UNIVERSITY OF SOUTHAMPTON

ZINC, COPPER AND SELENIUM STATUS IN HEALTH AND DISEASE, WITH EMPHASIS ON THE USE OF LEUCOCYTE ANALYSES

By
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August 1987

Submitted in accordance with the requirement of the University of Southampton for the degree of Master of Philosophy

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ABSTRACT

FACULTY OF MEDICINE

CHEMICAL PATHOLOGY AND HUMAN METABOLSIM

MASTER OF PHILOSOPHY

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A technique for the isolation of leucocytes from 15ml of whole blood, using a modified dextran sedimentation procedure, was developed and evaluated. The mean recovery of leucocytes was 62%, without differential white cell loss, and there was no contamination from red cells, platelets or plasma. Atomic absorption spectrophotometric methods for the determination of zinc, copper and selenium in the isolated leucocytes, and copper in erythrocytes and whole blood, were evolved. Using these procedures, and established trace element methodology, reference ranges for zinc, copper and selenium in leucocytes, erythrocytes and plasma were obtained in a study of healthy adult subjects under the age of 65 years. These data provided a baseline for the assessment of the body status of zinc, copper and selenium in various disease states in which deficiencies of these trace elements might be present.

A study of the zinc and copper status of an elderly population was also undertaken. Housebound elderly people were found to have significantly reduced leucocyte zinc and copper concentrations compared with apparently healthy elderly subjects. Five-day metabolic balance studies for zinc and copper were also carried out on these subjects at the same time as the present study (for references see text). Negative zinc and copper balances were observed in the housebound elderly people, and there was a significant correlation between zinc balance and leucocyte zinc concentration in this subject group.

Patients with Crohn's disease, coeliac disease, and those receiving total parenteral nutrition, alcoholic subjects with and without established liver disease, and patients with eczema and psoriasis were investigated for possible deficiencies of zinc, copper and selenium. In all of the patient groups, significantly decreased concentrations of selenium in leucocytes and plasma were observed, indicating a lowered selenium status in these subjects. In addition, patients with alcoholic liver disease demonstrated significantly reduced levels of leucocyte zinc. The importance of these findings is discussed.

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ACKNOWLEDGEMENTS

I am extremely grateful to Professor Barbara Clayton for her guidance, support and encouragement throughout this study. My thanks also go in particular to Dr H. Trevor Delves for his advice relating to the development of the atomic absorption spectrophotometric methods; and Mr Ronald S. Lloyd contributed helpful discussions regarding the development of the leucocyte separation procedure. Professor M. Gardiner and Mr D. Machin provided similarly helpful advice concerning the statistical analysis.

A considerable proportion of the work presented in this thesis concerns investigations of a clinical nature, and I would like to express my gratitude to the fourth-year medical students, Manfred Colmsee, Karen Inwards and Susan Young, who played a part in the execution of these studies.I would also like to thank Dr Valda Bunker and Mrs Barbara Lloyd who contributed to the studies reported in Chapters 6 and 7, by performing specific analyses. During the course of this study, financial assistance was received from the Peel Medical Research Trust, to whom I am grateful.

My penultimate thanks go to Iain (Jessop), for his steadfast support during the preparation of this thesis, and for his practical contribution towards the graphic work. Finally, I am grateful to Mrs Jennifer Murphy who maintained cheerfulness and good humour whilst carrying out the onerous task of processing this thesis.

STATEMENT

I declare that during the course of this study, with the exceptions listed below, I carried out all of the practical work myself.

For some of the investigations, collection of blood samples and subsequent separation of leucocytes were carried out by fourth-year medical students who were working under my close supervision i.e. by Manfred Colmsee for some of the healthy human subjects under the age of 65 years; by Karen Inwards for many of the patients with Crohn's disease and treated coeliac disease; by Susan Young for many of the patients with eczema and psoriasis. Collection of blood samples only, from some of the healthy human subjects, and all of the alcoholic subjects, was performed in conjunction with Mrs Barbara Lloyd and Ivan Bantock (a fourth-year medical student) respectively.

Plasma and whole blood selenium measurements on the healthy human subjects were carried out by Mrs Barbara Lloyd. The balance samples for the healthy and housebound elderly subjects were collected by Miss Margaret Lawson and Mrs Maureen Stansfield respectively, and the zinc and copper estimations on these samples were performed by Dr Valda Bunker.

The differential white cell counts on the whole blood samples and corresponding leucocyte preparations were carried out by the staff of the NHS Haematology Laboratory, Southampton General Hospital. Routine liver function tests on the alcoholic subjects were performed by the staff of the NHS Chemical Pathology Laboratory, Southampton General Hospital.

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LIST OF SOME ABBREVIATIONS USED IN THIS THESIS

BMI	Body mass index
CI	Confidence interval
Cu	Copper
EDTA	Ethylenediamine tetra-acetic acid
LEM	Leucocyte endogenous mediator
PCV	Packed cell volume
Se	Selenium
SD	Standard deviation
TPN	Total parenteral nutrition
Zn	Zinc

LIST OF E.C. NUMBERS FOR ENZYMES STATED

Alcohol dehydrogenase	E.C. 1.1.1.1
Alkaline phosphatase	E.C. 3.1.3.1
Aspartate aminotransferase	E.C. 2.6.1.1
Cytochrome C oxidase	E.C. 1.9.3.1
Ferroxidase	E.C. 1.12.3.1
Gamma-glutamyltranspeptidase	E.C. 2.3.2.2
Glutathione peroxidase	E.C. 1.11.1.9
Lactate dehydrogenase	E.C. 1.1.1.27
Ribonuclease	E.C. 3.1.27.5
Superoxide dismutase	E.C. 1.15.1.1

INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION AND THE OBJECTIVES OF THE PROJECT

1.1 GENERAL INTRODUCTION

In the past few decades considerable progress has been made in the field of biological trace element research. Within this time span there has been an increasing realisation of the role of trace elements in intermediary metabolism: as constituents of metalloenzymes, or as co-factors for enzymic reactions. When Vallee (1959) wrote his review of the biochemistry, physiology and pathology of zinc in 1959, only seven zinc metalloenzymes were known. At the present time, over 200 zinc-dependent enzymes are recognised (Vallee and Falchuk, 1983).

An element is considered essential when deficient intake consistently results in impairment of function from optimal to sub optimal, and when supplementation of physiological levels of this element, but not of others, prevents or cures this impairment (Mertz, 1972, 1981). Although the definition of a trace amount is arbitrary, trace elements are commonly regarded as elements found in the human body in amounts that constitute less than 0.01% of total-body weight (Solomons, 1986). For a 70kg man, this represents 7 g or less. Zinc, copper and selenium are essential trace elements and many physiological functions of zinc and copper have been described in humans. It is only in recent years that the nutritional importance of selenium in man has been recognised.

Absorption of trace elements appears to take place principally in the small intestine, and depends upon their release from dietary complexes and subsequent presentation to the intestinal mucosa in a form suitable for uptake. Many dietary factors can affect the bioavailability of trace elements, and include the intake level of the element, the chemical form, promoters or inhibitors and mineral-mineral interactions. Bioavailability refers to the proportion of the nutrient in food which is absorbed and utilised.

Recognition of trace element deficiency states in man, both nutritional and accompanying some inborn errors of metabolism, has resulted in extensive knowledge of the clinical and biochemical manifestations that occur. Disorders of trace element metabolism may be present in a broad spectrum of clinical situations. Deficiency of zinc, for example, may be a complication of many gastrointestinal malabsorptive states (McClain, 1985).

A brief account of some of the important aspects of the physiology of zinc, copper and selenium is presented in the following chapter. The aetiology and manifestations of zinc, copper and selenium deficiency are also discussed.

Adequate instrumentation is currently available for analysis of most trace elements. At present however, the state of diagnosis for trace element deficiency is unsatisfactory, and no single test can be considered as ideal. The advantages and limitations of the laboratory investigations most commonly used to determine zinc, copper and selenium status are discussed in Chapter 3. Particular reference is made to the use of leucocyte zinc as an index of zinc status.

A high standard of analytical performance is essential for the reliable analysis of trace elements in body fluids and tissues. Mertz (1985) proposed that specificity, accuracy and precision of the methods used formed the foundation for trace element status assessment. Errors may be introduced during the preparation of the sample prior to analysis. An example of this is the technical difficulties associated with the separation of leucocytes from whole blood, which have not been totally resolved. The present study has attempted to solve these problems. Whatever technique is employed in the assessment of trace element status, a limiting factor is the numerous sources of contamination which can lead to erroneously high values. Contamination can occur during sample collection, processing and analysis. Stringent precautions must therefore be taken to ensure that there is no contamination of the appropriate trace element present at any of these stages.

In summary, the reliability of trace element results is dependent on analytical expertise in sample collection, preparation for analysis, control of contamination, and on the use of modern instruments.

1.2 THE OBJECTIVES OF THE PROJECT

The overall aim of the project described in this thesis was to investigate the zinc, copper and selenium status in patients with disorders in which deficiencies of these trace elements might be a complication, by the determination of zinc, copper and selenium in leucocytes, erythrocytes and plasma.

To achieve the above objective it was necessary to develop and evaluate a number of new analytical procedures. These were: a method for the isolation of leucocytes from whole blood that was practicable and gave efficient recovery of the leucocytes without differential white cell loss, and that was free from contamination from red cells, platelets and plasma; and atomic absorption spectrophotometric methods which made available accurate analyses for zinc, copper and selenium in the isolated leucocytes, and copper in erythrocytes and whole blood.

It was then intended to use these analytical methods, and also established methodology, to determine concentrations of zinc, copper and selenium in leucocytes, erythrocytes, plasma and whole blood in healthy human subjects in order to provide control data for use in subsequent clinical investigations.

The development of these analytical procedures and the clinical studies undertaken are described in separate chapters of this thesis.

CHAPTER 2

THE PHYSIOLOGY AND PATHOLOGY OF ZINC, COPPER AND SELENIUM

2.1 ABSORPTION, DISTRIBUTION AND EXCRETION OF ZINC, COPPER AND SELENIUM

2.1.1 ZINC

Absorption of zinc

Conflicting data from animal studies indicate that the main site of absorption of zinc in the intestine is either the duodenum (Van Campen and Mitchell, 1965) or the more distal parts of the small intestine (Sahagian et al, 1966; Kowarski et al, 1974). A study on human subjects with and without jejuno-ileostomy suggests the jejunum as the major site of intestinal zinc absorption (Andersson et al, 1976).

Apparent increases in zinc absorption have been observed during fasting (Quarterman and Morrison, 1981) and pregnancy and lactation (Davies and Williams, 1977) in rats, and in men fed low zinc intakes (Wada et al, 1985), indicating that the absorption of zinc may be homeostatically regulated. Although the actual mechanisms of zinc uptake by the enterocyte are poorly understood, experiments using metabolic inhibitors indicate that an active transport system is involved in the absorption of zinc (Kowarski et al, 1974). Some zinc-amino acid ligand mixtures have been shown to stimulate zinc absorption in animals (Giroux and Prakash, 1977, Wapnir and Stiel, 1986). Much interest has focussed on the ligands present in human milk as the bioavailability of zinc is greater from human milk than cow's milk (Johnson and Evans, 1978). A low molecular weight zinc-binding ligand from human milk has been isolated and identified as citrate by Lonnerdal et al (1980) and picolinic acid by Evans and Johnson (1980). However, Rebello et al (1982) detected picolinic acid in extremely low concentrations in human milk, insufficient to bind a significant proportion of zinc. Alternatively, it has been proposed that the bioavailability of zinc in milk may be related to the binding affinity of the constituent proteins which differ in human and cow's milk (Cousins and Smith, 1980). Blakeborough et al (1983) reported that most of the zinc in these two milks was associated with protein

complexes of high molecular weight, although the individual proteins making up the complexes differed, and suggested that in human milk, lactoferrin may be involved in the uptake of zinc.

Various other dietary constituents may either enhance or impair zinc absorption. Phytate and indigestible fibre which are present in plant foods form insoluble complexes with zinc in the intestinal lumen, and are generally considered to be the primary factors responsible for the poorer absorbability of zinc from plant proteins and cereal products than from foods of animal origin (Reinhold et al, 1976; Ismail-Beigi et al, 1977; Kies et al, 1979; Solomons, 1982; Navert et al, 1985). Animal experiments have shown that copper and cadmium may reduce the uptake of ^{65}Zn (Evans et al, 1974), and zinc absorption may also be impaired by tin (Johnson et al, 1981a) and calcium (Forbes, 1960). The inhibitory effect of phosphorus on apparent zinc absorption in men was reduced when the protein content of the diet was increased (Greger and Snedeker, 1980), and the addition of calcium and phosphorus supplement to a mixed diet did not significantly affect the excretion or apparent retention of zinc in male subjects (Snedeker et al, 1982). Administration of ferrous and zinc sulphates to fasting human subjects demonstrated an inhibitory effect of iron on zinc absorption as the iron:zinc ratio was increased (Solomons and Jacob, 1981; Sandstrom et al, 1985), although when this supplement was given with a test meal (Sandstrom et al, 1985) or when haem chloride was used as the iron source and Atlantic oysters as the zinc source (Solomons and Jacob, 1981), no such inhibitory effect was observed. Some congeners in wine may enhance the absorption of zinc (McDonald and Margen, 1980).

A response of intestinal metallothionien to dietary zinc intake in rats has been observed (Richards and Cousins, 1976; Menard et al, 1981), and a regulatory role of the small intracellular protein in zinc absorption has been proposed (Cousins, 1979; Menard et al, 1981). Zinc and copper have been reported to compete for binding sites on metallothionien in the intestinal mucosal cells (Hall et al, 1979). Although zinc induces the synthesis of metallothionien, copper binds more avidly to the protein and it has been suggested that once copper has displaced zinc from metallothionien it is unavailable for utilisation (Ogiso et al, 1979).

Distribution of zinc

Animal studies have shown that albumin is the protein which is involved in the removal of zinc from intestinal mucosal cells and in the subsequent transport of the element in the portal blood to the liver (Smith et al, 1978). Approximately 15% of zinc in the plasma is bound to α_2 macroglobulin and approximately 85% to albumin, and this latter pool almost entirely accounts for the variations in total zinc concentrations (Foote and Delves, 1984). Only a small percentage of the total plasma zinc pool is bound to low molecular weight ligands (Prasad and Oberleas, 1970).

A 70kg man contains 20-35 mmol (1.4-2.3g) of zinc (Widdowson et al, 1951). Of the total body zinc pool the highest concentrations are found in the retina and prostate gland (Halsted et al, 1974).

Excretion of zinc

Zinc is excreted almost entirely in the faeces (Spencer et al, 1965). Perfusion studies suggest that biliary and pancreatic secretions contribute significantly to the zinc found in human faeces (Matseshe et al, 1980). Daily losses of zinc by urinary excretion account for usually less than 9.2 µmol (600µg) (Roman, 1969) and losses of zinc through the body surface are approximately 7.7 µmol (500µg) per day (Jacob et al, 1979).

2.1.2 COPPER

Absorption of copper

A portion of the dietary copper supply may be absorbed from the stomach as the acidic environment may promote copper solubility and enhance its transport across the gastric mucosa (Marceau et al, 1970). Animal experiments have demonstrated that the major site for absorption of copper in the small intestine is the duodenum (Van Campen and Mitchell, 1965; Marceau et al, 1970).

Many dietary factors influence the uptake of copper by the gastrointestinal tract. Decreased copper retention has resulted from high levels of dietary fibre in fruit and vegetables (Kelsay et al, 1979) and dietary phytate (Davies and Nightingale, 1975). Animal studies have demonstrated that intestinal absorption of copper is depressed by high levels of ascorbic acid (Van Campen and Gross, 1968) and cadmium (Davies and Campbell, 1977). High intakes of dietary

protein have been reported to increase the retention of copper (Greger and Snedeker, 1980).

Endogenous secretions also appear to influence copper absorption. Hepatic and gallbladder bile both inhibited copper absorption in rats (Gollan and Deller, 1973; Gollan, 1975), and a similar effect in rats has been noted with pancreatic protein (Jamison et al, 1981). It has been proposed that endogenous binding components from these secretions may act as regulators of absorption by influencing the amount of copper available for uptake (Gollan, 1975; Jamison et al, 1981).

Distribution of copper

Hepatic uptake studies in rats have indicated that albumin is the major binding ligand that transports copper from the intestine to the liver (Weiner and Cousins, 1980). It has been shown that hepatic uptake of intravenously injected copper is rapid and efficient with an intial liver uptake of approximately 80% in humans (Osborn et al, 1963). At least 90% of the total plasma copper is tightly bound to caeruloplasmin, the remainder being mainly loosely bound to albumin, and a small amount to amino acids (Epstein, 1983; Laurie, 1983). Approximately 1.3-2.4 mmol (80-150mg) of copper is contained in the body of a normal adult with the highest concentration present in the liver (Solomons, 1985).

Excretion of copper

The major excretory route of copper in man is via the bile, and this amounts to about 32µmol (2mg) of copper per day (van Berge Henegouwen et al, 1977). In human bile, the major low molecular weight copper-binding ligands have been identified as conjugated bilirubin, peptides and amino acids, and it has been proposed that the peptide fraction may play a role in elimination of copper from the body (Martin et al, 1986). The urinary loss of copper is approximately 0.9µmol (60µg) per day (Klevay et al, 1980) and up to 5.4µmol (0.34mg) may be lost through the body surface (Jacob et al, 1979).

2.1.3 SELENIUM

Absorption of selenium

Studies in animals with ^{75}Se indicate that selenium is absorbed throughout the small intestine and that there appears to be no absorp-

tion from the stomach (Wright and Bell, 1966). Selenomethionine appears to share a common absorptive mechanism with methionine in the small intestine (McConnell and Cho, 1967; Ochoa-Solano and Gitler, 1968). It has been proposed that absorption of amino acid-bound selenium is accelerated by specific amino acid active transport mechanisms in the gut mucosa, whereas sodium selenite is absorbed by simple diffusion through the intestinal mucosa (Reasbeck et al, 1985). After oral administration of ^{75}Se to humans in the form of selenomethionine or selenite, the plasma ^{75}Se concentration reached a maximum at 3-4 hours (Griffiths et al, 1976) and 7-12 hours (Thomson and Stewart, 1974) respectively.

Little information is available on dietary factors affecting the bioavailability of selenium. High dietary copper intake markedly inhibits the physiological utilisation of absorbed selenium in rats (Rahim et al, 1986). In the same study, no antagonistic effect of calcium, iron, molybdenum or manganese on selenium utilisation was observed. An investigation into the availability of selenium in some human foods demonstrated that in rats with an initially low selenium status, selenium in cooked beef kidney, boiled wheat and canned tuna was 97%, 83% and 57% available respectively when compared to selenite which was regarded as being 100% available (Douglass et al, 1981). The reason for the low bioavailability of selenium from tuna is not clear. In a study of Finnish men with a low selenium status, the bioavailability of selenium in the form of Se-rich wheat or Se-rich yeast compared well with that of sodium selenate (Levander et al, 1983). Selenomethionine has been identified as the major selenium - containing compound in both wheat (Olson et al, 1970) and selenium - yeast (Korhola et al, 1986). In animal tissues, selenium is present as selenocysteine, selenomethionine and selenopersulphides (Burk, 1976).

Distribution of selenium

Selenium in plasma is mainly protein-bound and is probably associated with α_2 and β - globulins (Dickson and Tomlinson, 1967) and also with lipoproteins (Burk, 1974). New Zealand subjects were estimated to contain about 76 µmol (6mg) of selenium (Stewart et al, 1978), less than half of another estimate of the whole body content of selenium of 165-253 µmol (13-20mg) of American subjects (Schroeder et al, 1970).

Excretion of selenium

Urine is the principal route of excretion of selenium, followed by faeces, with trivial respiratory and dermal losses (Thomson and Stewart, 1974). In humans, approximately 55% to 67% of the total excreted selenium is in the urine (Stewart et al, 1978; Levander et al, 1981; Levander and Morris, 1984). Experiments in rats have indicated that selenium homeostasis is achieved by the regulation of urinary selenium excretion (Burk et al, 1972).

2.2 HUMAN REQUIREMENTS OF ZINC, COPPER AND SELENIUM

Human requirements of zinc

Estimates of zinc requirements using the factors of zinc retention, losses and availability have suggested that if the efficiency of zinc absorption was 100%, an adult would only require 34 µmol (2.2mg) of zinc per day, whereas if the zinc content of available zinc was only 10% or 40%, this requirement would be increased to 337 µmol (22mg) or 84 µmol (5.5mg) per day respectively (World Health Organisation, 1973). Clearly, food selection is of extreme importance in supplying the daily requirement for zinc. The currently recommended dietary allowance for zinc in humans 11 years old and over is 230 µmol (15mg) (National Research Council, 1980).

Human requirements of copper

The estimated safe and adequate dietary daily intake of copper in humans of 11 years old and over is 31-47 µmol (2.0-3.0mg) (National Research Council, 1980), although this may well be in excess of requirements. Surveys of a variety of diets in the United States have indicated that intakes substantially below 16 µmol (1mg) of copper per day are not unusual (Klevay, 1975). In addition to this, a New Zealand study of self-selected diets showed that half of them provided less than 31 µmol (2.0mg) of copper and 5% provided less than 16 µmol (1.0mg) of copper per day (Guthrie, 1973). Sandstead (1982) found the requirement for copper to be highest when dietary zinc was high and protein was low.

Human requirements of selenium

Published estimates of human daily intakes of selenium vary widely among different countries. Daily selenium intakes have been reported to be 0.8-1.9 µmol (60-150 µg) in the United States

(Schroeder et al, 1970), 1.4-2.8 µmol (110-220µg) in Canada (Thompson et al, 1975), 0.08-0.9 µmol (6-70µg) in New Zealand (Robinson, 1976) and 0.03-0.45 µmol (2.3-35.5µg) in China (Luo et al, 1985). The average daily selenium intake in Britain was estimated to be 0.8 µmol (60µg) (Thorn et al, 1978b). As chronic selenium toxicity has been demonstrated in animals at relatively low dietary intakes of selenium (World Health Organisation, 1973), a maximal daily intake of 2.5 µmol (200µg) of selenium has been suggested in humans of 11 years old and over (National Research Council, 1980).

Selenium balance studies have indicated that a daily selenium intake of about 0.9 µmol (70µg) and 0.3 µmol (20µg) was needed to maintain balance in North American males (Levander et al, 1981) and New Zealand women (Stewart et al, 1978) respectively. New Zealand subjects appear to excrete selenium more sparingly than North American subjects, and it has been suggested that this could be due to an adaptation to the low intakes of selenium (Robinson et al, 1985). Although the substantially smaller selenium intake of the New Zealand residents is associated with low selenium concentrations in blood, no detrimental effects to health have been reported (Griffiths and Thomson 1974; Thomson and Robinson, 1980). Low selenium intakes of healthy men from a low selenium area in China were sufficient to maintain a positive selenium balance (Luo et al, 1985).

2.3 FUNCTIONS OF ZINC, COPPER AND SELENIUM

Functions of zinc

Over 200 zinc-dependent enzymes have been identified and they are involved in a wide variety of metabolic processes including nucleic acid, protein, carbohydrate and lipid metabolism (Prasad, 1985). Zinc is indispensable to the enzymes which are critical to deoxyribonucleic acid, ribonucleic acid and protein synthesis and their degradation (Vallee and Falchuk, 1983). This element is therefore related to functions such as growth and development, tissue repair after injury and regeneration of rapidly proliferating tissue such as skin and hair (Arlette, 1983). Despite extensive studies there are still no clear links between many of the manifestations of zinc deficiency and the involvement of the element in enzyme systems. Studies using the organism Euglena gracalis have indicated a role for zinc in the

regulation of gene expression (Vallee and Falchuk, 1983) although these findings have yet to be confirmed in higher species.

It has been proposed that zinc plays a critical role in the structure and function of biomembranes (Bettger and O'Dell, 1981). Zinc has been shown to stabilise rat liver lysosomes (Ludwig and Chvapil, 1980). The extracellular concentration of zinc appears to influence both the function of the circulating erythrocytes (Bettger et al, 1980) and the chemotaxic response of leucocytes (Weston et al, 1977). Zinc has also been demonstrated to facilitate binding of insulin to cell membrane receptors (Arquilla et al, 1978).

The importance of zinc for the maintenance of vision, taste and smell has been reviewed (Russell et al, 1983). At present the bases of these functions are not fully understood.

Functions of copper

Copper exerts its biochemical role largely as a component of metalloenzymes (Solomons, 1985). Some of the possible relationships between cuproenzymes and specific pathological signs have been proposed. Development and maintenance of cardiovascular and skeletal integrity is dependent on the biosynthesis of the crosslinks in both collagen and elastin which are derived from lysine, and lysyl oxidase is required to catalyse the oxidation of specific lysyl and hydroxylysyl residues (O'Dell, 1976). The oxidase activity of caeruloplasmin has been implicated in the normal oxidation of ferrous iron to ferric iron (Osaki et al, 1966) and it has been postulated that a defect in this activity may result in an inability to mobilise and transport storage iron, thus producing the anaemia of copper deficiency (Solomons, 1985). Most of the recognised cuproenzymes catalyse reactions of physiological significance. Cytochrome C oxidase is of key importance in mitochondrial energy metabolism (O'Dell, 1976) and superoxide dismutase provides intracellular protection against oxidative damage from free radicals (McCord and Fridovich, 1969). No specific pathology has been attributed to these enzymes. Copper also appears to play a role in central nervous system structure and function in animals (Everson et al, 1968; Dipaolo et al, 1974) although the biochemical basis of this function is not clear.

Functions of selenium

The biochemical function of selenium as an essential component of glutathione peroxidase was not discovered until 1973 (Rotruck et al, 1973). No other specific biochemical functions of selenium have been reported since this time. The form of selenium in the active site of glutathione peroxidase appears to be selenocysteine (Forstrom et al, 1978) and the enzyme has a wide distribution in animal cells and is present in mitochondria and in the cytosol (Burk, 1983).

Glutathione peroxidase, along with other cellular antioxidant defence mechanisms, helps protect the cell against damage that may be caused by free radicals and hydroperoxides (Hoekstra, 1975). A free radical is any species containing one or more unpaired electrons (Dormandy, 1980). Exposure of cell membranes to oxygen free radicals stimulates the process of peroxidation of polyunsaturated fatty acids (Tappel, 1973). Lipid peroxidation diminishes membrane fluidity, increases non-specific permeability to ions and may inactivate membrane-bound enzymes (Halliwell, 1987). It is now widely accepted that vitamin E, an important lipid-soluble antioxidant, reacts rapidly with peroxy radicals formed during lipid peroxidation and as such can be classified as a chain-breaking antioxidant (Willson, 1987). Selenium and vitamin E function together in a complex system that protects cells from the detrimental effects of oxygen free radicals (Gross, 1979; Diplock, 1984).

It is of interest that selenium has also received much recent attention as an anticarcinogenic agent (1p, 1986). Animal studies have shown that supplementation of selenium above dietary requirement inhibits tumour development induced by a wide spectrum of carcinogens (1p, 1981; Jacobs, 1983; El-Bayoumy, 1985; Birt et al, 1986). The mechanism by which the element functions as an anticarcinogen is not clear.

2.4 AETIOLOGY OF ZINC, COPPER AND SELENIUM DEFICIENCY

Deficiencies of trace elements in humans are the result of reduced intake, decreased absorption, increased excretion or increased requirements.

Reduced intake

Dietary inadequacy related specifically to geographical location has been reported and, in humans, a resulting zinc deficiency has been observed in Iran (Eminians et al, 1967) and selenium deficiency in the Chinese province of Keshan (Keshan Disease Research Group, 1979b). A low nutritional status for selenium has also been reported in residents of New Zealand (Thomson et al, 1977; Rea et al, 1979) and Finland (Westermarck et al, 1976).

