly (strawberry)	28
	Sugar	10
	Coffee or	3
	Tea	6
Lunch:	Frankfurter	56
	Bun	40
	Ketchup	28
	Corn chips	28
	Chocolate milk	125
	Vanilla creme cookies	28
	Pear	182
Dinner:	Chicken breast (sliced)	44
	Corn	105
	Broccoli	77
	Carrot (raw)	50
	Lettuce	50
	Tomato (sliced)	30
	Salad dressing	30
	Fruit cocktail	256
	Vanilla wafers	28
	Roll	28
	Margarine	14
	Sugar	5

[a] Deionized water available ad libitum with all meals.

APPENDIX III

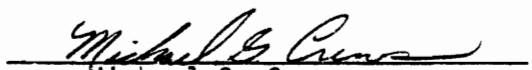
Reagents Used in Trace Mineral Analysis

1. Carbon disulfide, Fisher Reagent grade, ACS.
2. Ethyl alcohol, U.S.P. Pure, Pharmco.
3. Hydrochloric acid, "Baker analyzed", reagent grade.
4. Hydrogen peroxide, 30%, stabilized with 0.0002 percent $Na_2SnO_3 \cdot 3H_2O$, "Baker Analyzed" reagent grade.
5. Methyl Isobutyl Ketone, Fisher Reagent grade, ACS.
6. Nitric Acid, "Baker Analyzed", reagent grade.
7. Perchloric acid, 70 percent, G. Frederick Smith, double vacuum distilled in Vycor.
8. Pyrrolidine, Fisher reagent grade, ACS.
9. Sulfuric acid, 98 percent, Fisher Reagent grade, ACS.
10. Deionized water, redistilled water passed through a Barnstead mixed bed deionization column and stored in polycarbonate vessels.

VITA

Michael Glen Crews was born on April 1, 1943 in Princeton, West Virginia. He received his Bachelor of Science degree in Metallurgical Engineering in 1972 and his Doctor of Philosophy degree in Human Nutrition and Foods in 1979, both from Virginia Polytechnic Institute and State University. Work experiences include six years as a laboratory technician with the Department of Human Nutrition and Foods at Virginia Polytechnic Institute and State University, and one year as Materials Engineer/Engineering laboratory supervisor for KDI-Electro-Tec Corporation, Blacksburg, Virginia. Currently, he is an assistant professor of Foods and Nutrition with the College of Home Economics at Texas Tech University located in Lubbock, Texas.

He is married to the former Georgia Mae Williams. They have one daughter, Naomi Elizabeth.



Michael G. Crews
4400 Avenue X
Lubbock, Texas 79412

THE EFFECT OF ORAL CONTRACEPTIVE AGENTS ON COPPER AND ZINC BALANCE IN YOUNG WOMEN

by

Michael Glen Crews

(ABSTRACT)

An 18-day balance study was designed to test the hypothesis that the use of oral contraceptive agents would change the metabolic balance of copper and zinc in young women. The subjects were divided into two treatment groups based on the use or non-use of oral contraceptive agents. The mean age was 21 years and 6 months for the non-oral contraceptive group and 21 years and 2 months for the oral contraceptive group. The mean weight was 57.2 kg for the non-oral contraceptive group and 59.7 kg for the group on oral contraceptive therapy.

The study was composed of three 6-day periods. The subjects of both groups consumed identical diets for the three experimental periods. The diet contained approximately 2 mg of copper, 9 mg of zinc, 50 g of protein, 181 mg of cholesterol, and 2000 kcal per day. The first experimental period served as an adjustment period with the latter two periods serving as the balance periods. Subjects were given a multi-vitamin supplement daily.

All urine and feces were collected and assayed for copper, zinc, and nitrogen. Food samples were measured for content of copper, zinc, and nitrogen. Blood samples were taken and the plasma fraction

analyzed for copper, zinc, and cholesterol content. Urinary copper was measured as an organic chelate by atomic absorption spectrophotometry. Copper and zinc content of the other samples were assayed in wet ashed samples by atomic absorption spectrophotometry. Food and fecal nitrogen was assayed by Kjeldahl analysis. Urine nitrogen and creatinine was measured by standard automated techniques.

No significant differences were found in the metabolic balances of copper, zinc, and nitrogen. Oral contraceptive users were found to have significantly ($p<0.01$) higher plasma cholesterol and copper levels, but plasma zinc concentrations were not significantly different. No difference was found in hemoglobin or hematocrit for the two groups. The urinary excretion of zinc was similar for the two groups. The excretion of copper and creatinine was significantly ($p<0.01$) higher for the oral contraceptive group. The urinary and fecal excretion of copper and zinc was not significantly different for the two groups. While the balance data was not different for the two groups, it is of interest that both groups maintained negative balance states on intakes similar to projected intakes for the general population. This indicated that both groups needed more copper and zinc than they received. It therefore appears that consideration should be given to a possible suboptimal nutritional state existing for copper and zinc nutrition for at least this sex-age group.