Long-term total parenteral nutrition with infusion fluids that have not been supplemented with trace elements have been associated with deficiency states of zinc (Principi et al, 1979), copper (Fleming et al, 1976) and selenium (Kien and Ganther, 1983; Brown et al, 1986). Intravenous nutritional solutions are reported to contain inadequate amounts of selenium in particular (van Rij et al, 1979). The trace element content of synthetic diets used to treat children with inborn errors of metabolism may also be inadequate (Alexander et al, 1974; Thorn et al, 1978a). Pre-term infants fed with unsupplemented human breast milk have been reported to be at risk of zinc and copper deficiency (Dauncey et al, 1977). Vegetarians may also be at risk of a low dietary intake of zinc (Bodzy et al, 1977; Freeland-Graves et al, 1980).

Malabsorption

Metabolic disorders affecting intestinal absorption can result in specific trace element deficiencies. Menkes' syndrome was first described in 1962 (Menkes et al, 1962) although the relationship to copper deficiency was not identified until a decade later (Danks et al, 1972). Parenteral copper therapy fails to bring about a clinical improvement (Garnica et al, 1974) and a basic defect in some component of the intracellular copper transport system has been proposed (Danks, 1985). Acrodermatitis enteropathica is regarded as a specific inborn error of zinc absorption and impaired intestinal absorption of ^{65}Zn in these patients has been demonstrated (Lombeck et al, 1975). Treatment with large doses of oral zinc has proved successful (Moynahan, 1974) and diodoquin appears to improve zinc absorption in these patients (Delves et al, 1975).

Children with various malabsorptive states have been shown to be at risk of deficiency of copper and zinc (Clayton, 1980), and severe copper deficiency has been reported in infants with chronic or

recurrent diarrhoea (Graham and Cordano, 1976). Malabsorption of zinc has been demonstrated in patients with exocrine pancreatic insufficiency (Aggett et al, 1979) and a lowered selenium status has been demonstrated in children and young adults with cystic fibrosis (Neve et al, 1983; Stead et al, 1985; Dworkin et al, 1987).

Increased body losses

Excessive losses of zinc and copper from the gastrointestinal tract may occur in fluids lost through fistulous discharge (Wolman et al, 1979; Shike et al, 1981). Increased losses of zinc in urine have been reported in cases of alcoholic cirrhosis (Allan et al, 1975) and in hypercatabolic states (Fell et al, 1973), and high urinary outputs of zinc and copper have been demonstrated in children following thermal injury (Carr and Wilkinson, 1975). Significantly increased losses of zinc and copper via exfoliation in patients with psoriasis may occur (Molin and Wester, 1973). Depletion of copper and zinc has been reported in patients receiving penicillamine therapy (Klingberg et al, 1976; Cutolo et al, 1982) and urinary zinc losses of clinical significance may encountered in patients on diuretics (Wester, 1980).

Increased requirements

Increased requirements for zinc are observed during growth, pregnancy and lactation (National Research Council, 1980). It is noteworthy however that a recent study of normal healthy primigravidae, who subsequently delivered normal babies, had a mean daily dietary zinc intake of only 139 µmol (9.1mg) at 30 weeks gestation (Tuttle et al, 1985) Increased utilisation of selenium in pregnancy has been reported (Swanson et al, 1983).

2.5 MANIFESTATION OF ZINC, COPPER AND SELENIUM DEFICIENCY

Manifestations of zinc deficiency

The clinical manifestations exhibited in zinc deficiency depend upon the severity and length of the imposed deficiency and are shown in Table 2.1. In addition, a high incidence of congenital abnormalities in the pregnancies of women with acrodermatitis enteropathica has been reported (Hambidge et al, 1975) and some studies have associated foetal growth retardation with low maternal plasma or serum zinc concentrations (Jameson, 1976; Crosby et al, 1977). Impaired

TABLE 2.I

CLINICAL EFFECTS OF ZINC DEFICIENCY*

Severe/Acute

- Cessation of growth
- Anorexia
- Characteristic skin lesion
- Hair loss - alopecia
- Diarrhoea - abdominal pain
- Behavioural changes - depression
- Immune suppression - infections
- Hypoproteinaemia

Marginal/Chronic

- Appetite loss
- Hypogonadism - dwarfism
- Testicular function - sexual maturation
  sperm production - fertility
  loss of sexual libido
- Skin changes - dry scaly appearance
- Hair growth reduced, body hair pattern abnormal
- Wound healing
- Phagocytic function - neutrophil chemotaxis
- Platelet aggregation
- Dark adaptation
- Sense of taste and smell impaired

* From: Fell, 1984.

immunocompetence associated with severe zinc deficiency has been demonstrated by delayed cutaneous hypersensitivty (Golden et al, 1978; Pekarek et al, 1979), and impairment of leucocyte chemotaxis (Weston et al, 1977).

The biochemical manifestations observed during experimental zinc deficiency in healthy human subjects (Prasad et al, 1978) are listed in Table 2.II. All changes were reversed when the zinc status of the subjects was returned to normal. Other zinc depletion studies have reported reduced serum concentrations of testosterone (Abbasi et al, 1980) and decreased activity of serum and leucocyte alkaline phosphatase (Baer and King, 1984).

Manifestations of copper deficiency

In most clinical situations, deficiency of copper is probably also associated with deficiency of other nutrients and information regarding the manifestations that occur as a result of copper deficiency alone is limited. Much of this information has been obtained from a series of case reports illustrating a dietary copper deficiency in malnourished infants during rehabilitation on milk-based, low copper diets (Cordano et al, 1964, 1966; Holtzman et al, 1970). The clinical effects of copper deficiency are listed in Table 2.III. Although the manifestations of anaemia and neutropenia are not specific for copper deficiency, they are most commonly associated with deficiency of this trace element in patients receiving total parenteral nutrition (Dunlap et al, 1974; Zidar et al, 1977). Anaemia has not been reported in Menkes' disease (Danks, 1985). Skeletal demineralisation occurs only in growing bone and is restricted to juvenile copper deficiency (Bennani-Smires et al, 1980). The earliest detectable biochemical manifestations of copper deficiency are a fall in the concentrations of serum copper and caeruloplasmin (Holtzman et al, 1970).

Manifestations of selenium deficiency

Very few clinical abnormalities have been reported in conjunction with biochemical signs of selenium deficiency. A low selenium status has been linked to Keshan disease, which is a cardiomyopathy that affects mainly young children in a district in China (Keshan Disease Research Group, 1979b). This disease is characterised by low blood and hair selenium levels, decreased activity of blood glutathione

TABLE 2.II

BIOCHEMICAL MANIFESTATIONS OF ZINC DEFICIENCY DURING EXPERIMENTAL ZINC DEPLETION IN HUMAN SUBJECTS*

Decreased

- Plasma zinc concentration
- Erythrocyte zinc concentration
- Leucocyte zinc concentration
- Urinary zinc concentration
- Plasma alkaline phosphatase activity
- Plasma lactate dehydrogenase activity

Increased

- Plasma ribonuclease activity
- Plasma ammonia concentration

* Adapted from: Prasad et al, 1978.

TABLE 2.III

CLINICAL MANIFESTATIONS OF COPPER DEFICIENCY IN HUMANS*

Microcytic, hypochromic anaemia
Neutropenia
Skeletal demineralisation
Arterial aneurisms
Hypotonia
Hypothermia

Pili Torti (kinky hair), and cerebral and cerebellar degeneration seen exclusively in the inborn error of copper metabolism (Menkes' syndrome) and not in acquired copper deficiency to date.

* From: Solomons, 1985.

peroxidase and decreased urinary excretion of selenium after a loading dose (Keshan Disease Research Group, 1979b). This fatal cardiomyopathy can be prevented by selenite supplementation (Keshan Disease Research Group, 1979a). Although selenium deficiency is a contributing factor to Keshan disease, a multifactorial aetiology is likely (Levander, 1982).

Two patients on total parenteral nutrition who demonstrated very low levels of plasma selenium (van Rij et al, 1979) and very low levels of plasma and red cell selenium as well as extremely low activities of glutathione peroxidase in plasma, red cells, white cells and platelets (Brown et al, 1986), also complained of muscular discomfort. In both cases the biochemical and clinical symptoms disappeared after intravenous supplementation with selenium.

Comment:

Deficiency states of zinc, copper and selenium which occur in many different clinical situations have been increasingly recognised. The clinical manifestations of deficiency states of copper and zinc are well documented although in many instances they are non-specific. Very few clinical abnormalities have been reported in association with biochemical signs of selenium deficiency, and the possible long-term clinical consequences of a reduced selenium status are at present not understood.

CHAPTER 3

ASSESSMENT OF BODY STATUS OF ZINC, COPPER AND SELENIUM

The most satisfactory procedures for assessing trace element status remain controversial. Much interest has centred on the investigation of marginal deficiency states of zinc, copper and selenium.

3.1 LABORATORY INVESTIGATION OF DEFICIENCIES OF ZINC, COPPER AND SELENIUM

Many biochemical and functional procedures have been used either clinically or experimentally in an attempt to diagnose deficiencies of zinc, copper and selenium. Although isotopic labelling techniques and balance studies have provided valuable data, these procedures are confined to special research units. At present, no single test may be considered as ideal for any element.

3.1.1 LABORATORY INVESTIGATION OF ZINC DEFICIENCY

The use and limitations of the concentration of zinc in skin and fingernail, and taste acuity, as indices of zinc status have been reviewed by Solomons (1979). None of these procedures has gained widespread acceptance. The use of leucocyte zinc in the assessment of zinc status is discussed in the following section of this chapter.

Plasma and serum zinc

There is no significant difference between values for plasma and serum zinc (Wei-jie et al, 1986). Studies involving experimental zinc deficiency in healthy subjects have shown a decrease in plasma zinc values within 4-6 weeks, and these correlated well with the severity of the dietary restriction (Prasad et al, 1978; Baer and King, 1984). A rapid fall in plasma zinc levels has been demonstrated in a group of 6 patients with acrodermatitis enteropathica whose zinc supplementation was temporarily stopped (Anttila et al, 1984). However, in the context of disease or even altered normal physiology as in pregnancy, problems in interpretation of marginally lowered levels of plasma zinc

arise. Animal studies have demonstrated that a low molecular weight polypeptide hormone, 'leucocyte endogenous mediator' (LEM), released from the granulocytes, mediates the redistribution of zinc by means of sequestration by the liver (Pekarek et al, 1972; Kampschmidt et al, 1973). In man LEM has been associated with a fall in plasma zinc in both infectious (Wannemacher et al, 1972, 1975) and inflammatory conditions (Solomons et al, 1978). Adminstration of corticosteroids causes a decrease in plasma zinc concentrations (Yunice et al, 1981). Some studies have shown a decrease in plasma zinc levels in women taking oral contraceptives (Halsted et al, 1968; Prasad et al, 1975) whilst others have reported no change (Crews et al, 1980; Vir and Love, 1981). It is possible that the decrease in oestrogen content of the newer contraceptive pills may be partly responsible for these conflicting observations. Endogenous gonadal hormones during pregnancy have been reported to depress circulating concentrations of zinc (Halsted et al, 1968). Plasma zinc concentrations are also affected by changes in albumin concentrations (Kahn et al, 1965; Walker et al, 1973), and it has been suggested that the affinity of albumin for zinc is decreased in pregnancy and hepatic cirrhosis (Giroux et al, 1976, 1977).

Erythrocyte zinc

Several studies have reported no change in erythrocyte zinc concentrations in patients with obvious zinc deficiency. Klingberg et al (1976) did not find reduced erythrocyte zinc levels in a patient with Wilson's desease receiving penicillamine therapy and showing classical signs of zinc deficiency which responded to zinc supplements. No significant change was observed in erythrocyte zinc concentrations in subjects who became zinc depleted after 4-6 weeks on an experimental diet (Baer and King, 1984) or in patients who developed zinc deficiency within 4 weeks of receiving total parenteral nutrition solutions not containing zinc (Takagi et al, 1986). In contrast to these reports however, an experimental zinc depletion study demonstrated a decrease in erythrocyte zinc levels, although only in 2 of the 4 subjects who were on the more severe zinc restricted diet, and only after 12 weeks (Prasad et al, 1978).

Urinary zinc

A reduced urinary zinc concentration has been proposed as an indication of zinc deficiency and experimental zinc-depletion studies have demonstrated decreases of about 50% in urinary zinc levels (Prasad et al, 1978; Baer and King, 1984). Excessive urinary losses of zinc have been reported to occur in starvation (Elia et al, 1984) or trauma (Fell et al, 1973), and these observations limit the usefulness of this measurement.

Salivary zinc

Whilst the zinc content of mixed saliva is not a satisfactory index of zinc status (Greger and Sickles, 1979; Freeland-Graves et al, 1981), a decreased concentration of zinc was noted in salivary sediment in subjects who had been fed a low zinc diet for 22 days, although daily fluctuations were not determined (Freeland-Graves et al, 1981). Collection of salivary samples however is subject to contamination with other oral sources of zinc (Mathur et al, 1977) and the usefulness of this measurement is doubtful.

Hair zinc

Low hair zinc concentrations have been reported in untreated patients with acrodermatitis enteropathica (Amador et al, 1975). However, problems with hair zinc as an indicator of zinc status exist (Bradfield and Hambidge, 1980) and it has been demonstrated that hair zinc concentrations were related to age and body size in children with acrodermatitis enteropathica (Anttila et al, 1984). A major problem with hair analysis is the many sources of contamination with exogenous zinc (Hambidge, 1982). Hair zinc determinations appear to be of no value in the diagnosis of severe zinc deficiency and do not have an established role in the detection of marginal zinc deficiency (Taylor, 1986).

Zinc metalloenzymes

Low plasma alkaline phosphatase activity, which was observed in a child with acrodermatitis enteropathica, returned to normal after simultaneous administration of zinc sulphate and amphotericin and was associated with clinical improvement and an increased plasma zinc concentration (Aggett et al, 1981). A decrease in serum alkaline phosphatase activity of approximately 11% was noted in subjects after

consuming a low intake of zinc for 28 days, and the activity of this enzyme increased after only 9 days following an increased intake of zinc (Herman et al, 1984). In the same study, total serum alkaline phosphatase activity was separated into two distinct isoenzyme fractions representing the intestinal and the liver isoenzymes although a portion of this latter fraction also appeared to be of bone origin. Despite the significant drop in total enzyme activity, neither isoenzyme was selectively affected and the relative percentages of the total activity remained unchanged. Notable changes in the activity of plasma ribonuclease have been observed during experimental zinc depletion (Prasad et al, 1978), and its measurement has been used as an index of zinc status in patients with chronic renal failure (Mahajan et al, 1979). The measurement of these plasma enzymes may be helpful in demonstrating uncomplicated zinc deficiency, particularly if their activity is further observed for a short period of time after zinc supplementation.

Zinc tolerance test

After ingestion of 765 µmol (50mg) of zinc as the sulphate, peak concentrations of zinc in the plasma occur at 2 hours in normal fasting subjects (Sullivan et al, 1979a). A reduction in the zinc response curve has been reported in patients with coeliac disease or dermatitis herpetiformis (Crofton et al, 1983b) and pancreatic insufficiency (Boosalis et al, 1983). The main disadvantage of this test is the nausea experience by fasting subjects during the first hour of the study, and attempts to overcome this drawback by adminstration of a light meal have failed to demonstrate a satisfactory increase in plasma zinc levels (Bunker et al, 1986). However, it has been proposed that if the test meal is standardised, the zinc tolerance test may be used as an index of zinc absorption when studying groups of subjects, although not individual patients (Valberg et al, 1985a). Fickel et al (1986) demonstrated an enhanced plasma zinc response to a zinc load in healthy human subjects after both zinc depletion and supplementation and suggested that this test was not a reliable indicator of zinc status.

3.1.2 LABORATORY INVESTIGATION OF COPPER DEFICIENCY

Deficiency of copper in human subjects has been observed much less frequently than that of zinc, and consequently laboratory investigations of copper deficiency have been less widely reported.

Plasma and serum copper

Patients receiving total parenteral nutrition without copper supplementation demonstrated a rapid decrease in serum copper concentrations (Fleming et al, 1976). Copper deficiency in Menkes' syndrome is also characterised by decreased plasma copper levels (Williams et al, 1977). Both these studies also reported decreased levels of caeruloplasmin in plasma or serum.

Changes in serum copper levels have been reported as a result of adminstration of synthetic glucocorticoids (Lifschitz and Henkin, 1971). LEM causes an increase of hepatic synthesis and release of caeruloplasmin (Pekarek et al, 1974).

Although plasma copper appears to be a sensitive index of the nutritional status of this element, its measurement may be unreliable in disease states.

Erythrocyte copper

There are few studies of erythrocyte copper in nutritional deficiency or disease states. Williams et al (1977) reported erythrocyte copper levels within the normal range in a patient with Menkes' syndrome. Reduced concentrations of erythrocyte copper were observed in female patients with rheumatoid arthritis (Scudder et al, 1976) although the clinical significance of this finding is not clear.

Hair copper

Hair analysis has proved unreliable in the assessment of copper status due to copper contamination from exposure to the environment (Hambidge, 1973). Despite careful sampling, hair copper levels in children with severe copper deficiency were no different from those of control subjects (Bradfield et al, 1980).

Urinary copper

Few studies of urinary copper in association with copper-deficient states have been reported. An infant with Menkes' syndrome

demonstrated a twenty-four hour urinary copper excretion within the normal range (Williams et al, 1977). Urinary excretion of copper was persistently elevated in some patients with major burns and scalds (Carr and Wilkinson, 1975).

Copper enzymes

A decrease in the plasma or serum ferroxidase activity (the oxidase activity of caeruloplasmin) is useful in detecting nutritional copper deficiency and responses of this metalloenzyme have been discussed in the preceding section on plasma and serum copper. Decreased erythrocyte superoxide dismutase activity has been reported in Menkes' syndrome (Williams et al, 1977) and in infants recovering from malnutrition (Uauy et al, 1985), and this enzyme has been reported to be a more sensitive index of marginal copper deficiency than serum ferroxidase activity (Fischer et al, 1984). Copper enzymes however have not been widely used in the assessment of copper status.

3.1.3 LABORATORY INVESTIGATION OF SELENIUM DEFICIENCY

Human studies involving both selenium repletion and depletion have proved extremely useful in demonstrating the sensitivity of the commonly used parameters of selenium status. Observations from these studies are therefore included in the following discussion.

Plasma and serum selenium

The concentration of selenium in plasma or serum is the most commonly used parameter of selenium status in man. A significant decrease in plasma selenium concentrations was observed in patients receiving total parenteral nutrition without selenium supplementation (van Rij et al, 1979). Conversely, supplementation of selenium deficient subjects with selenium is accompanied by a rise in plasma or serum selenium concentrations usually after several weeks (Fell et al, 1980; Kien and Ganther, 1983), although the return to normal levels is dependent upon the extent of depletion and the chemical form of the selenium compounds given (van Rij et al, 1981). These studies indicate that the measurement of plasma selenium is a reliable indicator of body selenium deficiency due to a low dietary intake of this element, and that it also reflects the changing selenium status when selenium supplementation is given.

Decreased serum or plasma selenium concentrations have been reported in pregnant subjects (Behne and Wolters, 1979; Perona et al, 1979). Alcohol consumption and cigarette smoking have also been associated with decreased plasma selenium levels in healthy male subjects, and a significant increase in plasma selenium concentration with the use of oral contraceptives has been reported (Lloyd et al, 1983). Conversely, a study of New Zealand residents found no significant differences in plasma selenium levels in association with cigarette smoking or the use of oral contraceptives (Robinson et al, 1983).

Erythrocyte selenium

Unlike plasma levels, erythrocyte selenium concentrations are not influenced by pregnancy or oral contraceptive use (Behne and Wolters, 1979; Capel et al, 1981; Lloyd et al, 1983).

Decreased concentrations of erythrocyte selenium have been observed in patients receiving long-term parenteral alimentation (Johnson et al, 1981b; Lane et al, 1981). However, several selenium depletion studies have indicated that erythrocyte selenium concentration is a less sensitive parameter than plasma selenium concentration to short-term changes in selenium status (van Rij et al, 1979; Janghorbani et al, 1984). This may probably be explained by the relatively long lifetime of red blood cells in the circulation. Supplementation of subjects with low selenium status with selenium in the form of Se-rich yeast or wheat demonstrated increases in erythrocyte selenium levels of 58-100% above the baseline values after 9-12 weeks of the trials (Levander et al, 1983; Thomson et al, 1985).

Erythrocyte selenium levels appear to be a reliable indicator of selenium status although they are less sensitive than plasma selenium levels to short-term fluctuations.

Urinary selenium

Selenium concentrations in human urine in health and disease states, and their medical implications have been reviewed (Robberecht and Deelstra, 1984). Although experimental depletion of selenium in humans results in a significant decrease of the concentration of this element in urine (Janghorbani et al, 1984), this parameter has not been widely used for the diagnosis of selenium deficiency.

Hair selenium

Little is known about the clinical significance of hair selenium levels. A significant increase in hair selenium concentrations in normal people was observed during ingestion of a selenium supplement for 6 weeks, although whole blood selenium levels did not change (Gallagher et al, 1984). The use of selenium-rich shampoos add to the problem of contamination from exogenous sources (Davies, 1982).

A recent review on the usefulness of elemental analysis of hair concluded that the trace element content of hair is not a reliable measurement and furthermore, in many instances it provides data that is misleading (Taylor, 1986).

Platelet selenium

Selenium concentration in platelets has been reported to exceed that of most other tissues including erythrocytes, muscle and liver (Kasperek et al, 1979). Decreased levels of selenium in platelets from dietetically treated children with low selenium intake have been observed (Kasperek et al, 1982). Further investigation of the use of platelets as an index of body selenium status is warranted.

Glutathione peroxidase

The activity of glutathione peroxidase is regarded as a good functional index of body selenium deficiency. Studies of the relationship between selenium levels and glutathione peroxidase activity in erythrocytes or whole blood have shown it to be linear at low levels of erythrocyte or whole blood selenium, but at higher levels it reaches a plateau of enzyme activity indicating that the requirement for selenium by glutathione peroxidase is met (Thomson et al, 1977; Rea et al, 1979). Patients receiving intravenous hyperalimentation demonstrated decreased erythrocyte glutathione peroxidase activity (Johnson et al, 1981b; Cohen et al, 1985). However, the activity of this enzyme in erythrocytes is not a sensitive indicator of short-term changes in selenium status (Cohen et al, 1985).

Subjects with a low selenium status supplemented with selenium in the form of sodium selenate or Se-rich yeast demonstrated a rapid increase of glutathione peroxidase activity in platelets (Levander et al, 1983; Menzel et al, 1983). Selenium supplementation studies with Se-rich yeast and sodium selenate have demonstrated an increase as

early as 2 days in glutathione peroxidase activity of plasma (Steiner et al, 1982).

Increased activity of this enzyme in erythrocytes has been reported in association with the use of oral contraceptives (Capel et al, 1981) and decreased plasma glutathione peroxidase activity has been demonstrated in pregnancy (Behne and Wolters, 1979).

3.2 LEUCOCYTE ZINC IN THE ASSESSMENT OF ZINC STATUS

Since the pioneering work of Vallee and Gibson (1948) much interest has been focused on the possible use of leucocyte zinc as an index of body status. Since leucocytes contain a nucleus, mitochondria and all other organelles, their zinc concentration may reflect body zinc content.

3.2.1 RELATIONSHIP BETWEEN LEUCOCYTE AND MUSCLE ZINC

As skeletal muscle contains a large proportion of body zinc, the relationship between the concentration of zinc in leucocytes and muscle has been investigated . Meadows et al (1981) reported an excellent correlation between the zinc concentration in leucocytes and rectus muscle in pregnant subjects undergoing caesarian section. No relationship was observed between the concentration of zinc in plasma and muscle or plasma and leucocytes. Significant correlations between the zinc concentration in leucocytes and muscle from the abdominal wall have also been reported in both control patients and those with disease undergoing elective surgery (Jones et al, 1981). In the same study, there was no significant relationship between the concentration of zinc in leucocytes and liver or muscle and liver. Leucocyte zinc concentrations therefore appeared to reflect the extrahepatic nucleated tissue zinc concentration in the patients investigated.

3.2.2 ZINC-DEPLETION STUDIES

Studies of severe zinc deficiency in rats have reported significant reductions in the concentration of zinc in bone (Jackson et al, 1982; Milne et al, 1985a), testicular tissue and liver (Jackson et al, 1982) but no decrease in the zinc concentration of skin, hair, heart muscle, three different skeletal muscles (Jackson et al, 1982),

mononucleated cells or erythrocytes (Milne et al, 1985a). Similar findings have also been reported in zinc deficient pigs, with significantly decreased concentrations of zinc in plasma, liver and pancreas but not in leucocytes or four different abdominal muscles (Crofton et al, 1983a). It has been postulated that in severe zinc deficiency in animals there is a mechanism for conserving zinc for the growth of tissue such as muscle, and in these circumstances mobilisation of zinc from bone occurs (Guigliano and Millward, 1984). In humans, zinc losses in starvation also probably occur from bone, not muscle, and it may be that a similar mechanism could apply (Elia et al, 1984). An 11 years old thalassaemic boy who was treated with a chelating agent for tranfusion haemosiderosis sustained very large losses of zinc in the urine, and analysis of tissues removed at post-mortem showed low zinc concentrations in liver, bone and testis, but normal zinc concentrations of hair, heart and skeletal muscle (Jackson et al, 1982). Leucocyte zinc concentration was not measured.

Moderate zinc deficiency states have been induced under experimental conditions in human subjects (Prasad et al, 1978). Two healthy male volunteers consumed a diet which provided only 54 µmol (3.5mg) of zinc per day for 50 weeks. Data from balance studies demonstrated that both subjects were depleted of approximately 7.7 mmol (500mg) of zinc during this period, which is approximately 30% of the normal body content of this element. The zinc concentration of leucocytes fell to about 40% of baseline values during the zinc-restriciton period, although no information is given on how quickly a significant decrease occurred. In another experimental zinc depletion study a marginal dietary zinc deficiency was produced in 5 human subjects, and at the end of 3 months a decrease of 31% in the zinc concentration of neutrophils was observed (Prasad and Cossack, 1982). Leucocyte zinc concentration therefore appears to reflect the decrease in body zinc content in human subjects caused by an inadequate dietary intake.

3.2.3 LEUCOCYTE ZINC MEASUREMENTS IN CLINICAL STUDIES

Leucocyte zinc concentration has been measured in a range of disorders in which zinc deficiency may be a complication. An association between low maternal leucocyte zinc concentration during pregnancy and production of low birthweight infants has been reported (Meadows et al, 1981; Patrick and Dervish, 1984; Salim et al, 1986;

Wells et al, 1987) and the leucocyte zinc concentration of the cord blood of babies with intrauterine growth retardation was lower than in normal babies (Meadows et al, 1983). Mahajan et al (1979) demonstrated a 20-25% decrease in leucocyte zinc concentrations in patients with chronic renal failure whether treated by dialysis or not. Zinc supplementation resulted in an increase in leucocyte zinc concentrations in haemodialysis patients which was associated with a significant improvement in abnormalities of taste and sexual function (Mahajan et al, 1982). In diabetic patients, normal leucocyte zinc concentrations were observed by Pidduck et al (1971) whereas Kumar and Rao (1974) demonstrated significantly reduced concentrations of zinc in leucocytes, erythrocytes and plasma. Leucocyte zinc concentrations have been reported to be normal in patients with Down's syndrome (Milunsky et al, 1970), and carcinoma of the prostate (Habib et al, 1980), and decreased in patients with leukaemia (Dennes et al, 1961; Fredericks et al, 1964; Carpentieri et al, 1986), liver disease (Fredericks et al, 1960; Keeling et al, 1980) and anorexia nervosa (Ainley et al, 1986). Little information is available concerning leucocyte zinc concentrations in malabsorption syndromes. Patrick and Dervish (1984) reported decreased levels of leucocyte zinc in children with cystic fibrosis when compared to adults. In view of the fact that the data was not age and sex matched, this study was considered inconclusive as evidence for body zinc deficiency in these children.

Comment:

Although the use of leucocyte zinc as an indicator of zinc deficiency has been questioned in animal studies, Prasad et al (1978) have clearly shown that leucocyte zinc concentrations provide evidence of experimental zinc depletion in humans. Furthermore, significant reductions in leucocyte zinc concentrations have been reported in a range of disorders in which zinc deficiency may be implicated.

METHODOLOGY

CHAPTER 4

DEVELOPMENT AND EVALUATION OF A TECHNIQUE FOR THE ISOLATION OF LEUCOCYTES FROM WHOLE BLOOD

4.1 INTRODUCTION

Despite numerous techniques that have been reported for the isolation of leucocytes from whole blood, the technical difficulties do not appear to have been totally resolved. Many methods are based upon the differences in sedimentation behaviour between leucocytes and erythrocytes. Erythrocyte aggregating agents which have been used include fibrinogen, phytohemagglutinin and dextran (Skoog and Beck, 1956; Baron and Ahmed, 1969). Procedures described for elimination of the erythrocytes remaining after preliminary separation include hypotonic shock with distilled water or saline (Fallon et al, 1962; Baron and Ahmed, 1969). Although this technique has been used by other workers (Nishi, 1980; Jones et al, 1981) it has the disadvantage that the leucocyte yield is low and a mean recovery of only 45% is obtained. Viability studies of isolated leucocytes obtained from differential sedimentation techniques demonstrated that isolated leucocytes were not damaged to any appreciable extent (Fallon et al, 1962; Baron and Ahmed, 1969). The use of density gradient layering techniques, which result in removal of erythrocytes from the upper layer leaving leucocyte-rich plasma, has been reviewed (Boyum 1968a, 1968b).

Separation of mononucleated and polymorphonucleated cells has been achieved using gradient centrifugation over a synthetic polymer of sucrose (Ficoll-Hypaque) (English and Andersen, 1974; Whitehouse et al, 1982; Purcell et al, 1986) or colloidal polyvinylpyrrolidone-coated silica (Percoll) (Milne et al, 1985b). However, various degrees of contamination of the mononucleated cellular fraction by platelets have been reported in these studies. Furthermore, polymorphonucleated cells separated by centrifugation over Ficoll gradients do not have normal sodium transport characteristics and may therefore have suffered membrane damage (Poston et al, 1982).

The main methodological problems encountered with all the leucocyte separation procedures is the occurrence of cell clumping,

which is extremely difficult to disperse, and in such circumstances white cell counts are unreliable. Some workers have advised removal of clumps of cells or discarding the specimen in cases of severe clumping (Whitehouse et al, 1982; Milne et al, 1985b).

This chapter describes the development and evaluation of a technique for the isolation of leucocytes from whole blood, which is suitable for the purpose of measuring zinc, copper and selenium in the separated white cells. Jones et al (1980) investigated the dynamnics of zinc transport in normal human leucocytes incubated in various media and buffers. They reported that zinc influx appeared to consist of two components which were an inital rapid phase and a subsequent slower phase, and suggested that the rapid uptake represented the binding of zinc to the leucocyte membrane. These observations emphasise the need for all wash solutions used in the leucocyte separation procedure to be free of zinc. In the same study, no significant efflux of zinc occurred when the media was zinc-free.

4.2 EXPERIMENTAL

4.2.1 ISOLATION OF LEUCOCYTES

4.2.1.1 Apparatus

An MSE, model 6L, centrifuge with a 20-cm head was used.
Blood cell counts were performed using a Coulter Counter, Model DN, in conjunction with the Coulter Dual Diluter III.

4.2.1.2 Reagents

Unless otherwise stated, reagents were Analar or Aristar grade materials (BDH Chemicals Ltd.)
Dextran 150, 6% m/V in 0.9% m/V saline (Dextravan 150, Fisons)
Sodium heparin (Injection BP, Weddel Pharmaceuticals Ltd.)
Sodium dihydrogen orthophosphate
Disodium hydrogen orthophosphate
Ammonium chloride
Tris (hydroxymethyl) aminomethane (Tris)
Sodium Chloride, 0.9% m/V in de-ionised, distilled water
Hydrochloric acid, 1M and 5M

Decon 75, 5% V/V in de-ionised water
Nitric acid, 16M
Repelcote water repellent

4.2.1.3 Procedure

De-ionised distilled water was used throughout.

Preparation of glassware

All glassware was first cleaned with 5% V/V Decon 75 solution, then soaked in 10% V/V nitric acid for approximately 12h, followed by six rinses with water. The 5-ml glass tubes used for the digestion procedure were further cleaned by addition of 2ml of 16M nitric acid, after which they were heated in a heating block at 155^{o}C for 30 min and this was followed by six rinses with water.

The calibrated 25ml glass tubes used for the separation procedure were siliconised with Repelcote.

De-ionised distilled water, acid-washed glassware, dextran. heparin and all other solutions were found to be free from zinc, copper and selenium.

Preparation of solutions

Phosphate-buffered saline (PBS): A stock PBS solution was prepared by dissolving 23.17g of sodium dihydrogen orthophosphate and 179.9g of disodium hydrogen orthophosphate in about 4l of water. The pH was adjusted to 7.2 with 5M hydrochloric acid, and after adding 701.3g of sodium chloride the solution was diluted to 8l. A working PBS solution was prepared by diluting the stock solution 1 + 9 with water.

Tris (hydroxymethyl) aminomethane (Tris)-ammonium chloride solution (TAC): A stock solution of 0.83% m/V ammonium chloride was prepared by dissolving 8.3g of ammonium chloride in 1 litre of water. 0.17M Tris stock solution was prepared by dissolving 20.6g of Tris in 900ml of water and adjusting the pH to 7.65 with 1M hydrochloric acid, after which the solution was diluted to 1 litre with water.

To obtain a working Tris-ammonium chloride solution, a dilution of 1 part of Tris solution to 9 parts of ammonium chloride solution was made and the pH was adjusted to 7.2 with 1M hydrochloric acid.

Collection of blood samples

A 17ml sample of venous blood was collected by venepuncture from an antecubital vein using minimum stasis and taking care to avoid frothing and haemolysis.

A 2ml aliquot of the blood was transferred to a blood sample tube containing ethylenediamine tetra-acetic acid (EDTA) as anticoagulant, for the determination of a white cell count. The remaining 15ml blood sample was placed into a calibrated 25ml stoppered, siliconised tube containing 300 units of heparin. This was mixed gently by inversion.

Red cell sedimentation

9.0ml of the saline solution and 6.0ml of dextran were added to the heparinised blood and gently mixed by inversion, taking care to avoid frothing. After allowing the tube to stand unstoppered and vertical at room temperature (23 ± 3°C) for 45 ± 5 min, the leucocyte rich supernatant was transferred with an acid washed plastic Pasteur pipette into a fresh 25ml siliconised tube. Care was taken to aspirate right down to the interface without drawing up any of the red cell sediment.

Washing procedure and elimination of remaining red cells

A volume of 9.0ml of PBS solution was added to the aspirate and gently mixed by inversion. This was centrifuged at 450g (1200 rev min^{-1}) for 6 min at 4°C, after which the supernatant fluid was discarded. A volume of 15ml of PBS solution was then added to the cell pellet and this was mixed thoroughly usng a vortex mixer until an even re-suspension of cells was obtained. The suspension was centrifuged at 200g (850 rev min^{-1}) for 10 min at 4°C and the supernatant fluid containing the platelets was removed and discarded.

To achieve lysis of the red cells, the cell pellet was resuspended by adding 4.5ml of TAC solution and vortex mixed, followed by incubation in a water bath at 37°C for 10 min. Immediately after this, a volume of 15ml of PBS solution was added and mixed by inversion. The suspension was centrifuged at 450g for 6 min at 4°C after which the pink supernatant fluid containing the red cell lysate was discarded, leaving behind the white cell pellet.

Finally, the white cells were washed by adding a further 15ml of PBS solution and mixing on a vortex mixer, then centrifuged at 450g

for 6 min at 4°C after which the supernatant solution was removed and discarded.

Final leucocyte preparation

A volume of 2ml of PBS solution was added to the cell pellet and after vigorous vortex mixing the suspension was transferred quantitatively into a calibrated 5ml glass tube. The volume was made up to 3.0ml using PBS solution and mixed thoroughly using a vortex mixer. A 200µl aliquot of the cell suspension was removed and retained for the purpose of white cell counting. The remaining 2.8ml of suspension was centrifuged at 450g for 6 min at 4°C and after discarding the supernatant solution the white cell pellet was stored at -20°C until required for the determination of zinc, copper and selenium.

Cell counting

Standard procedures according to the manufacturer's instruction manual were used for the determination of white cell counts on whole blood and the leucocyte preparation.

Counting errors were minimised by processing samples of whole blood and of the corresponding leucocyte fraction in the same batch. Duplicate counts were obtained from separate duplicate dilutions of each sample and the mean of all four values was calculated. This procedure gave a within-batch coefficient of variation of 2.3% at a level of 8.7×10^9 white cells per litre and a between-batch coefficient of variation of 4.6% at a level of 8.8×10^9 white cells per litre.

Calculation

The yield of leucocytes obtained from the blood sample was calculated as follows:

$$\text{Recovery\%} = \frac{\text{Total final leucocyte count} \times \text{Volume of original suspension (3.0ml)}}{\text{Total original leucocyte count} \times \text{Volume of heparinsed blood (15.0ml)}} \times 100$$

4.3 RESULTS AND DISCUSSION

4.3.1 OPTIMISATION OF LEUCOCYTE SEPARATION PROCEDURE

Optimum conditions for the isolation of leucocytes were established by investigation of the effect of dextran concentration on leucocyte losses during sedimentation of red cells, the use of ammonium chloride as a red cell lysing agent, and the effect of delay before initiating sedimentation on leucocyte recovery.

4.3.1.1 Effect of dextran concentration on leucocyte losses during sedimentation of red cells

Although increasing concentrations of dextran are known to result in an increased sedimentation rate, entrapment of leucocytes in the red cell sediment may also be increased. It was therefore necessary to establish the optimum concentration of dextran which gave the greatest recovery of leucocytes in the supernatant. Rapid sedimentation of red cells has been demonstrated with 3% m/V dextrans of molecular weights of 139,000 and 228,000 and using these dextrans, good leucocyte yields in the supernatant have been obtained with a volume ratio of 2:1 dextran-blood mixture (Skoog and Beck, 1956). In the present study, a volume of 15ml of blood was required in order to achieve a large enough yield of white cells to enable the measurement of zinc, copper and selenium in the isolated leucocytes. As 25ml tubes were used for the separation procedure, varying concentrations of dextran were investigated using a dextran-blood mixture of a volume ratio of 1:1. A solution of 6% m/V dextran 150 in 0.9% m/V saline was chosen as the starting point as this was readily available and found to be free of zinc, copper and selenium.

A volume of 61ml of blood was withdrawn from each of 3 healthy subjects (A,B,C), and divided into 4 aliquots of 15ml which were placed into 25ml siliconised tubes containing 300 units of heparin. A 1ml aliquot of blood was transferred into a blood sample tube containing EDTA, as anticoagulant, for the determination of a white cell count. Table 4.I shows the amounts of 6% m/V dextran 150 and 0.9% m/V saline that were added to each aliquot in order to achieve the desired concentration of dextran 150. The total amount added was kept at a constant volume of 15ml so that a dextran-blood mixture of

TABLE 4.I

SEDIMENTATION REGIMES EMPLOYED TO INVESTIGATE THE EFFECT

OF DEXTRAN CONCENTRATION ON LEUCOCYTE RECOVERY IN SUPERNATANT

Blood ml	Dextran added ml	Saline added ml	Conc. of dextran 150 added % m/V
15	12	3	4.8
15	9	6	3.6
15	6	9	2.4
15	3	12	1.2

volume ratio 1:1 was ensured. Sedimentation was considered achieved after the agglutinated erythrocytes had become separated from the top layer containing the leucocytes, such that the character of the interphase boundary was well differentiated. After sedimentation, the leucocyte-rich supernatants were removed and the volume of each aspirate was noted. White cell counts were performed on each supernatant and the whole blood specimen. Table 4.II shows the leucocyte recoveries observed and the time taken to achieve sedimentation with the varying concentrations of dextran 150. Although the shortest sedimentation time was noted with the use of 4.8% m/V dextran 150, the most efficient leucocyte recovery in the supernatant was demonstrated with the use of 2.4% m/V dextran 150. A decrease in the volume of supernatant obtained with decreasing concentrations of dextran was observed.

4.3.1.2 Elimination of remaining red cells

Elimination of remaining red cells has commonly been carried out by hypotonic shocking but this subsequently leads to white cell clumping which is extremely difficult to disperse. An alternative method was therefore sought. Although red cells can be destroyed by lysis with ammonium chloride alone, this has the disadvantage that the pH alterations involved are likely to damage and destroy the white cells. In the present study, the white cell losses which occur when using ammonium chloride were minimised by addition of tris-(hydroxymethyl)-methylamine (Tris) to the ammonium chloride to form a buffer adjusted to pH 7.2. The Tris-ammonium chloride solution was not effective as a red cell lysing agent at room temperature (23 ± 3°C) over a period of 30 min and an incubation at 37°C for 10 min was necessary for complete lysis of the remaining red cells. No subsequent clumping of the white cells was observed.

4.3.1.3 Effect of delay before initiating red cell sedimentation on leucocyte recovery

Baron and Ahmed (1969) reported that delay of more than 20 min before addition of the dextran solution to freshly drawn heparinised blood which had been kept at 37°C resulted in a marked loss of leucocytes. The effect of this was therefore studied.

TABLE 4.II

LEUCOCYTE RECOVERIES OBTAINED IN THE SUPERNATANT AFTER SEDIMENTATION OF RED CELLS WITH VARYING CONCENTRATIONS OF DEXTRAN 150

Conc. of dextran 150 % m/V	Subject	Leucocytes in 15ml blood x 10^7	Leucocytes in supernatant x 10^7	Volume supernatant ml	Recovery %	Sedimentation time mins.
4.8	A	12.1	8.8	22	73	30
4.8	B	10.5	7.3	24	69	27
4.8	C	10.7	8.4	24	78	29
3.6	A	12.1	9.4	21	78	36
3.6	B	10.5	8.1	22	77	34
3.6	C	10.7	8.8	21	82	35
2.4	A	12.1	10.1	19	84	47
2.4	B	10.5	8.5	20	81	45
2.4	C	10.7	9.3	20	87	48
1.2	A	12.1	8.4	14	70	63
1.2	B	10.5	8.0	16	77	60
1.2	C	10.7	8.4	16	78	65

A volume of 61ml of blood was withdrawn from each of 3 healthy subjects (A,B,C) and divided into 4 aliquots of 15ml. One aliquot underwent the separation process immediately and of the other 3 aliquots, one was left for 15 min, one for 30 min and one for 60 min at room temperature (23 ± 3°C) before sedimentation was initiated. To determine any variation in recovery of leucocytes from different aliquots of the same sample of whole blood which had not been subjected to any delay before sedimentation was initiated, a further 61ml of blood was taken from another healthy subject (D), and all 4 aliquots were separated immediately. White cell counts were performed on the final leucocyte preparations and the remaining 1ml aliquots of blood from subjects A,B,C and D. These data, together with the leucocyte recoveries obtained are shown in Table 4.III. Recovery of leucocytes from the 4 aliquots of blood from subject D, which had all undergone the separation process without delay, ranged between 64-65%. Although lower leucocyte recoveries were not observed in the blood samples from subject B until 30 min delay before sedimentation (60% at 0 min, 57% at 30 min), a decrease in leucocyte recoveries was noted after only 15 min delay in the blood samples from subjects A and C (64 and 70% at 0 min respectively, 61 and 67% at 15 min respectively). On the basis of these observations, it was concluded that the separation process should be started immediately after withdrawal of the blood in order to achieve maximum recovery of leucocytes.

4.3.2 EVALUATION OF LEUCOCYTE SEPARATION PROCEDURE

The method described was evaluated for efficiency of absolute white cell recovery, for leucocyte recovery without differential losses of any particular white cell line and for any possible losses of zinc, copper or selenium immediately after the separation process.

4.3.2.1 Absolute leucocyte recovery

Figure 4.1 illustrates red and white cell recovery at the individual stages of the separation process. Only about 10% of the leucocytes present are lost at the stage of lysis by TAC, which is a substantial improvement over the hypotonic shock method. The white cell losses involved at the stage of plasma and platelet separations and washings are small, the largest identifiable losses occuring

TABLE 4.III

THE EFFECT OF DELAYING THE START OF THE SEDIMENTATION PROCEDURE ON RECOVERY OF LEUCOCYTES

Subject	Leucocytes in whole blood $\times 10^9\ l^{-1}$	Delay mins.	Leucocytes in final prep. $\times 10^9\ l^{-1}$	Recovery of leucocytes %
A	5.2	0	16.5	64
A		15	15.9	61
A		30	15.1	58
A		60	13.7	53
B	6.2	0	18.4	60
B		15	18.6	61
B		30	17.7	57
B		60	15.6	51
C	4.9	0	17.1	70
C		15	16.3	67
C		30	15.7	64
C		60	14.6	60
D	5.9	0	18.8	64
D		0	19.0	65
D		0	18.8	64
D		0	18.7	64

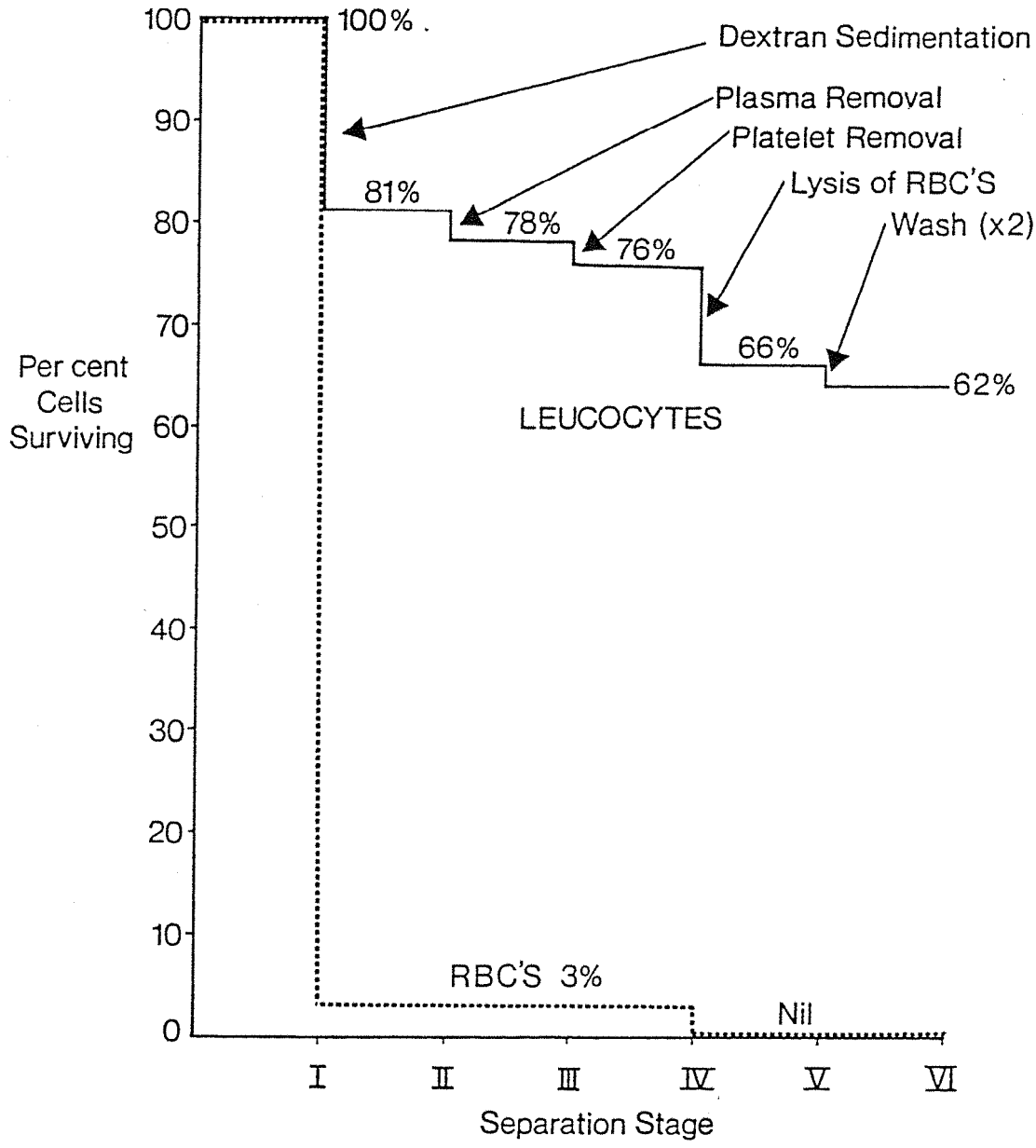


Figure 4.1

Red and white cell survival at the individual stages of the separation process

during dextran sedimentation. Absolute white cell recovery has been plotted in Figure 4.2. The linear response of recovery with the intial white cell count and reasonably close fit of the data points to the regression line (r = 0.85) suggests a well controlled separation process. A mean recovery of leucocytes of 62% (range 51-75%) was obtained for 120 leucocyte separations.

4.3.2.2 Differential leucocyte recovery

Microscopic examination of smears confirmed complete elimination of red cells and platelets. Morphologically, the white cells appeared normal in shape and stained smears showed intact nuclei, well stained cytoplasm and granules similar to those seen in fresh smears.

200-cell differential white cell counts were performed by standard techniques which discerned polymorphonucleated cells from mononucleated cells on stained whole blood films and white cell preparation smears. The percentages of mononucleated cells and polymorphonucleated cells in the corresponding preparations for 12 leucocyte separations are shown in Table 4.IV. The observed differences in the percentages of white cells from the two preparations were less than 5% for 7 subjects, between 5 and 10% for 2 subjects and between 10 and 15% for 3 subjects. These differences are within the inherent errors of differential white cell counting (Dacie and Lewis, 1984) and therefore it was concluded that the mononucleated cells and polymorphonucleated cells appeared to be in the same proportions in the whole blood and corresponding leucocyte preparation.

4.3.2.3 Leakage of zinc, copper and selenium from leucocytes after the separation process

It was not possible to investigate for any leakage of zinc, copper and selenium during the separation process because of the contamination with the red cells that were present. Even after lysis of the residual red cells with TAC, two subsequent washings were necessary to remove contamination from the red cell lysate in the supernatant liquids. Leakage from the leucocytes was therefore investigated immediately after the separation procedure.

A 45ml blood sample was withdrawn from each of 3 subjects (1,2,3) and divided into aliquots of 15ml which were subjected to the

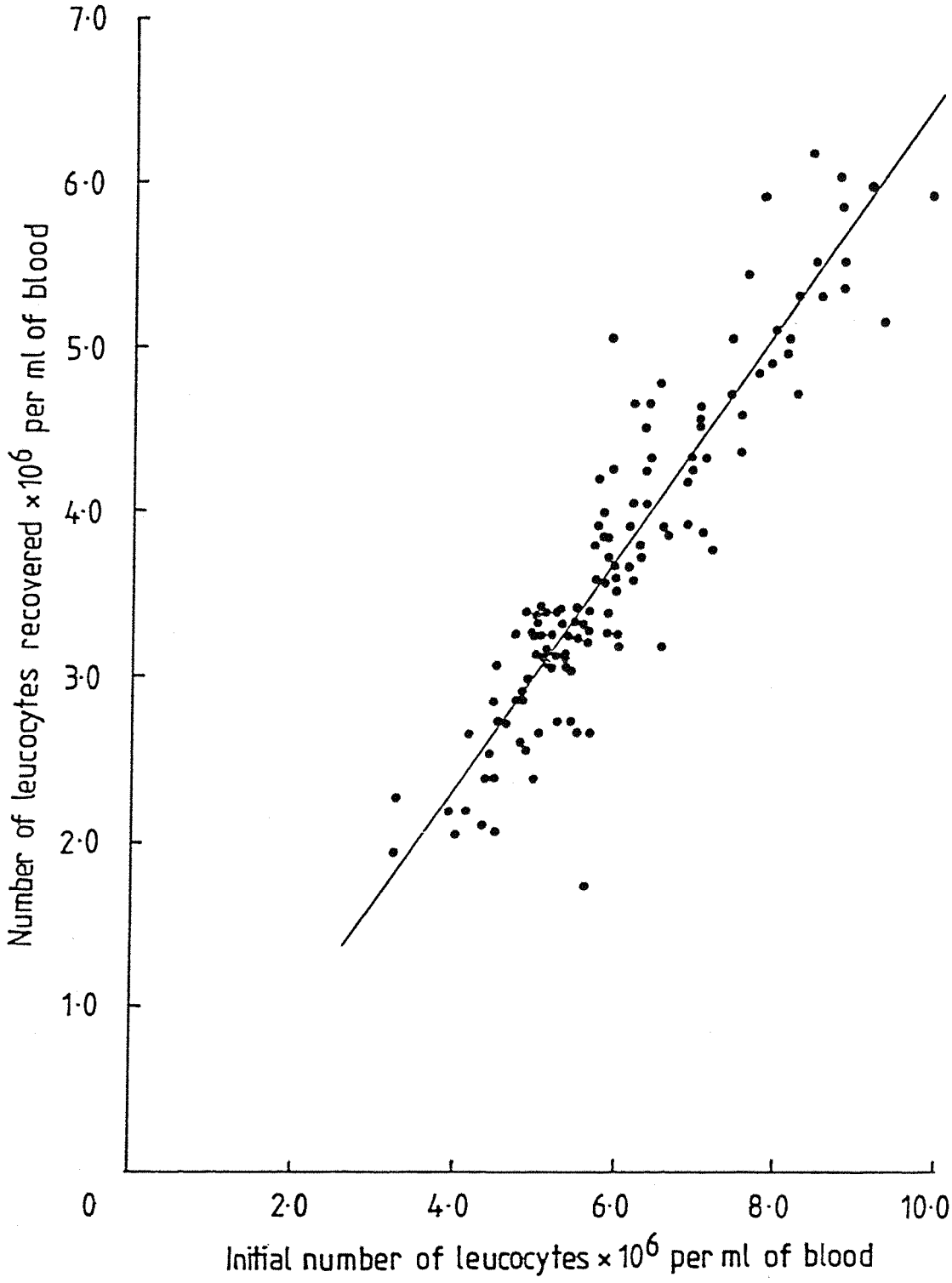


Figure 4.2

Leucocyte recoveries

TABLE 4.IV

PERCENTAGE OF MONONUCLEATED AND POLYMORPHONUCLEATED CELLS IN WHOLE BLOOD AND CORRESPONDING LEUCOCYTE PREPARATION

Whole blood		Leucocyte preparation	
Mononucleated cells %	Polymorphonucleated cells %	Mononucleated cells %	Polymorphonucleated cells %
27	73	24	76
38	62	44	56
32	68	45	55
47	53	43	57
41	59	48	52
39	61	43	57
51	49	40	60
30	70	34	66
39	61	29	71
53	47	51	49
46	54	49	51
52	48	50	50

TABLE 4.V

LEAKAGE OF ZINC, COPPER AND SELENIUM AFTER THE SEPARATION PROCESS

Trace element	Subject	Supernatant liquid			Leucocytes		
		A	B	C	A	B	C
Zinc	1	0.15	0.15	0.15	3.5	3.6	3.6
µmol l^{-1}	2	ND	ND	ND	2.6	2.5	2.4
	3	ND	ND	ND	4.0	4.2	4.0
Copper	1	ND	ND	ND	0.31	0.31	0.31
µmol l^{-1}	2	ND	ND	ND	0.48	0.46	0.49
	3	ND	ND	ND	0.22	0.20	0.21
Selenium	1	ND	ND	ND	41	38	40
nmol l^{-1}	2	ND	ND	ND	35	35	37
	3	ND	ND	ND	29	31	30

ND = none detected

i.e. less than 0.14 µmol l^{-1} for zinc

less than 0.006 µmol l^{-1} for copper

less than 1.85 nmol l^{-1} for selenium

separation procedure in parallel, yielding 3 separate leucocyte suspensions in PBS, each of 3.0ml volume (A,B,C). These were centrifuged at time intervals of 0 min (A), 30 min (B) and 60 min (C), and the supernatant liquids were poured off and retained immediately after centrifugation. Cells were digested with nitric acid and the volume made up to 3.0ml with water. The zinc, copper and selenium contents of the leucocyte digest samples and corresponding supernatant liquids were determined by the procedures described in Chapter 5. The results given in Table 4.V show that the leakage of zinc from white cells in one of the leucocyte suspension samples was 4% at a time of 0 min with no further increase in leakage when left in contact with the PBS for up to 60 min. There was no detectable leakage of zinc from the leucocytes in the other two samples or of copper or selenium from the leucocytes in any of the samples during the time of 0-60 min.

Comment:

The method described for the isolation of leucocytes has been shown to be practicable and to give an efficient recovery of leucocytes with no differential white cell loss. In addition, leucocyte suspensions were found to be free of clumps, red cells and platelets.

CHAPTER 5

ANALYSIS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES, PLASMA AND WHOLE BLOOD USING ATOMIC ABSORPTION SPECTROPHOTOMETRY

5.1 INTRODUCTION

Since the introduction of atomic absorption spectrophotometry (Walsh, 1955), and with the subsequent process in this field, particularly the development of atomic absorption with electrothermal atomisation, many reliable and sensitive techniques for the measurement of trace elements in biological samples have become available (Delves, 1981). The choice between flame and electrothermal atomisation techniques is usually determined by the concentration range of the analyte and the sample volume available. Flame-atomic absorption is faster than furnace and interference problems are less severe and often easier to overcome on the flame.

The major interference in flame-atomic absorption spectrophotometry is matrix interference. If the sample solutions are more viscous or have considerably different surface tension characteristics than the calibrating standards, there may be a difference in the rate of sample uptake or nebulisation efficiency. Diluent solutions such as butan-1-ol help to overcome the viscosity effects of the sample for atomisation in flames (Meret and Henkin, 1971). Simultaneous determination of zinc and copper in plasma, involving dilution of the sample with organic solvents has proved simple, quick and reproducible and the established accuracy of the procedure renders it the method of choice (Delves, 1981). Matrix problems have also been overcome for the determination of plasma zinc by preparing zinc standards in zinc-free pooled human plasma (Wawschinek, 1984).

In furnace-atomic absorption, the sample is dispensed into a small graphite tube which can be heated electrically. By increasing the temperature stepwise, the processes of drying, ashing and atomisation can be separated. Care must be taken in selecting the optimum conditions. The greater sensitivity of electrothermal-atomic absorption spectrophotometry has been applied to the measurement of zinc in microlitre volumes of serum, and an excellent agreement

(r = 0.94) between the results obtained by this procedure and a routine flame-atomic absorption method has been reported (Foote and Delves, 1982). No significant difference was demonstrated in whole blood zinc values obtained from electrothermal - and flame-atomic absorption techniques (Ward et al, 1979). Determination of copper in erythrocytes has received little attention. Methods employing pre-treatment of the sample by acid digestion (Robbins et al, 1975) or dilution with Triton X-100 (Velghe et al, 1982) have been described. Most of the zinc measurements in leucocytes have been performed by atomic absorption spectrophotometry using flame or electrothermal atomisation after digestion of the cells with nitric acid (Keeling et al, 1980; Whitehouse et al,1982; Patrick and Dervish, 1984; Milne et al, 1985b). Between-batch coefficients of variation of less than 6% have been reported by these workers.

The high background absorption at 196nm and the relatively poor sensitivity precludes the use of conventional flame-atomic absorption spectrophotometry for determination of selenium. Fluorimetric methods (Lalonde et al, 1982; Koh and Benson, 1983), or atomic absorption spectrophotometry using either electrothermal atomisation (Pleban et al, 1982; Paschal and Kimberly, 1986) or hydride formation techniques (Lloyd et al, 1982; Tam and Lacroix, 1982) are common. Complete digestion of the sample is required for hydride methods and fluorimetric techniques. The use of a combination of nitric, perchloric and sulphuric acids have been reported to be the most efficient wet digestion mixture (Neve et al, 1982). However, the use of perchloric acid requires an exhaust hood designed specifically for handling perchloric acid fumes, and is therefore a limiting factor with this digestion procedure. Lloyd et al (1982) reported a quantitative recovery of added selenium using a mixture of nitric and sulphuric acids only for digestion of plasma or whole blood followed by hydride generation and atomic absorption spectrophotometry.

This chapter describes the development and evaluation of methods for the analysis of zinc, copper and selenium in leucocytes, and copper in erythrocytes and whole blood. Established methodology for trace element analysis which was used in the present study is also reported.

5.2 METHOD DEVELOPMENT

5.2.1 ANALYSIS OF ZINC, COPPER AND SELENIUM IN ISOLATED LEUCOCYTES

5.2.1.1 Reagents

Unless otherwise stated, reagents were Analar or Aristar grade materials from BDH Chemicals Ltd.
Zinc chloride standard solution containing 5mmol l^{-1} of zinc
Copper (11) chloride standard solution containing 5mmol l^{-1} of copper
Selenous acid standard solution containing 12.7mmol l^{-1}(1mg ml^{-1}) of selenium
Nitric acid, 16M
Hydrochloric acid, 12M
Sulphuric acid, 4.5M
Sodium hydroxide solution, 0.25M
Sodium tetrahydroborate (111), 6% m/V solution in 0.25 M sodium hydroxide solution
Antifoam emulsion DB 110A (Dow Corning), 1% V/V aqueous solution

5.2.1.2 Instrumentation

Perkin-Elmer 560 atomic absorption spectrophotometer. Used for the measurement of zinc by atomic absorption spectrophotometry using an air-acetylene flame.

Perkin-Elmer 4000 atomic absorption spectrophotometer. Equipped with an HGA-500 graphite furnace and AS-40 autosampler; used for measurement of copper using the conditions given in Table 5.I.

Perkin-Elmer 2380 atomic absorption spectrophotometer. Equipped with an MHS 20 hydride generation system. Used for the measurement of selenium. Instrumental conditions are given in Table 5.II.

5.2.1.3 Apparatus

A Techne Dri-block heater, Model DB 3H, was used for digestion of the white cell pellet. The heater was fitted with three alloy blocks,

TABLE 5.I

INSTRUMENTAL CONDITIONS FOR THE DETERMINATION OF COPPER IN THE LEUCOCYTE DIGESTS

Model 4000 Atomic absorption spectrophotometer:-

Wavelength : 324.7 nm
Lamp current : 15 mA
Slit width : 0.7 nm (low)
Deuterium background correction
Peak height measurement
Absorbance mode

Model AS-40 Autosampler:-

Injection volume : 20 µl
Replicates : x2

Model HGA-500 Furnace:-

	Step			
	1	2	3	4
Parameter	Dry	Ash	Atomise	Clean
Temperature/^{o}C	120	900	2500	2700
Ramp time/s	60	25	1	1
Hold time/s	20	25	5	4
Internal gas argon/ml min^{-1}	300	300	20	300

TABLE 5.II

INSTRUMENTAL CONDITIONS FOR THE DETERMINATION OF SELENIUM IN THE LEUCOCYTE DIGESTS

Model 2380 Atomic absorption spectrophotometer:-

Wavelength : 196.0 nm
Lamp[+] power : 6 watts
Slit width : 2.0 nm
Deuterium background correction
Peak height measurement
Absorbance mode

Model MHS 20:-

Tube temperature : 900^{o}C
Purge 1 (argon) : 32s
Purge 11 (argon) : 20s
Reaction time : 10s
Reaction volume : 20ml

[+] Electrodeless discharge lamp with power supply

each drilled with 12 holes to a depth of 48mm and a diameter of 16.75mm.

All glassware was prepared and cleaned as previously described.

5.2.1.4 Procedure

De-ionised distilled water was used throughout.

Digestion of leucocytes

The white cell pellets in the 5ml glass tubes were dissolved by adding 100µl of 16M nitric acid and placing each tube in a heating block at 155°C for 30 min. For the studies carried out it was convenient to digest the samples in batches of 30 and run 5 blank tubes concurrently with each batch. After allowing the test and blank tubes to cool, the digested cells were diluted to 2.0ml with water and mixed. These solutions were transferred into 2.0ml trace-element-free polycarbonate tubes and stored at -20°C until required for the determination of zinc, copper and selenium.

Preparation of zinc standard solutions

A stock zinc standard solution containing 200 µmol l^{-1} of zinc in 5% V/V hydrochloric acid, as preservative, was prepared by diluting 2.0ml of the BDH Chemicals concentrated standard solution and 2.5ml of 12M hydrochloric acid to 50.0ml with water. This solution was stable for up to six months at 4°C.

Working standard solutions containing 0, 1, 2, 3, 4 and 5 µmol l^{-1} of zinc in 5% V/V nitric acid were prepared by adding 0, 0.5, 1.0, 1.5, 2.0 and 2.5 ml of the stock standard solution to 5ml of 16M nitric acid and making the volume of each up to 100ml with water.

Measurement of zinc

The atomic absorption instrument was set up for the determination of zinc according to the manufacturer's instruction manual. Standards and samples were aspirated directly into the flame. A series of working standard solutions were run before and after each batch of samples, and two different standard solutions were run after every six samples. An internal quality control sample was run with each batch of test samples.

Preparation of copper standard solution

A stock copper standard solution containing 10µmol l^{-1} of copper in 5% V/V hydrochloric acid, as preservative, was prepared by diluting 200µl of the BDH Chemicals concentrated standard solution and 5ml of 12M hydrochloric acid to 100ml with water. This solution was stable for up to six months at 4°C.

Working standard solutions containing 0, 0.2, 0.4, 0.6 and 0.8 µmol l^{-1} of copper in 5% V/V nitric acid were prepared by adding 0, 2.0, 4.0, 6.0 and 8.0 ml of the stock standard solution to 5ml of 16M nitric acid and making the volume of each up to 100ml with water.

Measurement of copper

Standard and sample solutions were transferred into the AS-40 sampling cups and atomic absorption measurements of copper were carried out using the conditions given in Table 5.I. Duplicate measurements were performed. A series of working standard solutions were run before and after each batch of samples and three different standard solutions were run singly after every sixth pair of duplicate samples. An internal quality control sample was run with each batch of samples.

Preparation of selenium standard solutions

A stock selenium standard solution containing 12.7 µmol l^{-1} (1µg ml^{-1}) of selenium in 5% V/V nitric acid, as preservative, was prepared by diluting 100µl of the BDH Chemicals concentrated standard solution and 5ml of 16M nitric acid to 100ml with water. This solution was stable for up to six months at 4°C.

Working standard solutions containing 0, 12.7, 25.3, 38.0, 50.7 and 63.3 nmol l^{-1} (0,1, 2, 3, 4, 5 µgl^{-1} respectively) of selenium were prepared by adding 0, 0.1, 0.2, 0.3, 0.4 and 0.5ml of the stock standard solution to 5ml of 16M nitric acid and making the volume of each up to 100ml with water.

Sample oxidation

Duplicate 500µl portions of leucocyte digest samples were transferred into borosilicate glass tubes, and after addition of 1ml of 16M nitric acid and 1ml of 4.5M sulphuric acid these tubes were placed into pre-heated aluminium blocks at 155°C for 60 min. The tubes were allowed to cool to room temperature before addition of 2ml of 6M

hydrochloric acid after which the solutions were mixed by tapping the test-tubes, and then returned to the aluminium block pre-heated at 95°C for 30 mins. After allowing the tubes to cool to room temperature, the contents were transferred quantitatively into the reaction vessels for hydride formation and diluted to a final volume of 20ml with water followed by an addition of 100µl of antifoam reagent.

Measurement of selenium

Selenium concentrations were determined using the instrumental conditions given in Table 5.II. 500µl volumes of the selenium working standard solutions were transferred into the reaction vessels containing 1ml of 16M nitric acid, 1ml of 4.5M sulphuric acid and 2ml of 6M hydrochloric acid and diluted to a final volume of 20ml with water after which 100µl of antifoam reagent was added. Standards and samples were analysed in duplicate and the working standard solutions were run after every fifth pair of duplicate samples. An internal quality control sample was run with each batch of test samples.

Calculation

The following equation was used to calculate the concentration of zinc, copper and selenium in the white cells and to express the results in picomoles per 10^6 cells.

$$\text{Zn/Cu/Se (pmol per } 10^6 \text{ cells)} = \frac{\text{Volume of leucocyte digest (2.0ml)} \times \text{Concentration of Zn,Cu or Se in leucocyte digest (pmol } l^{-1}) \times 10^6}{\text{Volume of cell suspension (2.8ml)} \times \text{Number of white cells in cell suspension } (10^9\ l^{-1})}$$

5.2.2 ANALYSIS OF COPPER IN ERYTHROCYTES AND WHOLE BLOOD

5.2.2.1 Reagents

All reagents were Analar or Aristar grade materials obtained from BDH Chemicals Ltd.

Copper (11) chloride standard solution containing 5mmol l^{-1} of copper

Triton X-100, 0.1% V/V aqueous solution

Sodium chloride, 0.9% m/V aqueous solution

5.2.2.2 Apparatus

A Perkin-Elmer, model 560, was used for the measurement of copper by atomic absorption spectrophotometry using an air-acetylene flame. A Teflon small-volume sampling cup (Perkin-Elmer Ltd.) was attached directly to the nebuliser for all sample analyses.

Trace-element-free, 2ml volume, polycarbonate tubes containing lithium heparin or ethylenediamine tetra-acetic acid (EDTA) (Teklab Ltd.) were used for blood collection.

Red blood cell counts were determined on the erythrocyte suspension samples using a Coulter Counter, model DN, in conjunction with the Coulter Dual Dilutor 111.

All glassware was cleaned as described in the previous chapter.

5.2.2.3 Procedure

De-ionised distilled water was used throughout.

Preparation of calibrating standards

Working standard solutions containing 0, 5, 10, 15, 20 and 30 µmol l^{-1} of copper were prepared by diluting 0, 0.1, 0.2, 0.3, 0.4 and 0.6ml of the BDH Chemicals concentrated standard solution to 100ml with water.

Sample collection and erythrocyte separation

A 4ml sample of whole blood was obtained by venepuncture and a 2ml aliquot was transferred to a heparinised tube for erythrocyte

separation, and the remaining 2ml sample was transferred to a tube containing EDTA for determination of whole blood copper.

After centrifugation of the heparinised blood and removal of the plasma, the red blood cells remaining were washed three times with 0.9% V/V sodium chloride. After the last wash, a volume of the sodium chloride solution approximately equal to the volume of red cells was added.

Prior to analysis, the samples were frozen at -20°C and subsequently thawed to produce complete haemolysis of the red blood cells. In this way, homogenous samples were obtained after dilution.

Stock blood sample

A stock sample of whole blood was obtained by venepuncture from a healthy adult and transferred to a series of tubes containing EDTA and stored at -20°C. A further sample of whole blood was taken to provide a stock suspension of red blood cells and aliquots of this were stored at -20°C in trace-element-free polycarbonate tubes.

Cell counting

Standard procedures according to the manufacturer's instruction manual were used for the determination of red cell counts on the erythrocyte suspension samples. Cell counts were performed prior to freezing of the samples. Duplicate dilutions of each sample were made and the mean value was calculated. This procedure gave a within-batch coefficient of variation of 2.5% at a level of 4.4×10^{12} red cells per litre and a between-batch coefficient of variation of 4.1% at a level of 4.8×10^{12} red cells per litre.

Analysis of copper

Working standard solutions containing 0, 5, 10 and 15 μmol l^{-1} copper and 0, 10, 20 and 30 μmol l^{-1} copper were used for the analysis of copper in the erythrocyte suspensions and whole blood respectively.

A calibration graph was prepared by diluting 100μl volumes of the stock whole blood sample 1 + 1 + 1 with Triton X-100 solution and each of the appropriate series of the working standard solutions. Duplicate 100μl samples of whole blood were diluted with 100μl of water and 100μl of Triton X-100 solution.

The atomic absorption instrument was set up for the determination of copper according to the manufacturer's instruction manual. After

adjusting the nebuliser uptake rate to approximately 8ml min^{-1}, diluted standards and samples were introduced into the flame by means of a PTFE small-volume sampling cup (Manning, 1975). This involved substituting a small plastic cup for the standard capillary tubing normally used and injecting 100µl volumes of the diluted samples into the cup using an automatic pipette fitted with plastic sampling tips. Water was added to the cup between samples to rinse away any carryover from the previous sample.

The copper content of the red blood cell suspensions was also determined as described for whole blood using the stock red blood cell suspension for preparing the calibration graph. Internal quality control samples were run with each batch of test samples.

Calculation

The following equation was used to calculate the concentration of copper in the red cells and to express the results in picomoles per 10^6 cells.

$$\text{Copper (pmol per } 10^6 \text{ cells)} = \frac{\text{Concentration of copper in erythrocyte suspension (pmol } l^{-1}\text{)}}{\text{Number of red cells in erythrocyte suspension } (10^{12} l^{-1})} \times 10^6$$

5.2.3 RESULTS AND DISCUSSION

5.2.3.1 Development of the furnace programme for determination of copper in leucocyte digest samples

Drying stage

A simple single stage drying procedure was found satisfactory. The sample was dried by increasing the temperature of the furnace to 120°C over 60 seconds and maintaining it at that temperature for a further 20 seconds.

Ashing stage

An ashing stage was incorporated into the furnace programme to destroy the sample matrix and decrease the possibility of the effect of absorption interferences on copper sensitivity. The relationship between the temperature used for ashing and the absorbance values obtained during the analysis of an aqueous standard sample in 5% V/V nitric acid and a leucocyte digest sample is shown in Figure 5.1. Absorbance readings for both samples remained constant as the ashing temperature was varied between 600°C and 900°C. Above 900°C, a loss in absorbance signal was noted which was associated with copper losses from both samples during the ashing process. The temperature of the ashing stage was therefore limited to 900°C.

Atomisation

Figure 5.1 also shows the relationship between the temperature of the atomisation stage and the absorbance signal found during the analysis of an aqueous standard sample in 5% V/V nitric acid and a leucocyte digest sample. Progressively larger amounts of copper were atomised as the temperature was increased from 1700°C to 2500°C. Temperatures greater than 2500°C did not result in further increases in absorbances indicating that all of the copper present in both samples was atomised under these conditions. A temperature of 2500°C was therefore selected to ensure complete atomisation of samples during routine analysis.

Internal gas flow-rate

The correlation between sensitivity and internal purge argon flow for copper was investigated. A threefold variation in sensitivity was obtained when the internal gas flow-rate was varied within the range of 0-300 ml min^{-1} (Figure 5.2). A setting of 20ml min^{-1} was selected to obtain good sensitivity.

5.2.3.2 Evaluation of the methods used for determination of zinc, copper and selenium in leucocyte digest samples

5.2.3.2.1 Calibration

Calibration graphs using aqueous standards were linear over the

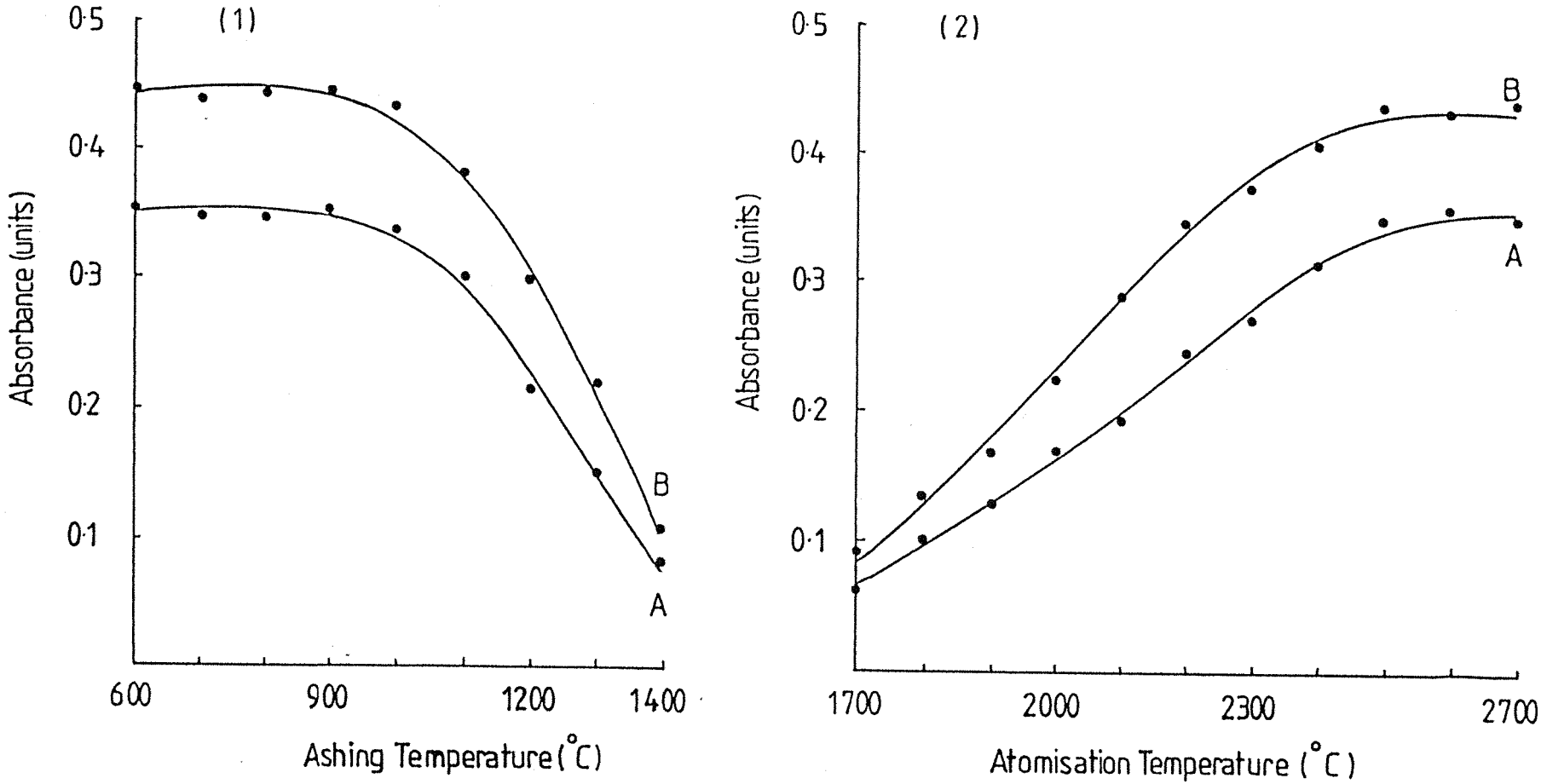


Figure 5.1

Effect of ashing temperature (1) and atomisation temperature (2) on the absorbance signal obtained during the analysis of a 20 µl digest sample containing 0.40 µmol l^{-1} of copper (A) and a 20 µl aqueous standard sample containing 0.60 µmol l^{-1} of copper (B)

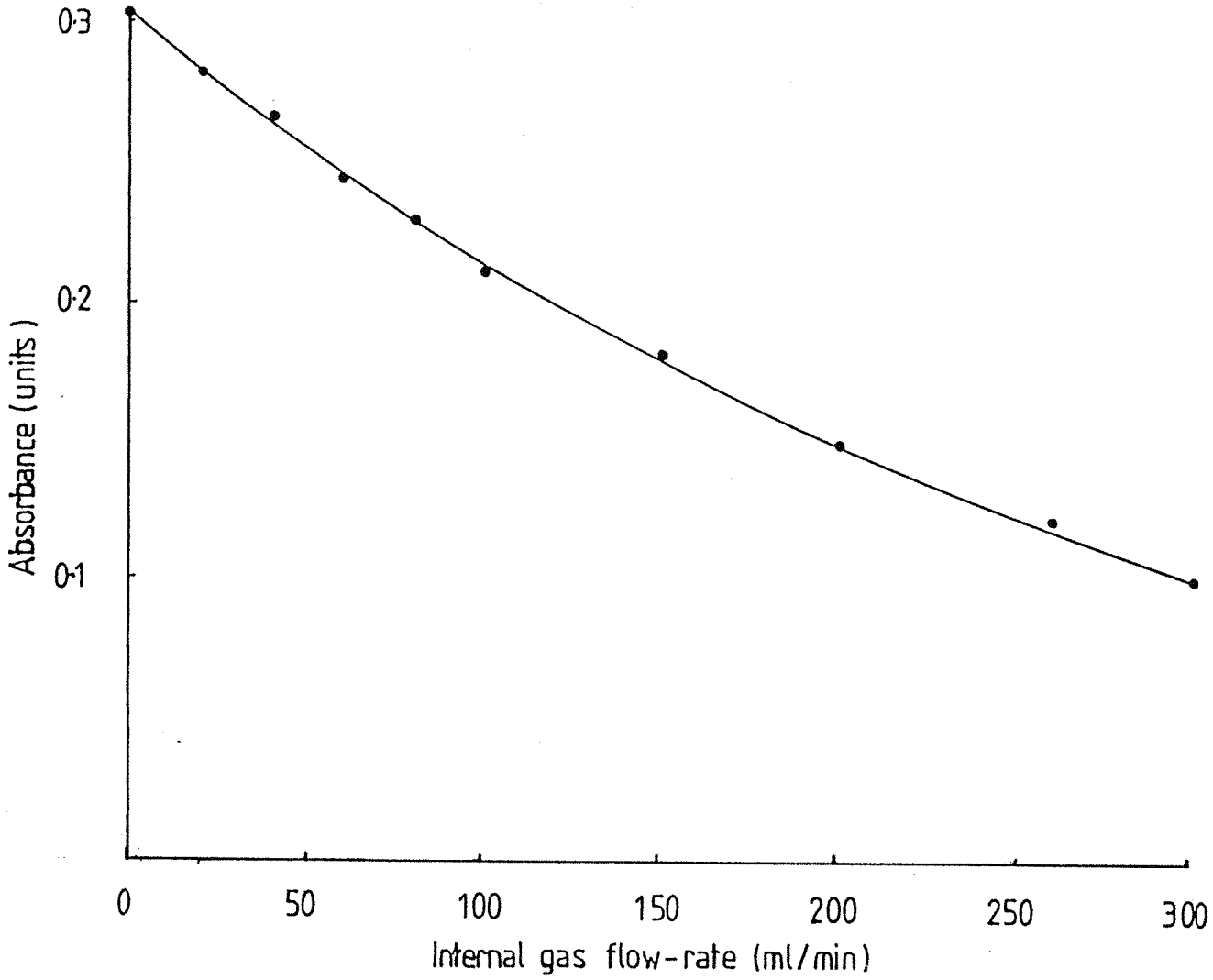


Figure 5.2

Effect of the internal gas flow-rate of argon on the absorbance signal obtained during the analysis of a 20 µl digest sample containing 0.34 µmol l^{-1} of copper

operating concentration ranges. The effect of matrix interference on analyte sensitivity was investigated for zinc, copper and selenium.

Zinc

A 45ml blood sample was withdrawn from each of 6 healthy subjects, and was divided into three aliquots of 15ml which underwent the separation process. The three digest samples obtained were combined to give the required volume (5.4ml) for the investigation. A series of working zinc standard solutions was prepared containing 0, 10, 20, 30, 40, and 50 µmol l^{-1} of zinc in 5% V/V nitric acid. 50µl portions of each working standard solution were added to 450µl of 5% V/V nitric acid (a) and to 450µl of each of the 6 digest solutions (b,c,d,e,f,g). Zinc determinations were carried out on duplicate dilutions as previously described.

Copper

A 15ml blood sample was withdrawn from each of 6 healthy subjects. Each blood sample underwent the separation process and 6 separate digest solutions were obtained. A series of working standard solutions was prepared containing 0,2,4,6 and 8 µmol l^{-1} of copper in 5% V/V nitric acid. 20µl portions of each working copper standard solution were added to 180µl of 5% V/V nitric acid (a) and to 180µl of each of the 6 digest solutions (b,c,d,e,f,g). Copper determinations were carried out on duplicate dilutions as previously described.

Selenium

Six individual digest samples were obtained in the same way as previously described for zinc. A series of working selenium standard solutions was prepared containing 0, 0.13, 0.25, 0.38, 0.51 and 0.63 µmol l^{-1} of selenium (0,10,20,30,40 and 50 µgl^{-1} respectively) in 5% V/V nitric acid. 50µl portions of each working standard solution were added to 450µl of 5% V/V nitric acid (a) and to 450µl of each of the 6 digest solutions (b,c,d,e,f,g). Duplicate dilutions were performed. The samples then underwent sample oxidation and subsequent determination of selenium as previously described.

For zinc, copper and selenium, the calibration graphs obtained by standard additions to the leucocyte digest samples were parallel with

those obtained from the aqueous standards (Figures 5.3, 5.4 and 5.5 respectively), indicating the absence of any significant matrix effect upon analyte sensitivity.

5.2.3.2.2 Precision

The within- and between-batch precisions of analysis for zinc, copper and selenium in leucocyte digest samples are shown in Tables 5.III, 5.IV and 5.V respectively. The between-batch coefficients of variation were obtained from determinations on 10 different days over a period of 3 months. Within- and between-batch coefficients of variation were 3.5 and 3.8% for concentrations of zinc of 2.3 and 4.0 $\mu mol\ l^{-1}$ respectively in the digest samples (67 and 124 pmol per 10^6 cells respectively), 2.3 and 4.6% for concentrations of copper of 0.47 and 0.39 $\mu mol\ l^{-1}$ respectively in the digest samples (14.2 and 11.9 pmol per 10^6 cells respectively) and 4.3 and 5.9% for concentrations of selenium of 29 and 41 $nmol\ l^{-1}$ respectively in the digest samples (1.3 and 1.9 pmol per 10^6 cells respectively).

5.2.3.2.3 Detection limit

The limits of detection given as three times the standard deviation of ten replicate blank determinations were 0.14 $\mu mol\ l^{-1}$ for zinc, 0.006 $\mu mol\ l^{-1}$ for copper and 1.85 $nmol\ l^{-1}$ (0.15 $\mu g\ l^{-1}$) for selenium. These were equivalent to concentrations of 5.8 pmol of zinc, 0.25 pmol of copper and 0.080 pmol (6.3pg) of selenium per 10^6 white cells for a leucocyte preparation containing $24 \times 10^9\ l^{-1}$ white cells.

5.2.3.3 Validation of ashing procedure used for digestion of selenium in leucocytes

The sample oxidation procedure described in the present chapter for digestion of selenium in leucocytes was performed as proposed by Lloyd et al (1982). This group reported that the use of nitric acid alone for oxidation of selenium in plasma and red cells was unsatisfactory as excessive frothing of the reaction mixture occurred during the addition of sodium tetrahydroborate (111) solution. In the same study a mixture of nitric and sulphuric acids gave a more

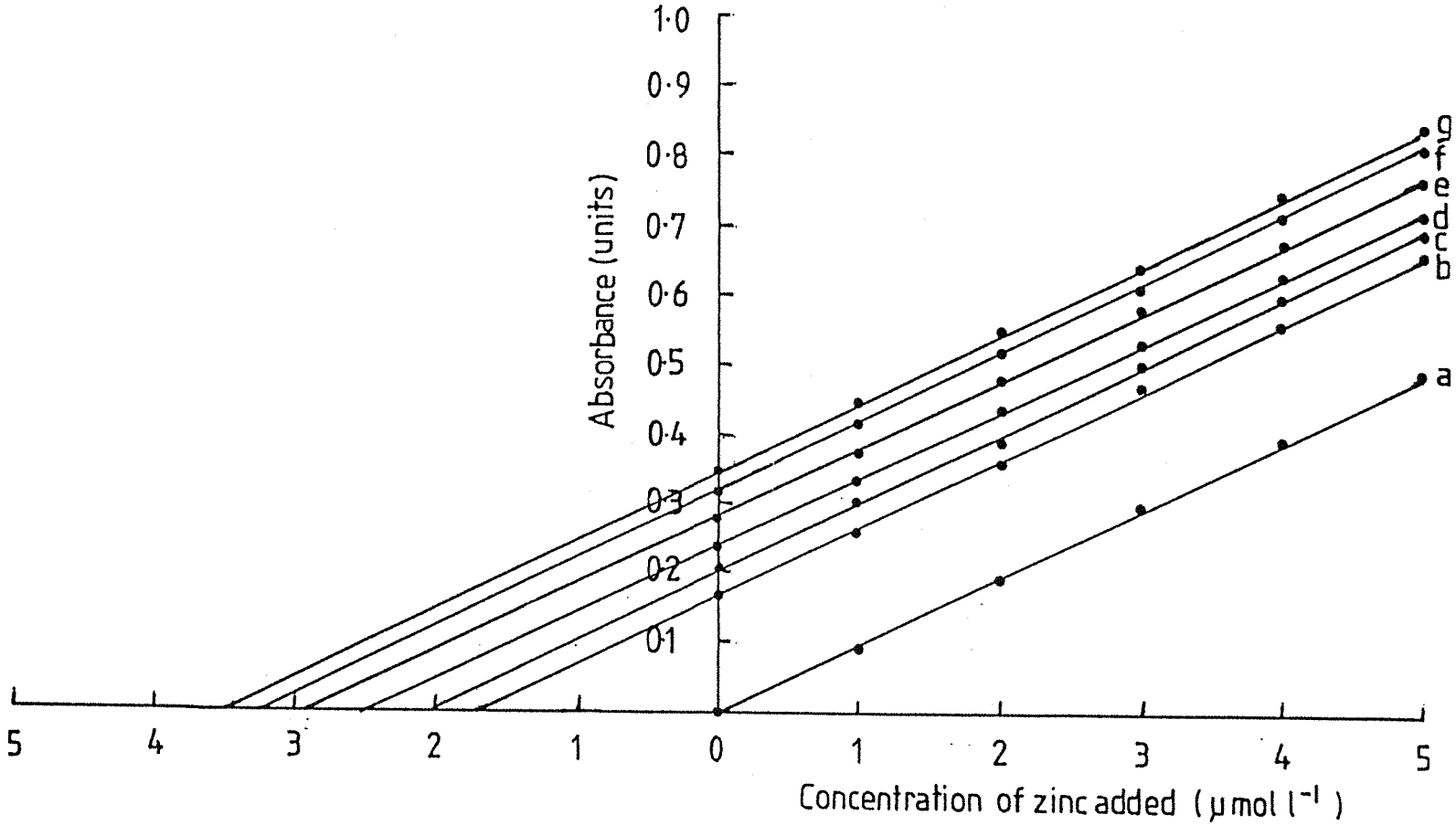


Figure 5.3

Calibration graphs for zinc. Aqueous standard solutions (a) obtained by dilution of 50 μl of working zinc standard solutions containing 0, 10, 20, 30, 40, and 50 μmol l^{-1} of zinc with 450 μl of 5% V/V nitric acid. Standard additions to leucocyte digest samples (b,c,d,e,f,g) obtained by dilution of 50 μl of working zinc standard + 450 μl leucocyte digest

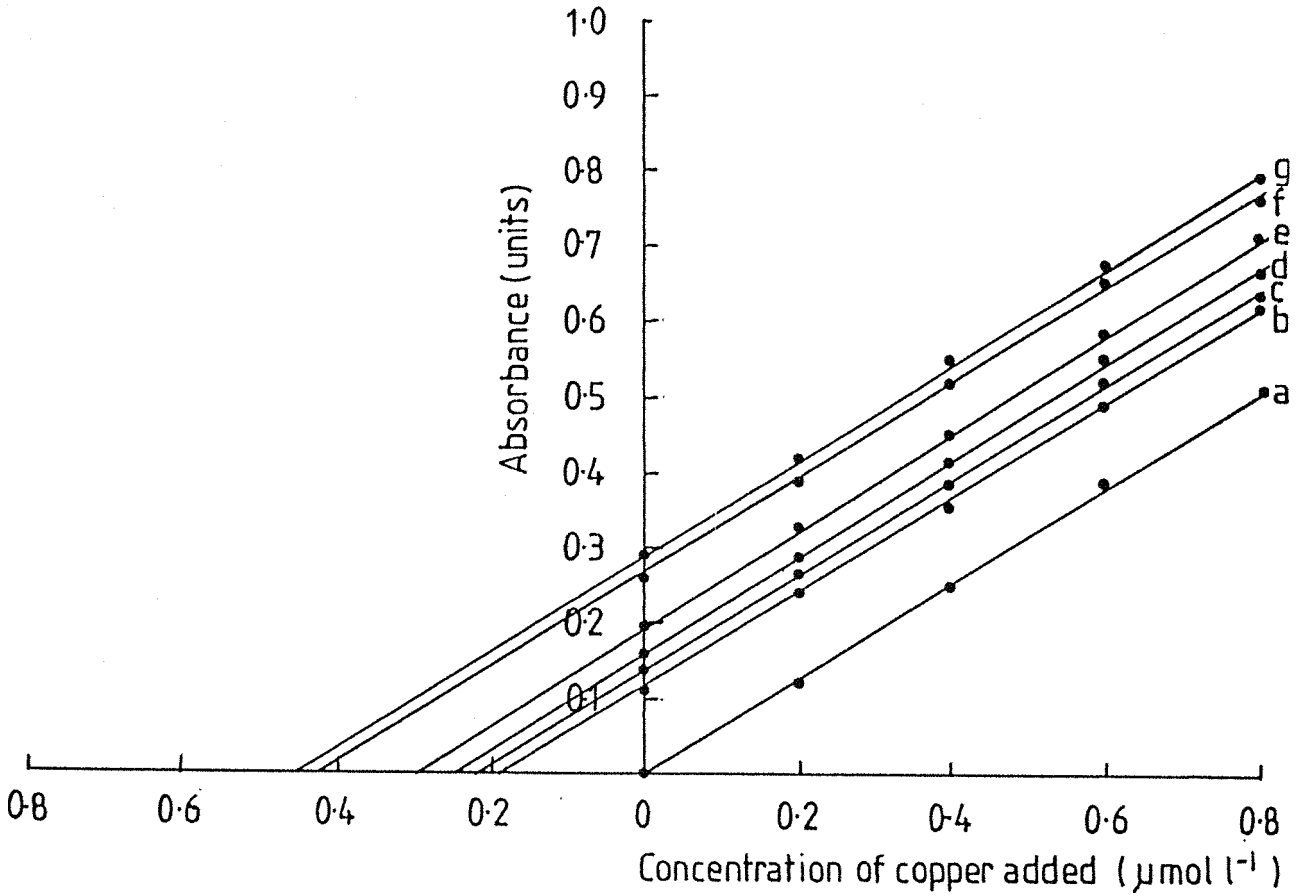


Figure 5.4

Calibration graphs for copper. Aqueous standard solutions (a) obtained by dilution of 20 μl of working copper standard solutions containing 0, 2, 4, 6, 8 μmol l^{-1} of copper with 180 μl of 5% V/V nitric acid. Standard additions to leucocyte digest samples (b,c,d,e,f,g) obtained by dilution of 20 μl of working copper standard +180 μl leucocyte digest

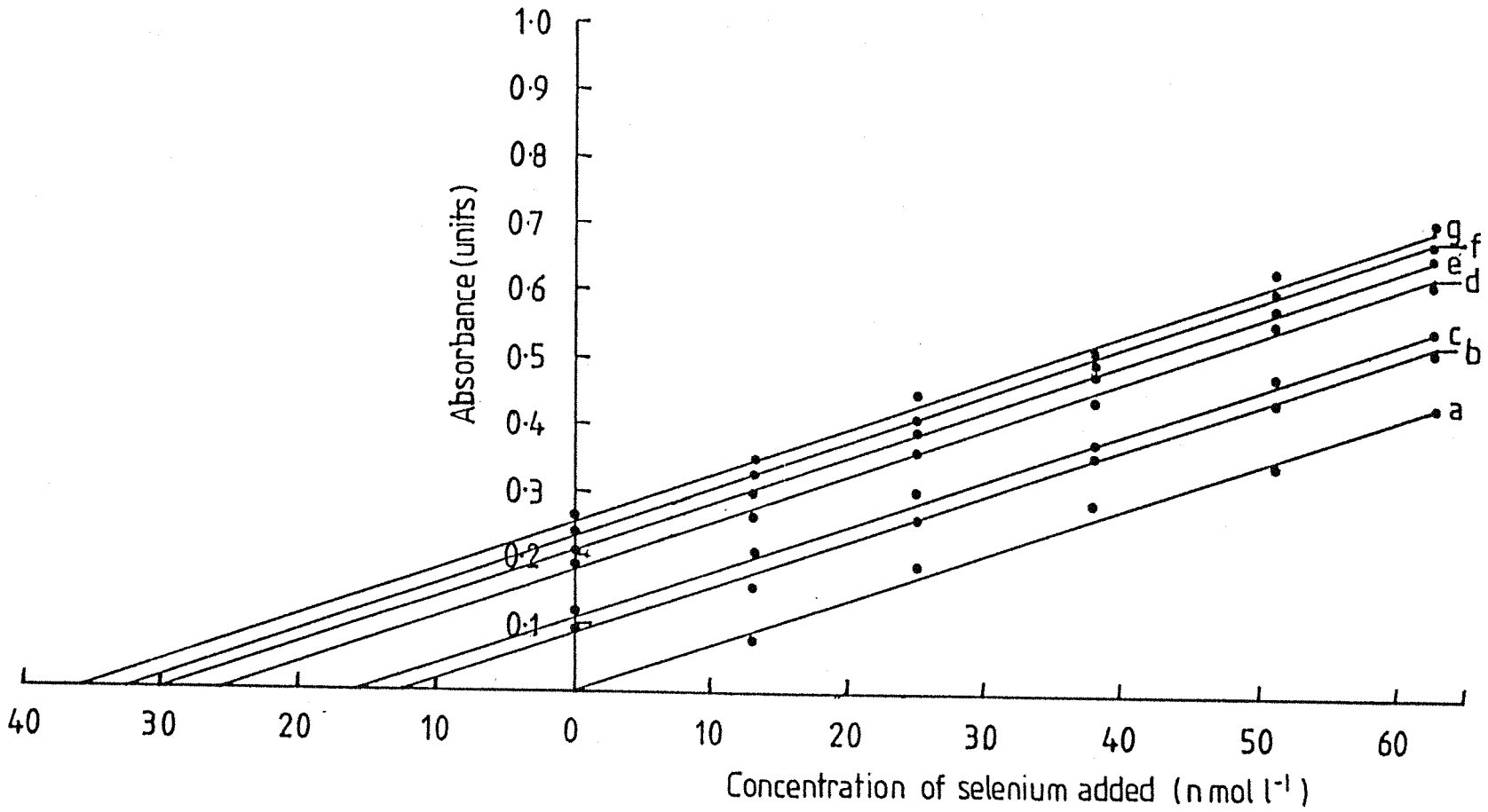


Figure 5.5

Calibration graphs for selenium. Aqueous standard solutions (a) obtained by dilution of 50 µl of working selenium standard solutions containing 0, 0.13, 0.25, 0.38, 0.51 and 0.63 µmol l^{-1} of selenium with 450 µl of 5% V/V nitric acid. Standard additions to leucocyte digest samples (b,c,d,e,f,g) obtained by dilution of 50 µl of working selenium standard + 450 µl leucocyte digest

TABLE 5.III

PRECISION OF ZINC DETERMINATIONS OF LEUCOCYTE DIGEST SAMPLES

Parameter	Within-batch	Between-batch
Number of analyses	10	10
Mean concentration of Zn μmol^{-1}	2.3	4.0
Range $\mu mol\ l^{-1}$	2.2-2.4	3.8-4.2
Standard deviation $\mu mol\ l^{-1}$	0.08	0.15
Coefficient of variation %	3.5	3.8

TABLE 5.IV

PRECISION OF COPPER DETERMINATIONS OF LEUCOCYTE DIGEST SAMPLES

Parameter	Within-batch	Between-batch
Number of analyses	10	10
Mean concentration of Cu μmol^{-1}	0.47	0.39
Range $\mu mol\ l^{-1}$	0.45-0.48	0.36-0.41
Standard deviation $\mu mol\ l^{-1}$	0.011	0.018
Coefficient of variation %	2.3	4.6

TABLE 5.V

PRECISION OF SELENIUM DETERMINATIONS OF LEUCOCYTE DIGEST SAMPLES

Parameter	Within-batch	Between-batch
Number of analyses	10	10
Mean concentration of Se $nmol\ l^{-1}$*	29	41
Range $nmol\ l^{-1}$*	28-32	38-44
Standard deviation $nmol\ l^{-1}$*	1.27	2.40
Coefficient of variation %	4.3	5.9

* Concentrations of selenium converted to $nmol^{-1}$ from $\mu g\ l^{-1}$ after calculation of precision values.

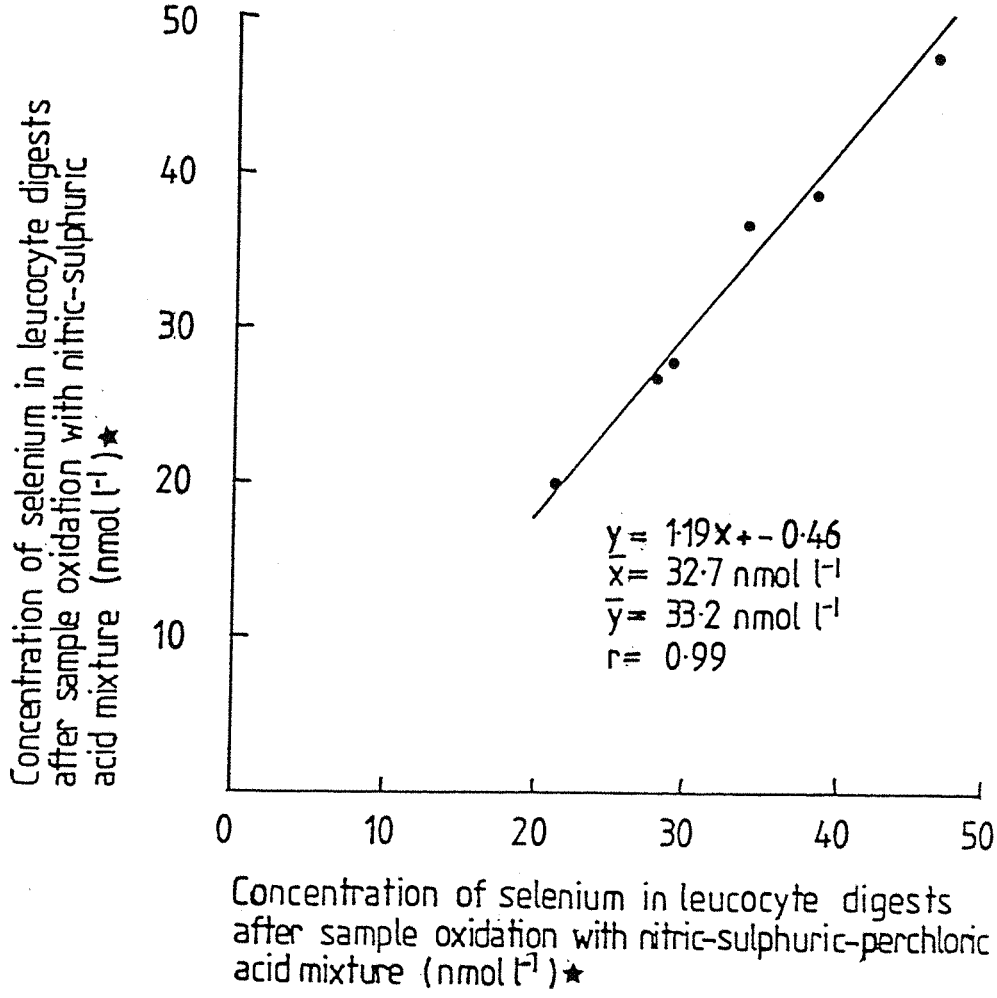
efficent oxidation of the samples and minimised frothing of the samples during the reduction stage. In the present study therefore, the leucocyte digest samples which had been digested with nitric acid alone underwent a further ashing procedure with a nitric-sulphuric acid mixture as described. All of the oxidised samples were heated with 6M hydrochloric acid prior to analysis in order to reduce any selenium (VI) present to selenium (IV).

In order to validate this ashing procedure, it was decided to compare values obtained using the present method with those obtained using a perchloric acid digestion stage. At the time this comparison was undertaken, it was possible to obtain 6 separate pooled samples of the required volume (4ml) by combining digest samples that were remaining from a previous study. The exact procedure was as follows: a volume (1.5ml) of each leucocyte digest sample was placed into Tecator Kjeldahl digestion flasks and 5ml of 16M nitric acid added. The flasks were placed into a Tecator digestion block fitted with an exhaust system. The temperature was raised over a period of 15min to 140^{o}C and held at this level for 25 min. After allowing the flasks to cool, 2.5ml of 18M sulphuric acid and 1ml of 11.6M perchloric acid was added. The flasks were returned to the digestion block and the temperature raised to 140^{o}C over a period of 15 min and held at this level for 15 min. The temperature was further increased to 205^{o}C over a period of 15 min and kept at this level until the contents of the flasks had reduced in volume to approximately 3.5ml. After allowing the flasks to cool, 30ml of 5M hydrochloric acid was added and the flasks were heated at 95^{o}C for 30 min. After the flask contents had cooled, they were diluted to 60ml, and 20ml aliquots were taken for selenium analysis. Duplicate determinations by both ashing procedures were performed.

An excellent agreement ($r = 0.99$) between the two sample oxidation procedures was obtained (Figure 5.6) thereby validating the procedure used in the present study. As the samples were pooled, it was not possible to present the data as concentration of selenium per number of white cells.

5.2.3.4 Reproducibility of the procedures used for measurement of zinc, copper and selenium in leucocytes

To assess the overall precision of isolation and digestion of the



★ Concentrations of selenium converted to nmol l^{-1} from $\mu g l^{-1}$ after calculation of regression values

Figure 5.6

Comparison of selenium values obtained using a sample oxidation procedure involving a perchloric acid digestion stage, and the method described in this chapter

white cells and analysis of the digest sample for zinc, copper and selenium, a 45ml blood sample was taken from each of 2 healthy subjects (A,B) and the samples were divided into 3 aliquots of 15ml (1,2,3). Each aliquot was subjected to leucocyte separation and to the determination of zinc, copper and selenium in the white cells as previously described. The results (Table 5.VI) show that the coefficient of variation of the whole method was within the range of 3.6 - 4.2% for zinc, 3.4 - 5.1% for copper and 5.9 - 6.8% for selenium.

5.2.3.5 Evaluation of the method used for determination of copper in erythrocytes and whole blood

Calibration

The effect of matrix interference on analyte sensitivity was investigated. Blood samples were obtained from 5 healthy subjects and processed as previously described. Duplicate 100µl aliquots of whole blood and erythrocyte suspensions were diluted with 100µl volumes each of the working standard solutions containing 0,10,20,30 and 0,5,10,15 µmol l^{-1} copper respectively, and 100µl volumes of Triton X-100 solution. Aqueous standard solutions were prepared by diluting duplicate 100µl volumes of each of the series of working standard solutions with 100µl volumes of water and 100µl volumes of Triton X-100 solution.

The calibration graphs obtained by standard additions to the whole blood samples were parallel with each other, but not with the calibration graph obtained from the aqueous standards. (Figure 5.7). Similar findings were observed for the calibration graphs obtained by standard additions to the erythrocyte suspension samples and from the aqueous standard solutions (Figure 5.8). All calibration graphs were linear over the operating concentration ranges.

Precision

The within- and between-batch precisions of analyses for copper in whole blood and erythrocyte suspension samples are shown in Tables 5.VII and 5.VIII respectively. The between-batch coefficients of variation were obtained from determinations on 10 different days over a period of 3 months. Within- and between-batch coefficients of variation were 2.6 and 4.1% for concentrations of copper of 12.6 and

TABLE 5.VI

REPRODUCIBILITY OF THE PROCEDURES USED FOR MEASUREMENT OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES

Subject	Aliquot	Concentration per 10^6 cells		
		Zinc pmol	Copper pmol	Selenium pmol*
A	1	120	20.8	1.70
A	2	129	21.7	1.83
A	3	124	20.3	1.63
Mean		124	20.9	1.72
Coefficient of variation %		3.6	3.4	5.9
B	1	91	14.2	1.18
B	2	99	15.2	1.25
B	3	96	15.7	1.35
Mean		95	15.0	1.26
Coefficient of variation %		4.2	5.1	6.8

* Concentrations of selenium converted to pmol from pg after calculation of precision values.

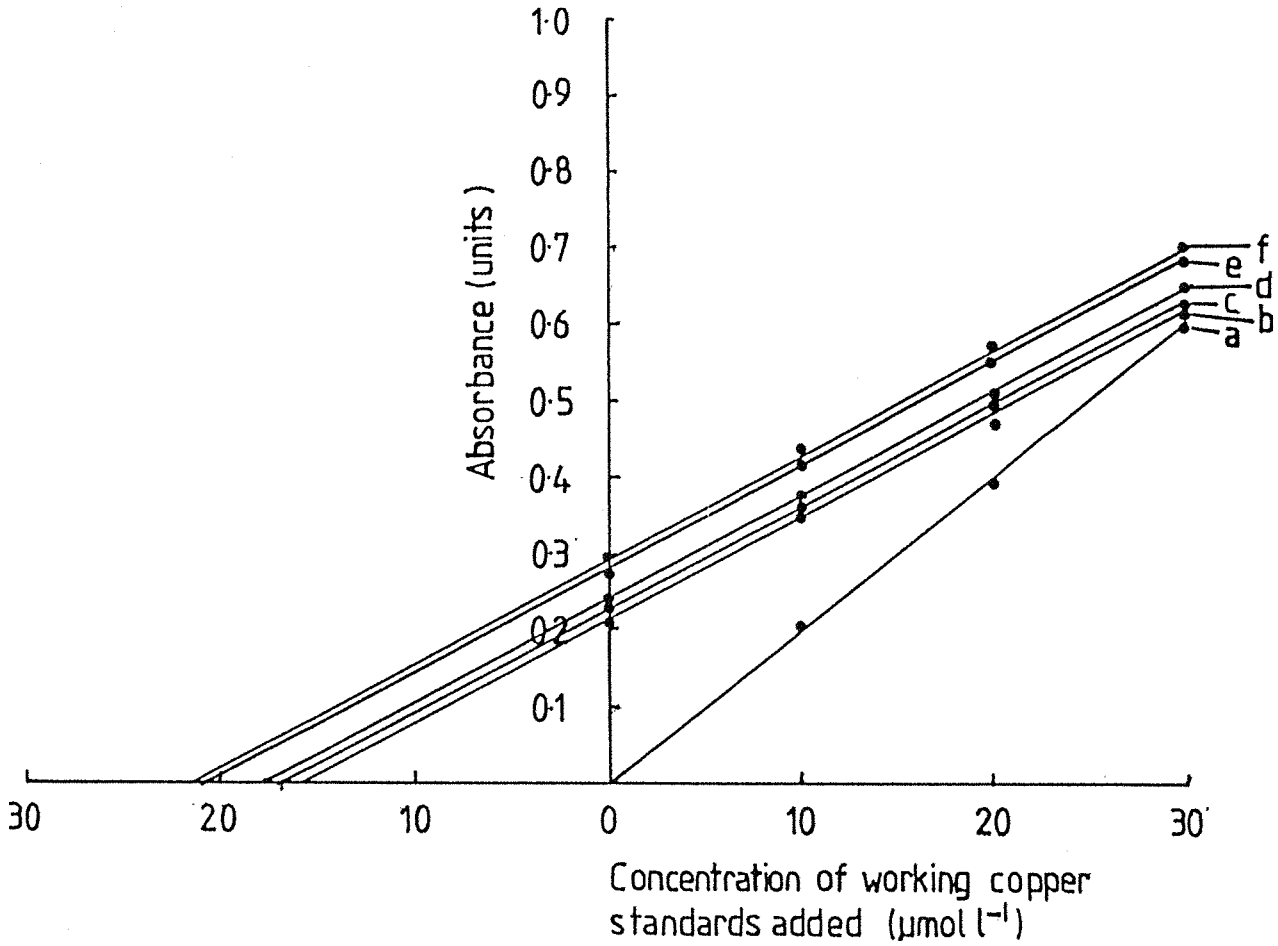


Figure 5.7

Calibration graphs for copper. Aqueous standard solutions (a) obtained by dilution of 100 µl of working copper standard solutions containing 0, 10, 20, and 30 µmol l^{-1} of copper +100 µl water +100 µl Triton X-100. Standard additions to whole blood samples (b,c,d,e,f) obtained by dilution of 100 µl working copper standard +100 µl whole blood +100 µl Triton X-100

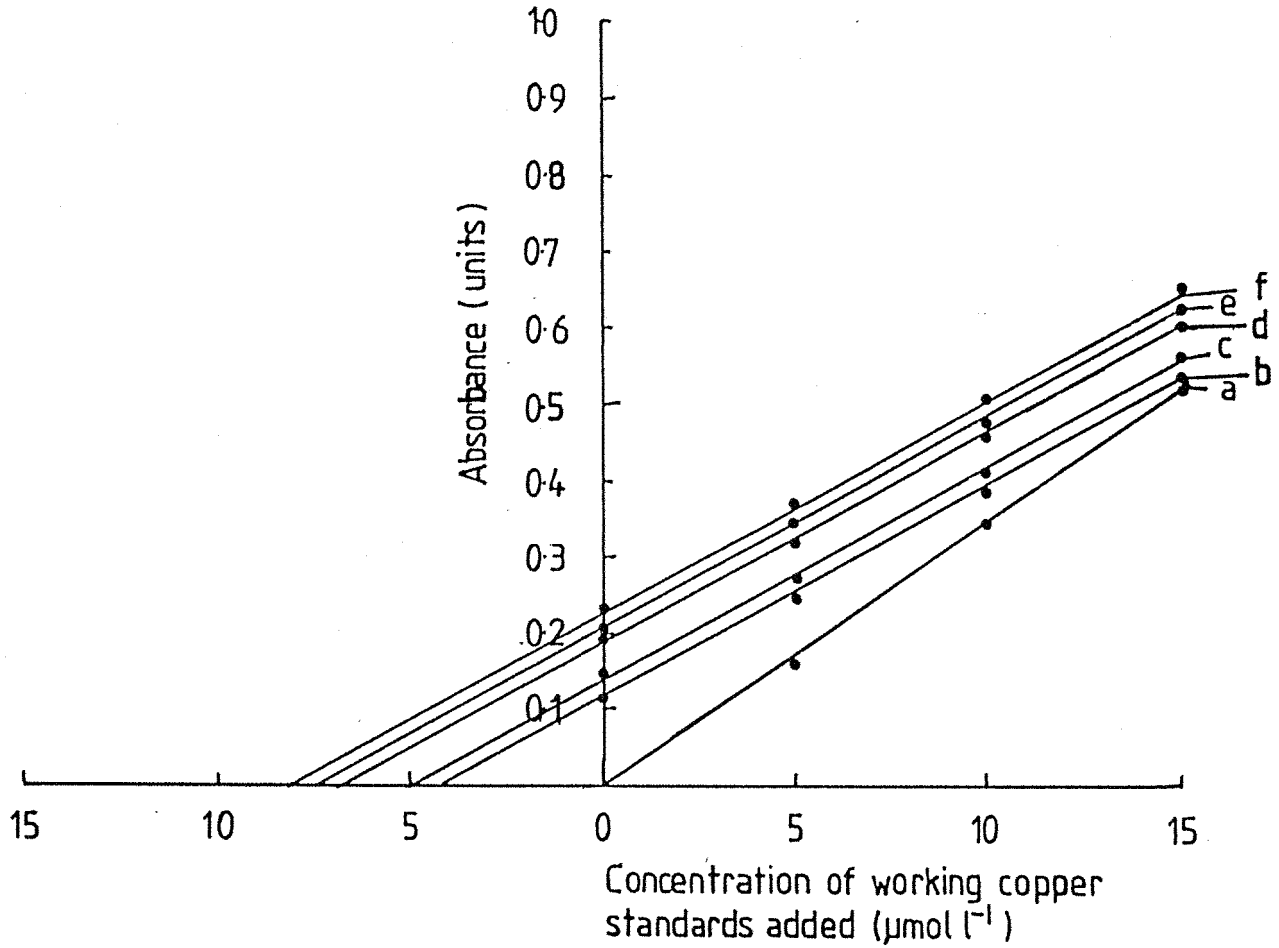


Figure 5.8

Calibration graphs for copper. Aqueous standard solutions (a) obtained by dilution of 100 μl of working copper standard solutions containing 0, 5, 10 and 15 μmol l^{-1} of copper + 100μl water +100 μl Triton X-100. Standard additions to erythrocyte suspensions (b,c,d,e,f) obtained by dilution of 100 μl working copper standard +100 μl erythrocyte suspension +100 μl Triton X-100

TABLE 5.VII

PRECISION OF COPPER DETERMINATIONS OF WHOLE BLOOD SAMPLES

Parameter	Within-batch	Between-batch
Number of analyses	10	10
Mean concentration of Cu μmol l^{-1}	12.6	11.3
Range μmol l^{-1}	12.2-13.0	10.8-11.9
Standard deviation μmol l^{-1}	0.33	0.46
Coefficient of variation %	2.6	4.1

TABLE 5.VIII

PRECISION OF COPPER DETERMINATIONS OF ERYTHROCYTE SUSPENSION SAMPLES

Parameter	Within-batch	Between-batch
Number of anlayses	10	10
Mean concentration of Cu μmol l^{-1}	5.1	4.6
Range μmol l^{-1}	4.8-5.3	4.1-5.0
Standard deviation μmol l^{-1}	0.18	0.27
Coefficient of variation %	3.5	5.9

11.3 μmol l^{-1} respectively in whole blood samples, and 3.5 and 5.9% for concentrations of copper of 5.1 and 4.6 μmol l^{-1} in the erythrocyte suspension samples (1.15 and 1.26 pmol per 10^6 cells respectively).

Detection limit

The limits of detection given as three times the standard deviation of ten replicate blank determinations were 0.96 and 0.51 μmol l^{-1} of copper for whole blood and erythrocytes respectively. This latter value was equivalent to a concentration of copper of 0.11 pmol per 10^6 red cells for an erythrocyte suspension containing 4.7×10^{12} l^{-1} red cells.

5.3 ESTABLISHED METHODOLOGY USED FOR TRACE ELEMENT ANALYSIS IN THE PRESENT STUDY

Unless otherwise stated, reagents were Analar or Aristar grade materials (BDH Chemicals Ltd.). All glassware was cleaned as previously described. De-ionised distilled water was used throughout. The tubes for blood collection were as described in the previous sections.

5.3.1 DETERMINATION OF ZINC AND COPPER IN PLASMA

The method used was based on that described by Meret and Henkin (1971). Plasma samples were obtained from heparinised blood. Determination of zinc and copper was carried out using flame-atomic absorption spectrophotometry.

Reagents

Zinc chloride standard solution containing 5mmol l^{-1} of zinc
Copper (11) chloride standard solution containing 5mmol l^{-1} of copper
Butan -1-ol, 6% V/V aqueous solution
Hydrochloric acid, 12M

Procedure

A combined zinc and copper stock standard solution containing 200 μmol l^{-1} of zinc and copper in 0.5M (5% V/V) hydrochloric acid was

prepared from the BDH Chemicals concentrated standard solutions. The stock standard solution was then diluted to give a series of working standard solutions containing 0,5,10,20,30 and 40 μmol l^{-1} of zinc and copper in 0.05M (0.5% V/V) hydrochloric acid.

Duplicate 200μl volumes of each plasma sample were diluted with 1.8ml of butan-1-ol diluent and mixed. A calibration graph was obtained by diluting each of the working standard solutions in exactly the same way as the samples.

Standards and samples were directly aspirated into the flame. Internal quality control samples were included in each run.

Precision

The between-batch coefficients of variation were 3.1% for plasma zinc analysis at a concentration of 12.9 μmol l^{-1} of zinc, and 3.9% for plasma copper analysis at a concentration of 16.2 μmol l^{-1} of copper.

5.3.2 DETERMINATION OF ZINC IN ERYTHROCYTES AND WHOLE BLOOD

The concentration of zinc was measured in erythrocytes and whole blood using a direct dilution procedure and flame-atomic absorption spectrophotometry (Method obtained from H.T. Delves).

Reagents

Zinc chloride standard solution containing 5mmol l^{-1} of zinc

Butan-1-ol, 6% V/V aqueous solution

Procedure

The BDH Chemicals concentrated zinc standard solution was diluted with water to give working standard solutions containing 0,50,100 and 150 μmol l^{-1} of zinc.

Collection of blood, separation and counting of erythrocytes were performed as described in the previous section. A stock sample of whole blood and a stock suspension of red blood cells were also obtained as previously described. Before zinc analysis the samples were frozen at -20°C and then thawed.

A calibration graph was prepared using 40μl volumes of each of the working standard solutions + 40μl of the stock whole blood sample + 1ml of butan-1-ol to give a 1 + 26 dilution. 40μl aliquots of whole

blood were added to 40µl of water and 1ml of butan-1-ol. Samples and standards were then directly aspirated into the flame.

The zinc concentrations of red blood cell suspensions were similarly determined, except that the stock red blood cell suspension was used for preparing the calibration graph.

Internal quality control samples were run with each batch of test samples.

Precision

The method gave a between-batch coefficient of variation of 2.1% and 2.6% at a concentration of zinc of 114 µmol l^{-1} in whole blood and 86 µmol l^{-1} (18.4 pmol per 10^6 cells) in erythrocyte suspension samples respectively.

5.3.3 DETERMINATION OF SELENIUM IN PLASMA AND WHOLE BLOOD

Hydride generation followed by atomic absorption spectrophotometry was used to determine the concentration of selenium in 100µl volumes of plasma and whole blood following acid digestion of the samples (Lloyd et al, 1982).

Instrumentation

A Perkin-Elmer MHS 20 hydride generation system was used for hydride generation. Instrumental settings for this were: tube temperature 900°C, purge 1 (argon) 42s, purge 11 (argon) 30s, reaction time 10s, and reaction volume 20ml.

Reagents

Selenous acid standard solution containing 12.7 µmol l^{-1} (1mg ml^{-1}) of selenium
Hydrochloric acid, 6M
Sulphuric acid, 4.5M
Nitric acid, 16M
Sodium hydroxide solution, 0.25M
Sodium tetrahydroborate (111), 6% m/V solution in 0.25M sodium hydroxide solution
Antifoam emulsion DB 110A (Dow Corning), 1% V/V aqueous solution

Procedure

The blood collection tubes were the same as those described in previous sections. Plasma samples obtained from heparinised blood, and whole blood samples containing EDTA were used for analysis of selenium.

Duplicate 100µl portions of plasma and whole blood were transferred into borosilicate glass tubes. After addition of 1ml of 4.5M sulphuric acid and 1ml of 16M nitric acid, the tubes were heated in aluminium blocks (Techne Dri-block heater, model DB 3H) at 155°C for 60 min. The tubes were then allowed to cool to room temperature. After addition of 2ml of 6M hydrochloric acid, the tubes were heated at 95°C for 30 min and then allowed to cool. The contents were transferred quantitatively into the reaction vessels and diluted to a final volume of 20ml with water. 100µl aliquots of the antifoam solution were finally added.

A stock standard solution containing 127 µmol l^{-1} (10 mg ml^{-1}) of selenium in 0.16M (1% V/V) nitric acid was prepared from the BDH Chemicals concentrated standard solution. This was diluted to give a series of working standard solutions containing 0, 0.6, 1.3, 1.9, 2.5 and 3.2 µmol l^{-1} (0,50,100,150,200 and 250 µg l^{-1} respectively) of selenium in 0.16M (1% V/V) nitric acid.

A calibration graph was established by adding duplicate 100µl volumes of the selenium working standard solutions to reaction vessels containing 1ml of 16M nitric acid, 1ml of 4.5M sulphuric acid and 2ml of 6M hydrochloric acid. These were diluted to a final volume of 20ml with water and 100µl portions of antifoam solution were added.

Samples were analysed in duplicate and internal quality control samples were included in each run. The selenium concentrations were calculated from the integrated absorbance values (integrated peak area 20s) and the calibration graphs.

Precision

The between-batch coefficient of variation was 4.9% for both plasma and whole blood analysis at concentrations of selenium of 1.4 µmol l^{-1} (112µg l^{-1}) and 1.7 µmol l^{-1} (133µg l^{-1}) respectively.

5.3.4 DETERMINATION OF SELENIUM IN FLUIDS USED FOR TOTAL PARENTERAL NUTRITION (TPN)

Samples were digested with a nitric:sulphuric acid mixture, prior to selenium determination, as described by Bunker and Delves (1987). These investigators demonstrated that a mixture of nitric and sulphuric acids was suitable for the digestion of composite diet, faecal and urine samples and standard reference materials prepared from bovine liver, rice flour, wheat flour and horse kidney. Selenium was measured in the digested samples by the method described in section 5.3.3.

Digestion procedure

Duplicate 5g amounts of the TPN fluids were weighed into 50ml glass conical flasks and 15ml of a concentrated nitric acid (16M): sulphuric acid (18M) mixture (1:1) added. When the initial reaction subsided, samples were gently mixed and left for approximately 20 hours to predigest. After mixing thoroughly, triplicate aliquots (1ml) of the chemical homogenate were transferred to borosilicate glass tubes and 2ml of the acid mixture added. The tubes were heated in a dry block at 150^{o}C for three hours, cooled and 2ml of 6M HCl added. The tubes were heated at 95^{o}C for 30 min, cooled and the contents transferred to reaction vessels and diluted to 20ml for subsequent analysis of selenium. A calibration graph was prepared by transferring 100µl volumes of the selenium working standard solutions described in section 5.3.3 into test tubes and adding 3ml of the nitric:sulphuric acid mixture. Thereafter the tubes were treated as for the sample ashing procedure described above.

Precision

The within-batch coefficient of variation was 2.6%.

5.4 STATISTICAL PROCEDURES USED IN THIS THESIS

The scatter of results obtained from the healthy men and women volunteers (Chapter 6) was visually examined to assess their closeness to a Normal distribution. Data that demonstrated a skewed distribution were subjected to logarithmic transformation to achieve approximate Normality.

Comparison of more than two samples of data was performed by a two-way analysis of variance test. Data sets consisting of two samples were compared using Student's paired or unpaired t-tests, or by the Mann-Whitney U-test or the Wilcoxon matched-pairs signed-ranks test in instances where the data was not regarded as having come from an approximately Normal distribution. The association between variables was assessed by calculation of Pearson's product-moment correlation coefficient (r), or Spearman's rank correlation coefficient (r_s) in situations where either of the variables was not drawn from an approximately Normal distribution. Multiple regression analysis was used to assess relationships between more than two variables. Regression lines were obtained by means of linear regression analysis.

Data are given in this thesis as arithmetic mean ± 1 standard deviation unless otherwise stated. The log transformed means were used to calculate the geometric means, and the interquartile distance is also given with this value. In these instances, the arithmetic mean ± 1 standard deviation is also given, where appropriate, to facilitate comparison with other published work.

Where the means of two sets of data are shown, for comparative purposes, 95% confidence intervals (CI) for the differences between arithmetic means, or for the ratio of geometric means are given. In instances of matched data comparisons, when data was regarded as having come from an approximately Normal distribution, 95% confidence intervals for the mean of the individual subject-subject differences are shown.

5.4.1 METHODS OF CALCULATING CONFIDENCE INTERVALS (CI)

5.4.1.1 Unpaired Case

5.4.1.1.1 Normal data

In the following example, $\bar{x}_1$ and $\bar{x}_2$ are the two sample means, s_1 and s_2 the corresponding standard deviations, and n_1 and n_2 the sample sizes.

Firstly, a 'pooled' estimate of the standard deviation is obtained by the following calculation:

$$s = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}$$

From this, the standard error of the difference between two sample means is:

$$SE_{diff} = S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}$$

The confidence interval is then:

$$\bar{x}_1 - \bar{x}_2 - (t^* \times SE_{diff}) \quad \text{to} \quad \bar{x}_1 - \bar{x}_2 + (t^* \times SE_{diff})$$

where t^* is the value from a t-table with $n_1 + n_2 - 2$ degrees of freedom, corresponding to 95% confidence.

Worked example

Plasma zinc levels were measured in 25 patients with malabsorption and 40 healthy control subjects. The plasma zinc levels were 9.0 μmol l^{-1} (SD 2.0) for the patient group and 13.0 μmol l^{-1} (SD 1.8) for the control subjects.

Using the formula given above the pooled estimate of the standard deviation is:

$$s = \sqrt{\frac{(24 \times 2.0^2) + (39 \times 1.8^2)}{63}} = 1.9 \text{ μmol } l^{-1}$$

and the standard error of the difference between sample means is

$$SE_{diff} = 1.9 \sqrt{\frac{1}{25} + \frac{1}{40}} = 0.5 \text{ µmol } l^{-1}$$

To calculate the 95% CI the appropriate value of t with 63 degrees of freedom is 2.0. Thus the 95% CI is given by:

$$-4.0 - (2.0 \times 0.5) \text{ to } -4.0 + (2.0 \times 0.5)$$

that is, from -5.0 to -3.0 µmol l^{-1}

5.4.1.1.2 Non-normal data

The above calculation is performed on data that has undergone logarithmic transformation. The confidence interval for the difference in means of the transformed data has to be transformed back. The anti-log of the difference in sample means on the transformed scale is an estimate of the ratio of the two population (geometric) means, and the anti-logged confidence interval for the difference gives a confidence interval for this ratio.

5.4.1.2 Paired Case

In the following example, $\bar{x}$ and SD are the mean and standard deviation of the individual subject-subject (patient-control) differences.

The standard error of the mean is determined by:

$$SE = SD/\sqrt{n} \quad , \quad \text{where } n = \text{sample size.}$$

The confidence interval is then:

$$\bar{x} - (t^* \times SE) \text{ to } \bar{x} + (t^* \times SE)$$

where t^* is the appropriate value from the t-table with $n - 1$ degrees of freedom.

CLINICAL STUDIES

CHAPTER 6

STUDIES ON HEALTHY HUMAN SUBJECTS UNDER THE AGE OF 65 YEARS

6.1 INTRODUCTION

Reports from the literature concerning the diurnal variation of plasma zinc levels and the possible effect of daily food intake are conflicting. These parameters are of relevance when establishing laboratory reference values. For practical purposes it is only necessary to assess any possible diurnal variation over a time period which would encompass the usual hours of blood sampling and which would also be associated with normal food intake. The diurnal variation of plasma concentrations of zinc, copper and selenium was therefore investigated in healthy volunteers, eating normally, between the hours of 0900 and 1700.

An investigation was also undertaken to obtain control data that would provide a baseline for the assessment of the body status of zinc, copper and selenium in various disease states in which deficiencies of these trace elements might be present. The effect of factors including age, sex and use of oral contraceptives on the parameters measured was determined.

The studies described in the present chapter and also in subsequent chapters were approved by the Joint Ethical Sub-Committee of the Faculty of Medicine of the University of Southampton and South West Hampshire Health Authority.

6.2 INVESTIGATION OF THE DIURNAL VARIATION OF PLASMA CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN HEALTHY ADULT VOLUNTEERS

6.2.1 SUBJECTS AND METHODS

Subjects

Nineteen normal healthy volunteers, 13 women and 6 men, aged 19-47 (mean 31.6) years, were studied.

The studies,samples and analyses

Samples of whole blood (2ml) were collected into trace-element-free heparinised tubes.

Two separate studies were undertaken.

In the first study, samples of blood were collected at 0900h (pre-breakfast), 1100h (post-breakfast), 1300h (pre-lunch), 1500h (post-lunch) and 1700h (pre-evening meal). All subjects consumed food immediately after samples were taken at 0900h and 1300h. Breakfast consisted of between 1 and 3 buttered wholemeal rolls filled with either cheese or marmalade. Lunch was varied; 15 subjects consumed packed meals which included sandwiches and the remaining 4 subjects ate cooked meals. All subjects drank beverages (no alcohol) at breakfast and lunch and also mid-morning and mid-afternoon. Snacks consisting of biscuits or fruit were eaten by 10 of the subjects between the hours of 1100 and 1300 and by 15 of the subjects between the hours of 1500 and 1700. Plasma was obtained from the whole blood samples and analysed for zinc, copper and selenium using the procedures described in Chapter 5.

In the second study, subjects remained in the fasting state throughout the period of sampling. Blood samples were taken at 0900h, 1000h, 1100h, 1200h and 1300h. Plasma samples obtained were analysed for zinc as previously described.

Statistical procedures

The statistical procedures used in this project are given in Chapter 5.

6.2.2 RESULTS

The data resulting from the analysis of plasma copper and selenium, obtained from the 19 subjects eating normally throughout the day, are given in full in Appendix 1; Appendix 2 shows the results obtained for plasma zinc from the 19 subjects eating normally, and also in the fasting state.

The plasma values obtained for zinc, copper and selenium from each of the 19 subjects, eating normally, between the hours of 0900 and 1700, are depicted in Figures 6.1, 6.2 and 6.3 respectively. The mean plasma zinc, copper and selenium levels observed at different

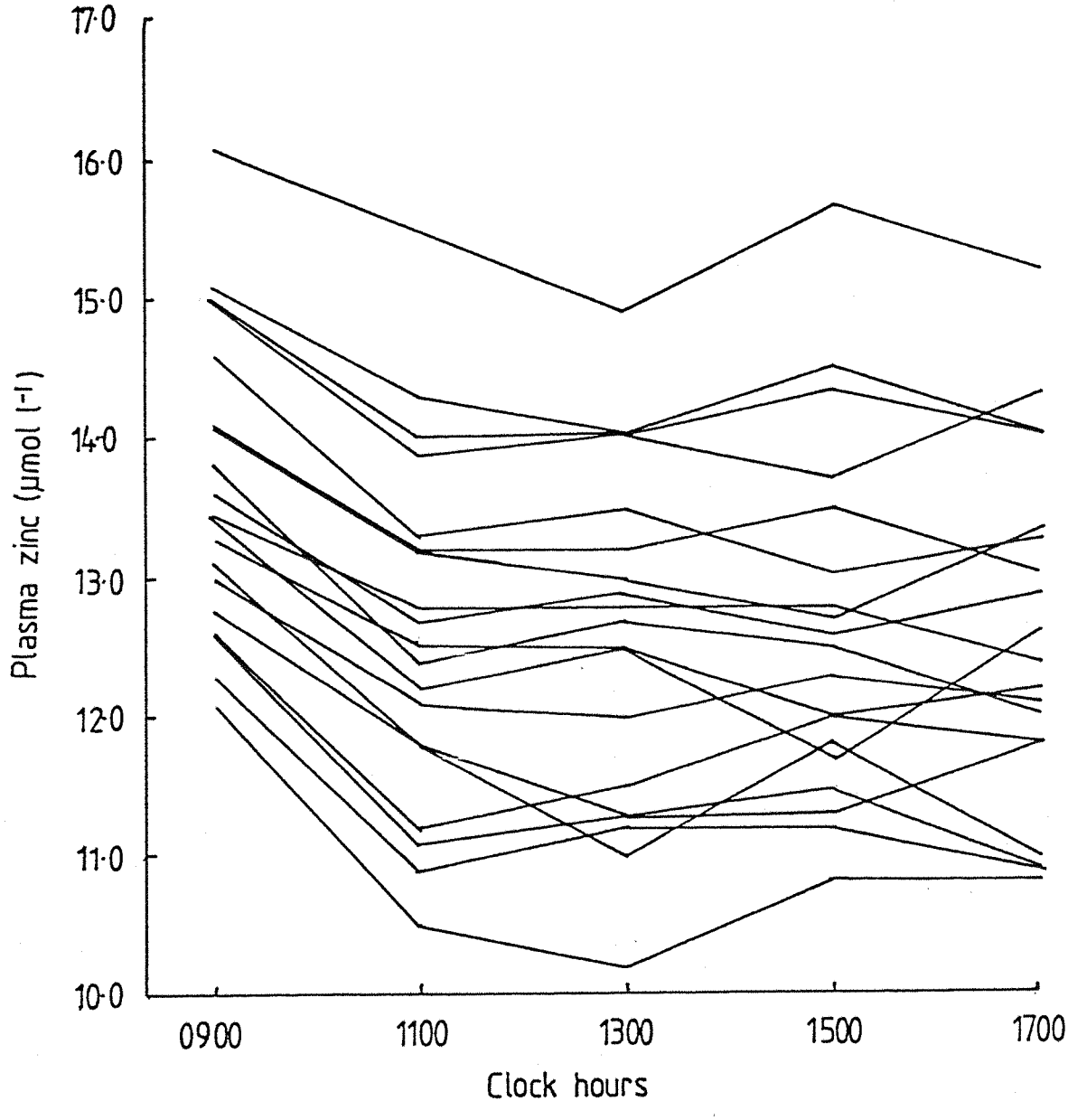


Figure 6.1

Concentration of zinc in plasma samples obtained during the day from healthy subjects eating normally. Subjects were in the fasting state at 0900h and ingested food immediately after blood samples were taken at 0900h and 1300h

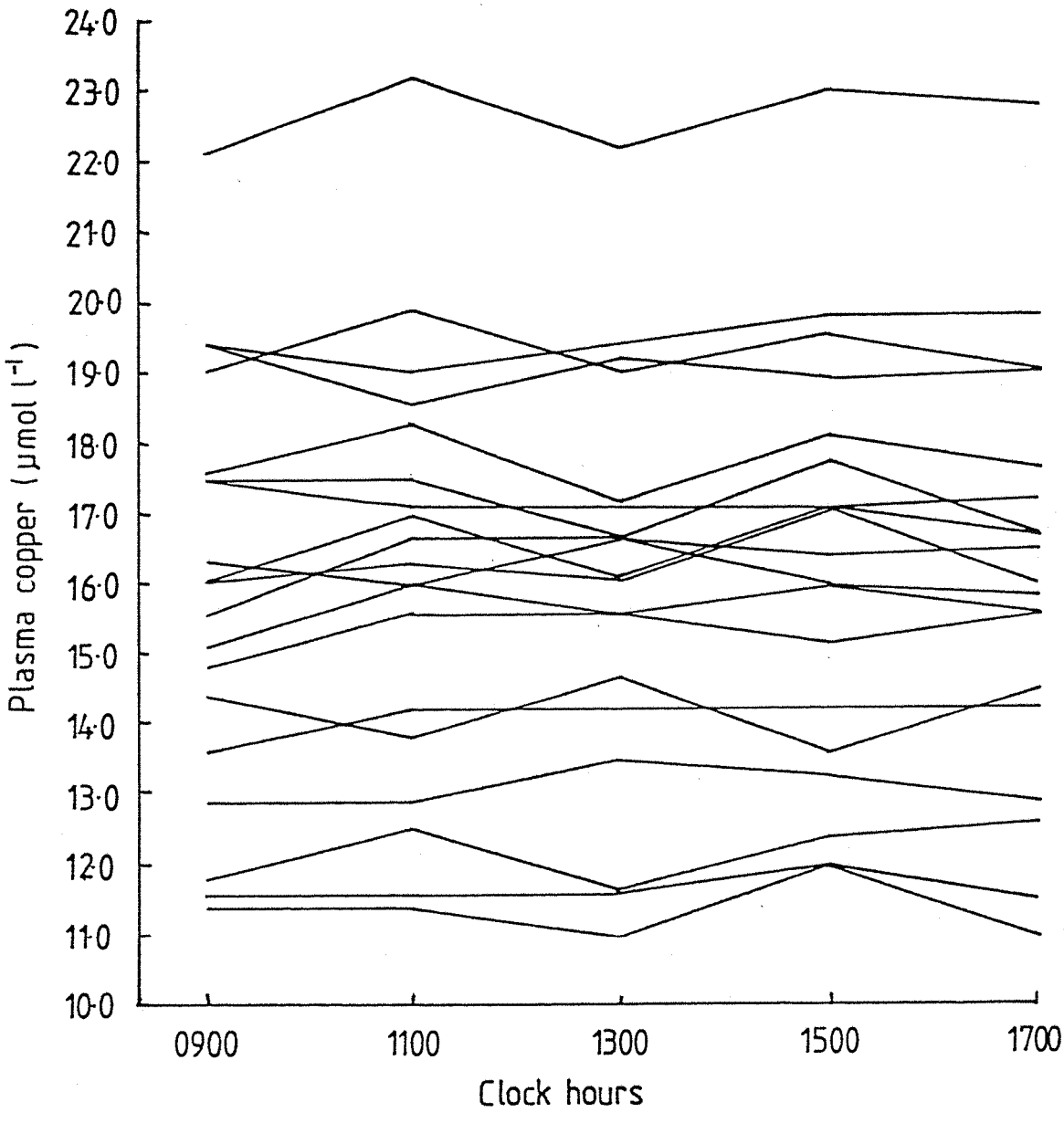


Figure 6.2

Concentration of copper in plasma samples obtained during the day from healthy subjects eating normally. Subjects were in the fasting state at 0900h and ingested food immediately after blood samples were taken at 0900h and 1300h

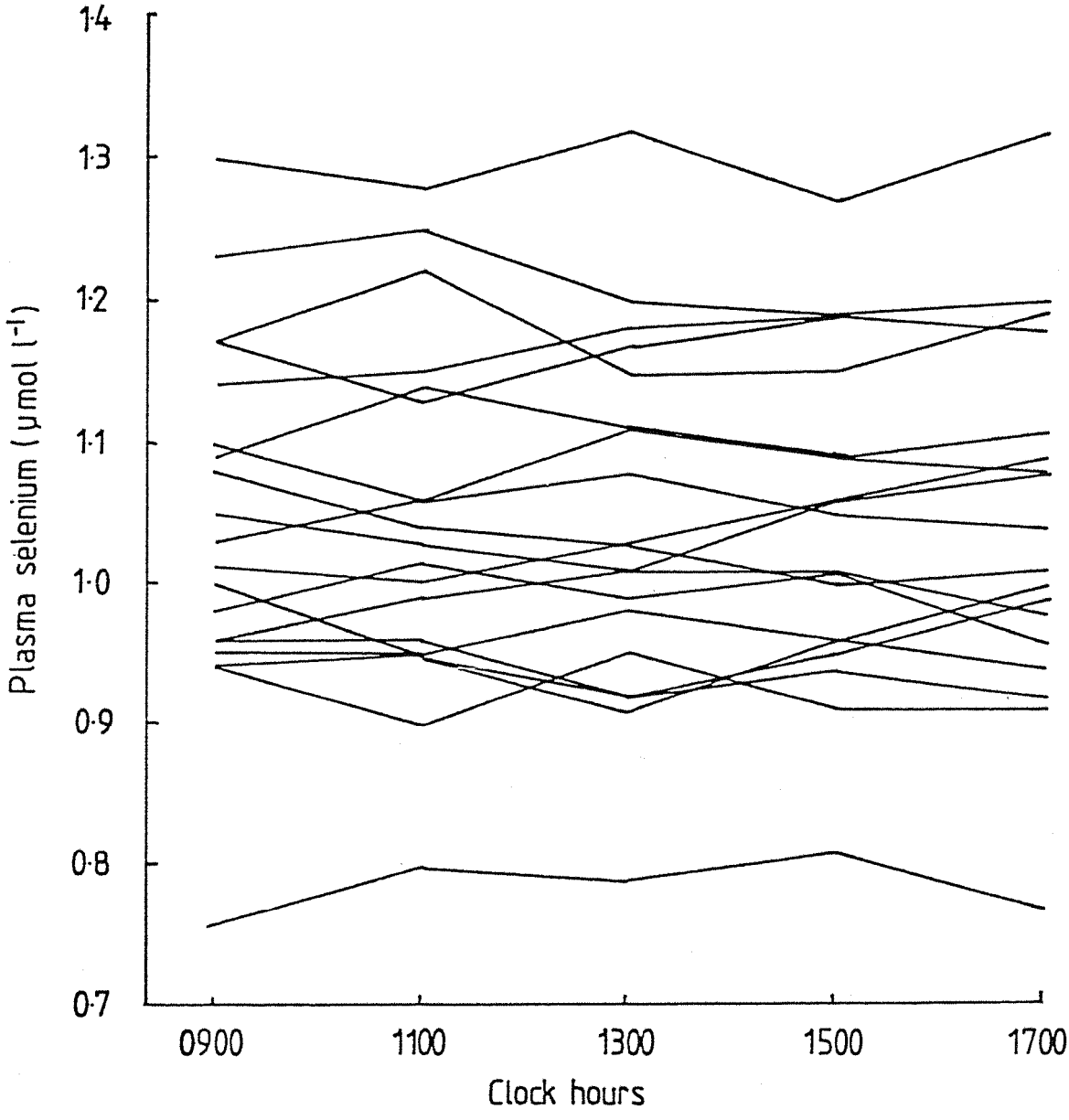


Figure 6.3

Concentration of selenium in plasma samples obtained during the day from healthy subjects eating normally. Subjects were in the fasting state at 0900h and ingested food immediately after blood samples were taken at 0900h and 1300h

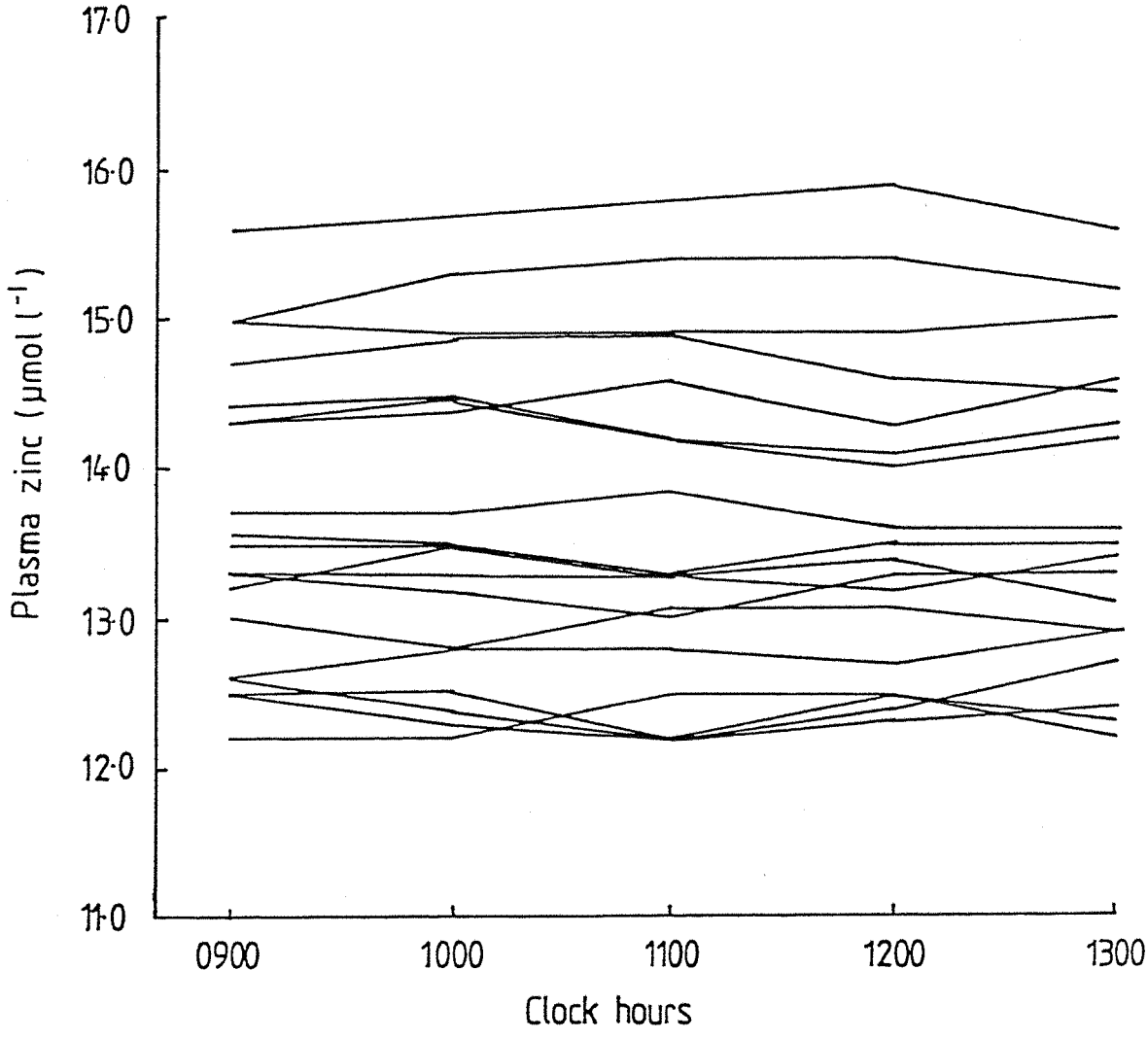


Figure 6.4

Concentration of zinc in plasma samples obtained at hourly intervals between 0900h and 1300h from healthy subjects in the fasting state

times of the day, and the Least Significant Difference (LSD) are given in Table 6.I. No significant variation was demonstrated between the concentrations of copper or selenium during the time of sampling. However, there was a significant variation ($p < 0.001$) between the plasma zinc concentrations, and the difference between the mean value of plasma zinc at 0900h and the mean value at 1100h (1.09 $\mu mol\ l^{-1}$), 1300h (1.17 $\mu mol\ l^{-1}$), 1500 (1.03 $\mu mol\ l^{-1}$) and 1700 (1.15 $\mu mol\ l^{-1}$) was larger than the LSD (0.17 $\mu mol\ l^{-1}$). There was no significant variation between the plasma zinc concentrations obtained between the hours of 1300 and 1700.

The plasma zinc values obtained from the fasting subjects during the period of 0900h and 1300h are shown in Figure 6.4. Table 6.II gives the mean plasma zinc values at the times of 0900h, 1000h, 1100h 1200h and 1300h. There was no significant variation between the plasma zinc concentrations demonstrated at these times.

6.3 CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES, PLASMA AND WHOLE BLOOD IN HEALTHY ADULT VOLUNTEERS

6.3.1 SUBJECTS AND METHODS

Subjects

Samples of whole blood were obtained from 153 normal healthy volunteers: 72 men aged 18-64 years, and 81 women aged 21-59 years. Twenty six of the women who participated, aged 21-32 years, were taking combined oral contraceptives. Precise details of the medications taken by this group of women are provided in Appendix 3.

Samples

Samples (19ml) of venous blood were collected between 0900 and 1100h from subjects who had eaten at least two hours previously. No fasting samples were taken.

A 15ml aliquot of the blood was transferred to a siliconised glass tube containing heparin, for leucocyte separation; a 2ml aliquot was added to a trace-element-free tube containing heparin, for erythrocyte and plasma separation; the remaining 2ml volume was added to a trace-element-free tube containing EDTA.

TABLE 6.I

MEAN CONCENTRATIONS OF PLASMA ZINC, COPPER AND SELENIUM OBTAINED DURING THE DAY FROM 19 HEALTHY SUBJECTS EATING NORMALLY

Mean plasma values ($\mu mol\ l^{-1}$) at times stated

	0900h	1100h	1300h	1500h	1700h	LSD($\mu mol\ l^{-1}$)
Zinc	13.69*	12.60	12.52	12.66	12.54	0.17
Copper	15.92	16.05	16.01	16.14	16.05	0.23
Selenium	1.045	1.046	1.046	1.047	1.050	0.014

LSD = Least Significant Difference

* Difference between mean value and the mean values at 1100h, 1300h, 1500h and 1700h larger than the LSD.

TABLE 6.II

MEAN CONCENTRATIONS OF PLASMA ZINC OBTAINED FROM FASTING SUBJECTS DURING A 4 HOUR PERIOD

Mean plasma zinc values ($\mu mol\ l^{-1}$) at times stated

0900h	1000h	1100h	1200h	1300h	LSD($\mu mol\ l^{-1}$)
13.6	13.67	13.63	13.65	13.64	0.09

LSD = Least Significant Difference

Analyses

Leucocytes were separated and analysed for zinc, copper and selenium using the procedures given in Chapters 4 and 5. Separation of erythrocytes and analysis of zinc, copper and selenium in plasma, whole blood and erythrocytes were performed by the methods described in Chapter 5. Haematocrit measurements on whole blood samples were made with a microhaematocrit centrifuge (Hawksley), spinning at 1300g for 5 min.

Statistical procedures

The statistical procedures used in this project are given in Chapter 5.

6.3.2 RESULTS

The data resulting from the analysis of zinc, copper and selenium in white cells, red cells, plasma and whole blood are given in full in Appendix 3, and in summarised form in Table 6.III.

The 153 normal healthy subjects investigated in the present study formed part of a much larger population (n = 391) who were investigated for the effect of age, sex, smoking and other factors on the concentration of selenium in erythrocytes, plasma and whole blood (Lloyd et al, 1983). Analysis of selenium in white cells was confined to the present study. The use of internal quality control samples ensured a satisfactory performance for the measurement of selenium by the investigators concerned. The erythrocyte selenium values reported by LLoyd et al (1983) were calculated from the difference between the concentration in whole blood and plasma, taking the haematocrit value into account. An excellent agreement between measured and 'calculated' whole blood selenium values had previously been demonstrated by these authors (Lloyd et al, 1982). Whereas Lloyd et al (1983) reported the erythrocyte selenium concentration in terms of µg/g Hb, this parameter is presented as pmol/10^6 cells in the present study.

Effect of oral contraceptives

Table 6.IV shows the values obtained for zinc, copper and selenium for the 26 women taking oral contraceptives and the 37 women, aged less than 40 years, who were not taking any medication.

TABLE 6.III

ZINC, COPPER AND SELENIUM CONCENTRATIONS IN LEUCOCYTES AND ERYTHROCYTES (per 10^6 cells), PLASMA AND WHOLE BLOOD ($\mu mol\ l^{-1}$)

	Zinc	Copper	Selenium
Number of subjects	153	153	153
Leucocytes	115(118)*	10.9	2.13
pmol/10^6 cells	(97-134)Ω	±3.8	±0.54
Erythrocytes	16.2	1.13	0.18
pmol/10^6 cells	±2.1	±0.16	±0.03
Plasma	12.7	17.5	1.42
$\mu mol\ l^{-1}$	±1.9	±3.9	±0.17
Whole blood	87.4	15.4	1.70
$\mu mol\ l^{-1}$	±10.5	±2.4	±0.22

* Geometric mean (arithmetic mean)

Ω Interquartile distance

TABLE 6.IV

EFFECT OF ORAL CONTRACEPTIVES ON ZINC AND COPPER CONCENTRATIONS IN LEUCOCYTES, ERYTHROCYTES, PLASMA AND WHOLE BLOOD, AND ON SELENIUM CONCENTRATIONS IN LEUCOCYTES

Analysis	Women aged <40yr not taking oral contraceptives n = 37	Women taking oral contraceptives n = 26	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	115(98-137)$^{\Omega}$	111(91-126)$^{\Omega}$	1.04 (a)	0.94 to 1.15 (a)
Erythrocyte Zn pmol/10^6 cells	16.1 ± 1.8	16.4 ± 2.4	-0.31 (b)	-1.43 to 0.81 (b)
Plasma Zn µmol l^{-1}	12.5 ± 1.7	12.8 ± 1.7	-0.31 (b)	-1.17 to 0.55 (b)
Whole blood Zn µmol l^{-1}	81.5 ± 7.0	84.2 ± 10.9	-2.71 (b)	-7.59 to 2.17 (b)
Leucocyte Cu pmol/10^6 cells	11.1 ± 3.7	10.9 ± 4.1	0.20 (b)	-1.81 to 2.21 (b)
Erythrocyte Cu pmol/10^6 cells	1.14 ± 0.15	1.12 ± 0.17	0.02 (b)	-0.06 to 0.10 (b)
Plasma Cu µmol l^{-1}	16.4 ± 3.2	23.6 ± 3.9^{+++}	-7.18 (b)	-9.03 to -5.33 (b)
Whole blood Cu µmol l^{-1}	14.6 ± 1.9	19.0 ± 2.7^{+++}	-4.37 (b)	-5.60 to -3.14 (b)
Leucocyte Se pmol/10^6 cells	2.04 ± 0.49	2.15 ± 0.52	-0.11 (b)	-0.36 to 0.14 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+++}$ $p < 0.001$

Significantly higher ($p < 0.001$) concentrations of copper in plasma and whole blood were found in women taking oral contraceptives. There were no significant differences between the two groups of women for any of the other parameters shown in Table 6.IV.

Correlations

Correlations were performed on data relating to men, and women not taking oral contraceptives ($n = 127$).

No significant correlations were found for zinc or copper between the leucocyte values and the plasma, whole blood or red cell values. A significant correlation was observed between the concentration of zinc in red cells and whole blood ($p < 0.001$, $r = 0.72$), but not in plasma and red cells or plasma and whole blood. There were significant relationships for copper between the concentration in red cells and plasma ($p < 0.05$, $r = 0.22$), red cells and whole blood ($p < 0.01$, $r = 0.27$) and plasma and whole blood ($p < 0.001$, $r = 0.69$).

For selenium, there were significant correlations between the leucocyte values and plasma ($p < 0.001$, $r = 0.39$), whole blood ($p < 0.001$, $r = 0.48$) and red cell values ($p < 0.001$, $r = 0.39$). Significant relationships were also noted between selenium concentrations in red cells and plasma ($p < 0.001$, $r = 0.44$), red cells and whole blood ($p < 0.001$, $r = 0.86$) and plasma and whole blood ($p < 0.001$, $r = 0.76$).

No significant correlations for men or women were shown between age and any of the other variables, or between cigarette smoking and leucocyte selenium concentrations.

Comparison of predicted and directly measured whole blood values

The data relating to men and to women not taking oral contraceptives was used for comparison.

Table 6.V shows the mean concentrations of copper and zinc measured in plasma, red cells and white cells expressed as the amount present per litre of blood, and a comparison of the mean predicted whole blood concentrations from the sum of these individual fractions and the concentrations obtained by direct analysis of whole blood is also given.

The mean predicted whole blood concentrations of zinc and copper were 99.4 and 100.8% respectively of that measured, with a range of 89.4-113.6% and 92.6-110.5% respectively. The predicted concen-

TABLE 6.V

MEAN ZINC AND COPPER CONCENTRATIONS PER LITRE OF BLOOD IN PLASMA, ERYTHROCYTES AND LEUCOCYTES MEASURED SEPARATELY, AND OF WHOLE BLOOD, PREDICTED AND DIRECTLY MEASURED

	Zinc	Copper
	Concentration (µmol) per litre of whole blood	
Number of subjects	127	127
Plasma	7.12	9.13
Erythrocytes	79.67	5.57
Leucocytes	0.72	0.06
Predicted whole blood	87.51	14.76
Measured whole blood	88.04	14.64

trations for zinc and copper were within ± 5% of the measured values for 91 and 89% of the samples respectively, with the largest differences showing a predicted concentration of 13 and 11% respectively higher than the measured concentrations.

For selenium, the mean concentrations (μmol) per litre of blood in plasma, red cells and white cells were 0.79, 0.89 and 0.013 respectively, and the measured whole blood concentration was 1.68 μmol l^{-1}.

Comparison of results obtained for men and women

Significantly higher ($p < 0.001$) red blood cell counts and packed cell volume measurements were found in men ($5.20 \pm 0.39 \times 10^{12}$ l^{-1} and 45.5 ± 2.6 respectively) when compared to women ($4.60 \pm 0.29 \times 10^{12}$ l^{-1} and 41.5 ± 2.4 respectively).

A comparison of the elemental concentrations in the various samples for men, and women not taking oral contraceptives (Table 6.VI) revealed significantly higher ($p < 0.001$) concentrations of zinc in whole blood for men. There were no significant differences between these two populations for any of the other parameters shown in Table 6.VI.

Reference ranges

The reference range for leucocyte zinc was derived from the 5th and 95th percentile points of the cumulative frequency distribution curve; reference ranges for copper and selenium in leucocytes, and zinc, copper and selenium in erythrocytes and plasma were calculated from the mean values ± two standard deviations (Table 6.VII). The range for plasma copper was calculated for men, and women not taking oral contraceptives.

6.4 DISCUSSION

6.4.1 DIURNAL VARIATION OF ZINC, COPPER AND SELENIUM CONCENTRATIONS IN PLASMA

A circadian pattern of variation for serum copper concentrations has been reported in subjects fed a liquid diet (Lifschitz and Henkin, 1971) but not in fasting subjects (Guillard et al, 1979). In the study by Lifschitz and Henkin (1971), variations in plasma copper concentrations of +6 to -8% of the overall mean were demonstrated over

TABLE 6.VI

COMPARISON OF MEN, AND WOMEN NOT TAKING ORAL CONTRACEPTIVES: ZINC AND COPPER CONCENTRATIONS IN LEUCOCYTES, ERYTHROCYTES, PLASMA AND WHOLE BLOOD, AND SELENIUM CONCENTRATIONS IN LEUCOCYTES

Analysis	Men n = 72	Women not taking oral contraceptives n = 55	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	117(97-139)$^{\Omega}$	114(98-136)$^{\Omega}$	1.01 (a)	0.93 to 1.09 (a)
Erythrocyte Zn pmol/10^6 cells	16.1 ± 2.3	16.3 ± 1.7	-0.17 (b)	-0.87 to 0.54 (b)
Plasma Zn µmol l^{-1}	12.8 ± 2.1	12.6 ± 1.6	0.20 (b)	-0.46 to 0.86 (b)
Whole blood Zn µmol l^{-1}	91.8 ± 10.7^{+++}	83.1 ± 7.4	8.68 (b)	5.61 to 11.85 (b)
Leucocyte Cu pmol/10^6 cells	11.0 ± 3.8	10.8 ± 3.9	0.20 (b)	-1.16 to 1.56 (b)
Erythrocyte Cu pmol/10^6 cells	1.12 ± 0.16	1.14 ± 0.17	-0.01 (b)	-0.07 to 0.04 (b)
Plasma Cu µmol l^{-1}	16.0 ± 2.1	16.6 ± 2.9	-0.61 (b)	-1.53 to 0.31 (b)
Whole blood Cu µmol l^{-1}	14.4 ± 1.4	14.9 ± 1.8	-0.43 (b)	-1.03 to 0.14 (b)
Leucocyte Se pmol/10^6 cells	2.09 ± 0.53	2.13 ± 0.55	-0.04 (b)	-0.23 to 0.15 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+++}$ $p < 0.001$

TABLE 6.VII

REFERENCE RANGES FOR ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA

		Leucocytes pmol/10^6 cells	Erythrocytes pmol/10^6 cells	Plasma µmol l^{-1}
Zinc	Total population	78-168	12-20.5	9-16.5
Copper	Total population	3.3-18.5	0.8-1.5	
	Men + Women not taking oral contraceptives			11.5-21
Selenium	Total population	1.04-3.20	0.12-0.25*	1.06-1.87*

* Data taken from Lloyd et al, (1983). Values for concentration of selenium in plasma converted from µg l^{-1} to µmol l^{-1}, and in erythrocytes, converted from µg/g Hb to pmol/10^6 cells.

a 24h period although it is not clear what proportion of these changes was due to analytical variation. The findings from the present study failed to demonstrate a significant variation in plasma copper levels between the hours of 0900 and 1700 in subjects eating normally. Similarly, no significant variation in plasma selenium concentrations was found during this period.

Plasma zinc concentrations remained constant in fasting subjects between 0900 and 1300h and this observation is in agreement with the findings of Kiilerich et al, (1980a). Although a significant variation in serum zinc levels over a 24h period in fasting subjects has been reported, there was no significant difference between the mean zinc concentrations at 0900 and 1200h (Guillard et al, 1979).

There are conflicting reports in the literature concerning the daily fluctuations in plasma zinc levels in subjects ingesting food. In the present study, a significant decrease in the fasting plasma zinc levels was noted two hours after food intake with no further fall in plasma zinc concentrations during the day. Busher et al (1982), working in this laboratory, found no significant variation in plasma zinc levels over a 24h period in a small number of elderly subjects (over 65 years) eating normally, and a study of healthy adults failed to demonstrate any significant difference between the plasma zinc concentrations in the fasting state and one hour after a meal (McBean and Halsted, 1969). Conversely, a significant decrease in the concentration of fasting plasma zinc levels in healthy subjects two hours after eating breakfast has been reported (Kiilerich et al, 1980a; McMillan and Rowe, 1982) and these observations are in agreement with the present study. It is interesting to note that although McMillan and Rowe (1982) found no further fall in plasma zinc levels over the following two hours, a further significant decrease in levels in the period following lunch, but not after food ingestion at 1700h, was demonstrated. Both Burr (1973) and Hetland and Brubakk (1973) reported a significant decrease in plasma and serum zinc concentrations throughout the day in subjects in the non-fasting state, although neither of these studies gave details of the overall precision of the method used for zinc determination. A circadian variation in serum zinc concentrations in healthy adult males given fixed calorie diets has been reported with peak levels occurring at 0930h and a mid-trough at 2000h, and as a high correlation was observed between the plasma zinc and ionised calcium patterns these

workers suggested that a common regulator was responsible for these two rhythms (Markowitz et al, 1985). In contrast to other reports, their study demonstrated small increases in serum zinc concentrations at 30-90 min after each meal.

The findings from the present study of a significant decrease in plasma zinc levels between the hours of 0900 and 1100 are suggestive of an effect mediated by food intake, as plasma zinc concentrations remained constant over the same period in fasting subjects. This observation is further supported by the fact that both studies were carried out on the same individuals. Whilst some investigators are in agreement with this finding (Kiilerich et al, 1980a; McMillan and Rowe, 1982), others have failed to document this effect (McBean and Halsted,1969; Busher et al, 1982; Markowitz et al, 1985), although the subjects studied by Busher et al (1982) were all aged over 65 years whereas the age range for the subjects in the present study was 19-47 years. It has been proposed that the effect of food ingestion on plasma zinc levels may be related to the meal size and content (Hetland and Brubakk, 1973). However, in the present study, subjects ate a varied lunch but failed to demonstrate a decrease in plasma zinc concentrations two hours after this meal. In this respect it would appear that the breaking of the fast was responsible for the decrease in plasma zinc levels rather than food intake per se. The reason for this apparent effect of food ingestion on fasting plasma zinc levels alone is not clear.

The data obtained shows that ideally blood for zinc determination should be drawn in the fasting state; however, this is not always practicable when carrying out clinical studies. It was decided therefore that blood should be withdrawn from subjects who had eaten breakfast at least two hours prior to the time of sampling.

6.4.2 CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES, PLASMA AND WHOLE BLOOD IN HEALTHY SUBJECTS

The small differences between the calculated and directly measured values for zinc and copper concentrations in whole blood demonstrated good internal control of the separation and analytical techniques performed. Furthermore, this validated the use of erythrocyte zinc and copper values obtained by calculation from the

difference between the concentration of the element in whole blood, and plasma and leucocytes.

Table 6.VIII shows a comparison of results for leucocyte zinc from workers who have reported in similar units of measurement to the present study. Precision values for the methods used are not stated by these investigators. Vallee and Gibson (1948) reported much higher values of leucocyte zinc than the other studies, whereas the present study shows the lowest values of all. These discrepancies may be due to the effect of methodological differences or to contamination with zinc from various sources. Measurement of zinc in the white cells was performed by neutron activation analysis (Dennes et al, 1961), dithiozone reaction and colorimetry (Vallee and Gibson, 1948; Fredericks et al, 1961) and flame-atomic absorption spectrophotometry (Prasad et al, 1978; Nishi, 1980). Nishi (1980) measured the concentration of zinc in the leucocytes by direct aspiration of the diluted leucocyte suspension into the flame with no prior digestion of the white cells and used a calibration graph prepared from aqueous working standard solutions. No information concerning the possible matrix effect on analyte sensitivity is given. Both Dennes et al (1961) and Fredericks et al, (1961) reported red cell contamination in their final leucocyte preparations and subsequently subtracted the calculated zinc contribution of the red cells from the zinc values obtained. The reliability of this procedure is questionable.

For the total population investigated in the present study the concentration of copper in white cells was 10.9 ± 3.8 pmol per 10^6 cells. Although there does not appear to be any other data relating to copper in total leucocyte populations in adults with which these results can be compared, it is interesting to note that Ishigame and Nishi (1985) reported concentrations of copper of 9.6 ± 3.5 pmol per 10^6 cells in granulocytes and 22.5 ± 7.6 pmol per 10^6 cells in lymphocytes. These authors however provide no details of the procedure used to separate lymphocytes and granulocytes from whole blood, or of the purity of these cell preparations. It is therefore not possible to comment on the validity of their results. No other studies have reported the selenium content of total leucocyte populations although the concentration of selenium in neutrophils has been reported in one subject during supplementation with sodium selenite (Johansson et al, 1983). However, as these results were

TABLE 6.VIII

COMPARISON OF LEUCOCYTE ZINC VALUES IN NORMAL SUBJECTS OBTAINED FROM THE PRESENT STUDY AND FROM PUBLISHED DATA

Data are given as mean (arithmetic) values ± standard deviation

Reference	Leucocyte zinc concentration ($pmol/10^6$ cells)	
Vallee (1948)	490 ± 199*	n = 31
Dennes et al (1961)	214 ± 29*	n = 29
Fredericks et al (1961)	158 ± 38*	n = 38
Prasad et al (1978)	171 ± 28*	n = 37
Nishi (1980)	197 ± 50*	n = 18
Present Study	118 ± 28	n = 153

* Values re-calculated from authors' data as $pmol/10^6$ cells.

presented as ppm dry weight, comparison of values with the present study was not possible.

The data for zinc demonstrates that approximately 90% of the blood zinc is associated with the red cells, with plasma contributing about 8%, and that less than 1% of the total blood zinc is associated with the white cells. Despite the low contribution to the total blood zinc concentration, the white cells contain about seven times as much zinc per cell as the red cells. This figure is at variance with previous conflicting reports of the ratio of fifteen times (Dennes et al, 1961) and nine times (Fredericks et al, 1961). Presumably these discrepancies may be attributed to differences in methodology for the various determinations carried out. The data obtained for copper demonstrates that approximately 60% of the copper in whole blood is present in the plasma with only about 0.4% associated with the white cells and approximately 40% with the red cells. Similarly, for selenium, plasma and red cells contribute about 45 and 55% respectively to the total blood selenium and less than 1% is associated with the white cells. Leucocytes were shown to contain about ten times as much copper per cell and twelve times as much selenium per cell as erythrocytes, but there are no other reports with which this data can be compared.

Significant increases in plasma copper concentrations were observed for women taking oral contraceptives and these findings are consistent with previous reports (Halsted et al, 1968; Schenker et al, 1971; Sing et al, 1978; Vir and Love, 1979a, 1981; Crews et al, 1980). The present study also demonstrated raised concentrations of copper in whole blood in women taking oral contraceptives; this observation is consistent with the positive correlation between plasma and whole blood copper. The increased copper concentration in plasma is most probably the result of an increased synthesis of caeruloplasmin in response to the ingestion of oral contraceptives (Carruthers et al, 1966). Experiments in rats utilising radioactive copper showed a significant increase in serum caeruloplasmin levels after oestradiol injections which was independent of the hepatic copper concentration (Evans et al, 1970). The significant elevation in serum copper concentrations has been reported to occur after usage of oral contraceptive agents for three months, with no further increase after a subsequent three month period (Vir and Love, 1979a, 1981). Increased endometrium copper levels during all phases of the menstrual

cycle have also been reported in women taking oral contraceptives (Sing et al, 1978). The physiological significance of these findings is not apparent. Studies have shown however that oral contraceptives do not alter the metabolic balance of copper (Crews et al, 1980) and it is interesting to note that in the present study no significant increase was found in the copper content of leucocytes or erythrocytes in the group of women taking oral contraceptives.

No significant effect of oral contraceptives on plasma zinc levels was observed. This finding does not agree with some earlier reports which have shown decreased levels of plasma and serum zinc (Halsted et al, 1968; Schenker et al, 1971; Prasad et al, 1975), but which is in general agreement with more recent publications (Sing et al, 1978; Vir and Love, 1979a, 1981; Crews et al, 1980). It is possible that the decrease in the oestrogen content of the newer oral contraceptives may be partly responsible for these conflicting findings. In the present study, most of the women concerned were regularly taking combination type (ethinyloestradiol 30µg + levonoresterol 150 or 250µg) oral contraceptives. No adverse effects on zinc absorption or increases in dietary zinc requirement have been reported with oral contraceptive usage (King et al, 1978). Although an increase in erythrocyte zinc content as a result of administration of oral contraceptives has been reported (Prasad et al, 1975), in the present study no significant effect was noted on the concentration of zinc in leucocytes, erythrocytes or whole blood.

Although Lloyd et al (1983) reported a significant increase in the concentration of plasma selenium in women taking oral contraceptives, no similar increase in leucocytes was observed in the present study. Other workers have failed to find significant differences in plasma selenium levels in association with the use of oral contraceptives (Robinson et al, 1983).

No significant difference in plasma zinc levels between the sexes was observed and this finding is in agreement with other investigators (Davies et al, 1968; Chooi et al, 1976) although some studies have reported significantly increased concentrations of plasma and serum zinc in men when compared to women (Lindeman et al, 1971; Rose and Willden, 1972; Kiilerich et al, 1980a). The lack of any sex-related difference for zinc in red cells, which was demonstrated in the present study, has also previously been reported (Lindeman et al, 1971). Furthermore, no significant difference between men, and women

not taking oral contraceptives was observed in the concentration of zinc in leucocytes, or copper in leucocytes, erythrocytes and whole blood. The significantly increased concentrations of zinc in whole blood found in men may presumably be attributed to the significantly higher red cell counts found in the male population. This evidence has been well documented and is further supported by findings from the present study. Lloyd et al (1983) reported a significant difference between men and women in the erythrocyte selenium concentration, but not in plasma or whole blood selenium levels. No significant difference between the sexes was found for leucocyte selenium concentration in the present study.

No significant correlations between age and the concentration of zinc or copper in leucocytes, erythrocytes, plasma or whole blood were observed in the subjects studied. Other studies have also shown no age dependency on the concentration of serum or plasma zinc in subjects aged 21-70 years (Kiilerich et al, 1980a) and 20-60 years (Davies et al,1968) or on red cell zinc levels in subjects aged 20-84 years (Lindeman et al, 1971) although a decrease in plasma zinc concentration with age has been reported in females aged 20-58 years (Lindeman et al, 1971).

Significantly lower concentrations of selenium in whole blood and erythrocytes in men and women aged over 55 years were reported by Lloyd et al (1983). In the present study, no relationship between age and leucocyte selenium levels in either men or women was found. It is interesting to note that although Lloyd et al (1983) also found significantly decreased levels of selenium in whole blood, plasma and erythrocytes in male cigarette smokers aged over 30 years compared with non-smokers, there was no significant relationship between smoking and leucocyte selenium concentrations in either men or women in the present study.

It is clear from the above discussion that selenium levels are influenced by age, sex and cigarette smoking. Furthermore, increased concentrations of plasma copper and selenium are associated with the use of oral contraceptives. Carefully matched data are therefore required to ensure correct interpretation of results on patients.

CHAPTER 7

LEUCOCYTE ZINC AND COPPER IN THE ELDERLY

7.1 INTRODUCTION

It has been suggested that malnutrition may be present in up to 10% of elderly patients admitted to hospital in Britain and in a significant number of old people in the community, the proportion rising with advance into very old age (Beaumont and James, 1985). Trace element deficiency may occur more commonly among the elderly than younger people as a result of a greater frequency of factors such as disease, drug-nutrient interactions, and reduced dietary intake due to decreased energy need or economic deprivation (Solomons, 1986). In addition, housebound elderly people usually eat less than active people matched for age (Beaumont and James, 1985). Marginal dietary zinc intakes in this population group may be particularly serious because zinc absorption appears to decrease with age in both men and women (Aamodt et al, 1981; Turnland et al, 1984). Certain manifestations of zinc deficiency such as impaired immune response, delayed wound healing and decreased taste acuity, are also features of old age (Sandstead et al, 1982). Evidence does not implicate copper deficiency in the deterioration of physiological functions which occur with aging (Watson, 1984). However, chronic low intake of copper may occur in the elderly (Henery and Smith, 1987), and they may therefore be at risk of copper deficiency.

The purpose of the present study was to investigate the zinc and copper status of a group of healthy and housebound elderly people. In addition to determination of these trace elemnents in leucocytes, metabolic balance studies for zinc and copper were performed on these subjects, and an opportunity was taken to compare the data obtained from the balance studies with the leucocyte values. These balance data were made available through the kindness of Dr V. Bunker.

7.2 SUBJECTS AND METHODS

Elderly subjects

Twenty-four apparently healthy subjects, 11 men and 13 women,

aged 70-86 (mean 76.9) years volunteered to participate. Details of this group of subjects are given in Table 7.I. One woman smoked an average of 20 cigarettes a day, but the other subjects were non smokers. All of them were living in their own homes and eating self-selected diets.

Twenty housebound elderly subjects, 7 men and 13 women, aged 70-85 (mean 78.8) years were also studied. All were classified as housebound in that they did not go out unaccompanied. This was generally due to physical infirmity although two subjects suffered from severe depression. Details of these subjects are given in Table 7.II. The participants were suffering from various stable chronic diseases and were taking a number of prescribed medicines including diuretics, tranquilizers and anti-inflammatory drugs. One woman smoked between 15-20 cigarettes a day and one man smoked an average of 30 cigarettes a day. None of the other housebound subjects were smokers. Sixteen of them received home help once or twice a week. Their food was bought by friends, relatives, home help or the subjects themselves when they were taken out. Seven of the subjects received meals-on-wheels five days a week.

Young control subjects

The data obtained for the healthy control subjects investigated as described in the previous Chapter were used for the purpose of comparison in the present study.

Blood samples

Blood samples were collected and aliquoted as decribed in Chapter 6. Samples were obtained for leucocyte analysis from all of the healthy elderly subjects and from 18 of the housebound subjects.

Analyses

Separation of white cells, analysis of zinc and copper in leucocytes and plasma, and cell counting were performed by the techniques given in Chapters 4 and 5.

Albumin was measured using an automated bromocresol green dye binding method. The between-batch coefficient of variation was 3.8% at a plasma albumin concentration of 44 g l^{-1}. Caeruloplasmin was determined by radial immunodiffusion using NOR-Partigen plates (Hoechst UK Ltd.), and the between-batch coefficient of variation was

TABLE 7.I

DETAILS OF APPARENTLY HEALTHY ELDERLY SUBJECTS

No.	Sex	Age (yrs)	Clinical details, medication and smoking habits
1	F	75	Arthritis; Rx oxprenolol hydrochloride
2	F	85	Very fit; Rx imipramine hydrochloride
3	F	74	Very fit and active; 20-30 cigarettes/d
4	F	83	Fit
5	F	85	Mild cerebral vascular accident 4 years ago; Rx phenytoin
6	F	70	Very fit
7	F	77	Very fit
8	F	73	Fit
9	F	71	Fit
10	F	71	Fit
11	F	79	Fit
12	F	71	Fit
13	F	73	Mild hyperglycaemia found during study
14	M	85	Very fit and active
15	M	73	Very fit
16	M	80	Fit
17	M	73	Very fit
18	M	83	Fit
19	M	75	Very fit
20	M	75	Slight arthritis
21	M	85	Prostatectomy 1 year ago for benign lesion
22	M	82	Fit
23	M	70	Osteoarthritis; Rx ibuprofen
24	M	78	Fit

TABLE 7.II

DETAILS OF HOUSEBOUND ELDERLY SUBJECTS

No.	Sex	Age (yrs)	Clinical details, medication and smoking habits
1	F	82	Disabled after unsuccessful hip replacement; Rx frusemide, cyclopenthiazide with potassium;15-20 cigarettes/d
2	F	82	Severe osteoarthritis; Rx cyclopenthiazide with potassium, triazolam
3	F	71	Depression, angina; Rx chlormethiazole, dihydrocodine tartrate, nitrazepam, cyclopenthiazide with potassium
4	F	75	Severe osteoarthritis, depression; Rx ibuprofen, lorazepam
5	F	77	Badly mended fractured radius, depression; Rx spironolactone, dextropropoxyphene hydrochloride + paracetamol(Distalgesic, Dista), triazolam
6	F	82	Severe osteoarthritis; Rx potassium chloride (Slow-k, Ciba), frusemide, mefenamic acid
7	F	79	Chronic depression; Rx nitrazepam
8	F	79	Recently bereaved, depression; Rx frusemide, potassium chloride (Slow-k, Ciba)
9	F	83	Osteoarthritis; Rx propanolol hydrochloride, amiloride hydrochloride + hydrochlorothiazide (Moduretic, Merck Sharp and Dohme Ltd), diazepam
10	F	81	Osteoarthritis; Rx ibuprofen, flurazepam hydrochloride
11	F	82	Recent cerebral vascular accident; Rx sennoside
12	F	75	Fractured femur 1 year ago, heart valve replacement; Rx nitrazepam, amiloride hydrochloride, digoxin, frusemide, warfarin
13	M	78	Chronic emphysema; Rx potassium chloride (Slow-k, Ciba), frusemide, digoxin
14	M	77	Recent heart valve replacement, fractured femur 1 year ago; Rx frusemide, spironolactone, theophylline, digonin; 30 cigarettes/d

TABLE 7.II continued

No.	Sex	Age (yrs)	Clinical details, medication and smoking habits
15	M	70	Disabled after cerebal vascular accident 4 years ago, recent fractured arm
16	M	75	Chronic obstructive airways disease
17	M	84	Osteoarthritis, very frail; Rx nitrazepam
18	M	85	Recurring bronchitis; Rx chlormethiazole, frusemide, amiloride hydrochloride
19	M	82	Recently bereaved, severe depression, hyperglycaemia found during study; Rx indomethacin, bendrofluazide
20	F	77	Osteoarthritis, severe depression; Rx fenoprofen, cyclopenthiazide with potassium, nitrazepam

6.7% at a concentration of plasma caeruloplasmin of 410 mg l^{-1}.

Statistical procedures

The statistical procedures used in this project are given in Chapter 5. A group of young control subjects (n = 50) consisting of 25 women who did not take oral contraceptives and 25 men, aged 21-64 (mean 37.2) years, were selected for comparison with the healthy elderly subjects.

Metabolic balance studies

Five-day metabolic balance studies were carried out by other investigators (Bunker et al, 1984; Bunker et al, 1987) on the subjects described in this chapter. These studies were carried out in the subjects' own homes and involved the collection of duplicate diets, faeces and urine into trace-element-free plastic containers taking care to avoid contamination of the samples. Where a subject received meals-on-wheels, two portions were delivered. These were combined and divided and any food not eaten was saved separately. Although it was necessary to spend much time with the subjects to ensure reliable collections, great care was taken not to encourage them to alter their normal eating habits.

Samples of diet, meals-on-wheels, rejected diet, rejected meals-on-wheels, faeces and urine were prepared and analysed for zinc and copper using atomic absorption spectrophotometry as described by Bunker et al (1984). The within-batch coefficients of variation for these procedures were 5% or less.

The mean values of the intake, total excretion and net retention of zinc and copper in the healthy and housebound elderly subjects are presented in the results section in order to facilitate comparison with the leucocyte zinc and copper results.

7.3 RESULTS

The individual zinc and copper results for the housebound and healthy elderly subjects are given in Appendix 4. Scattergrams of the plasma and leucocyte zinc values, and the plasma and leucocyte copper values are shown in Figures 7.1, 7.2, 7.3 and 7.4 respectively, for the housebound and healthy elderly subjects and young controls.

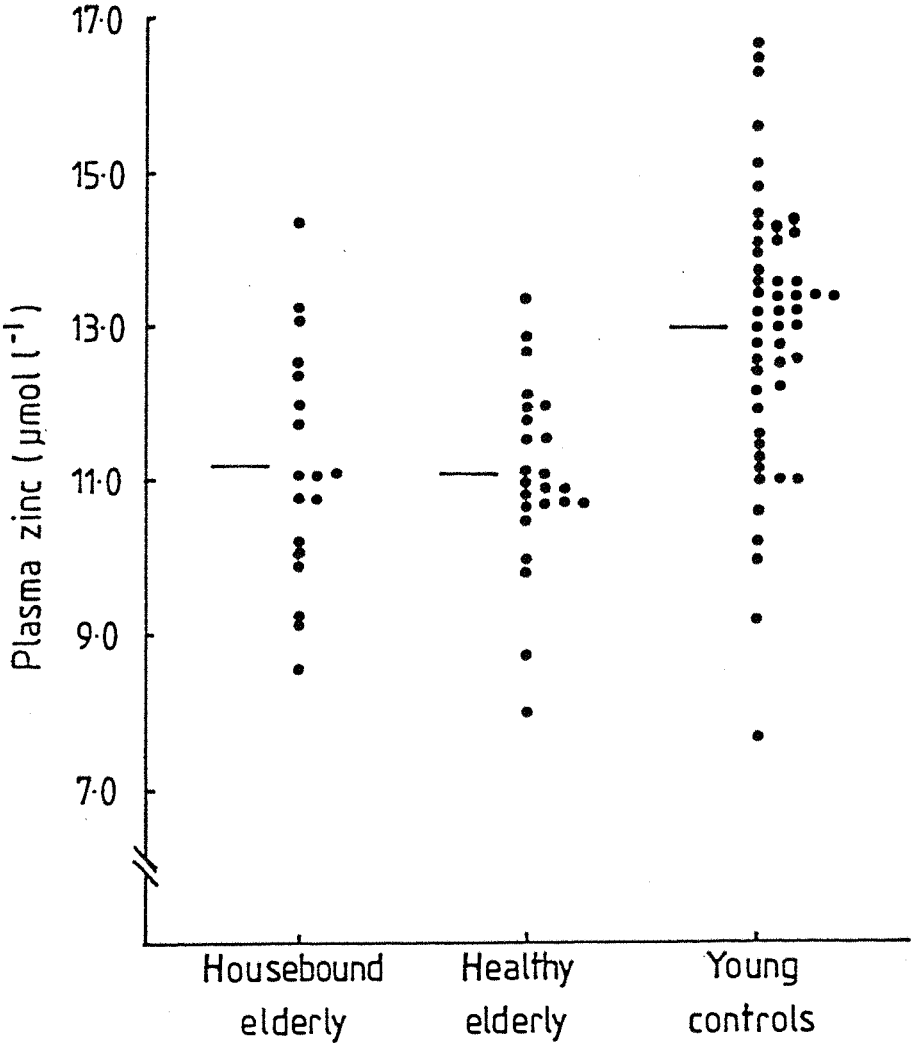


Figure 7.1

Scattergram of plasma zinc concentrations in housebound and healthy elderly subjects and young controls. Horizontal lines show the mean values of the data sets

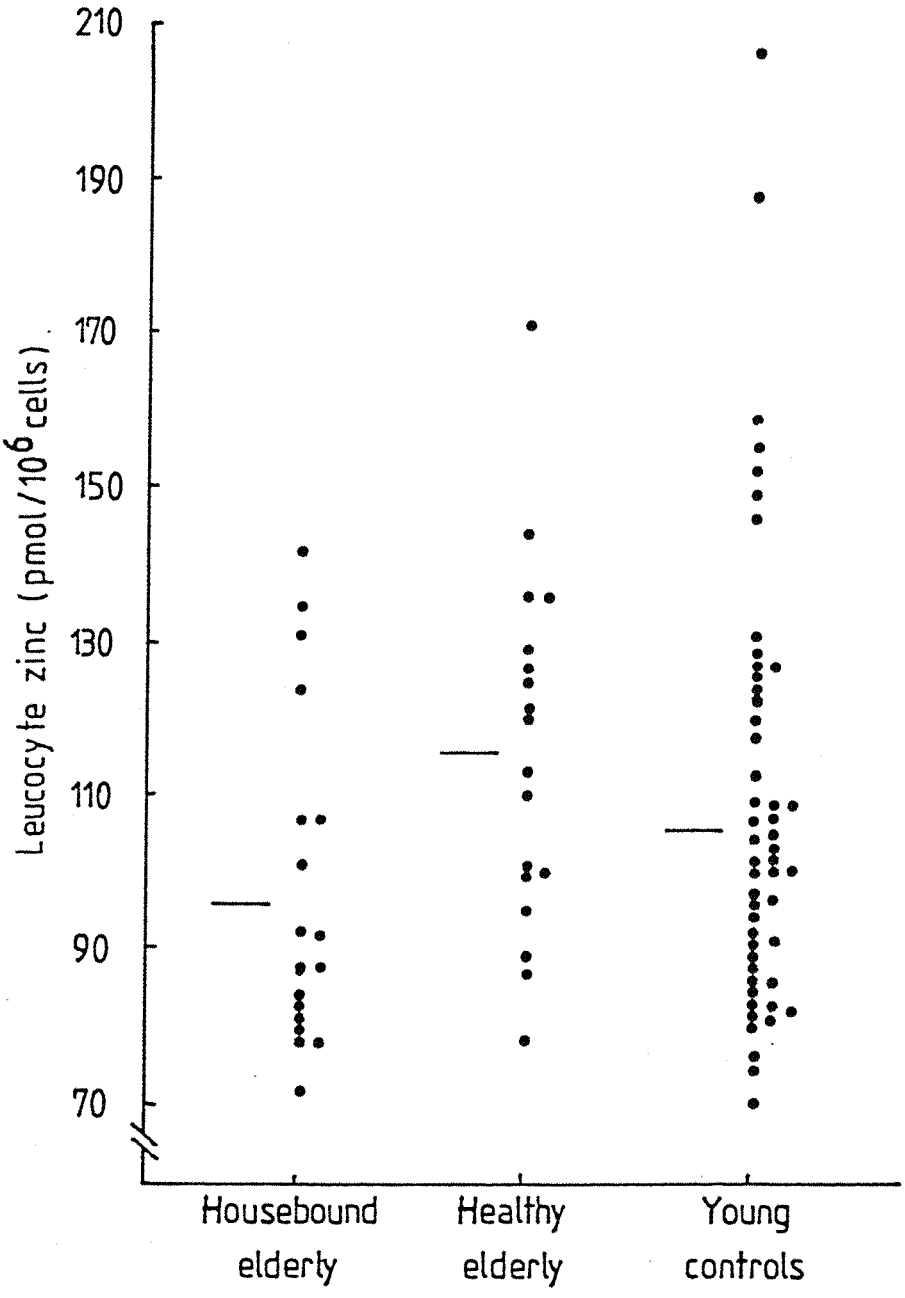


Figure 7.2

Scattergram of leucocyte zinc concentrations in housebound and healthy elderly subjects and young controls. Horizontal lines show the geometric mean values of the data sets

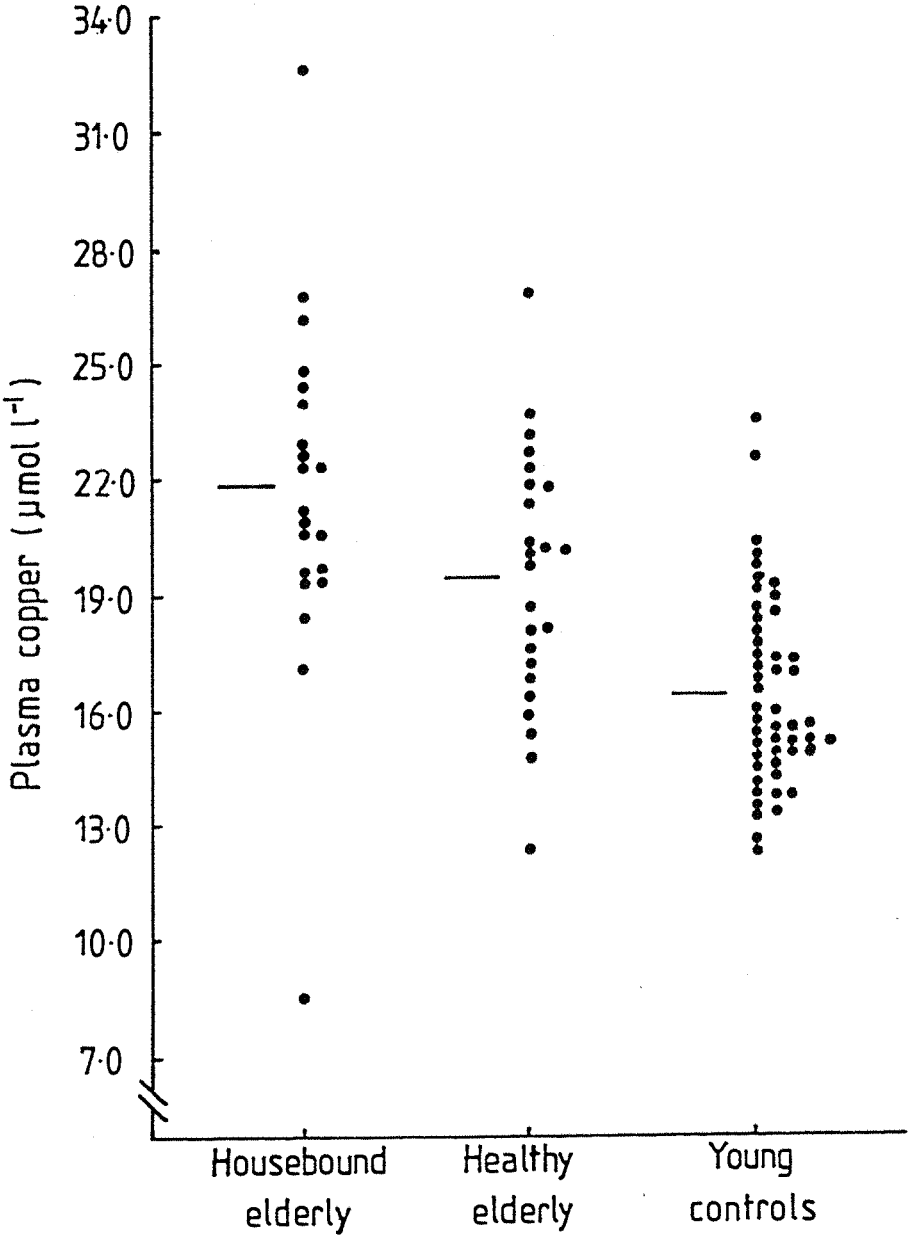


Figure 7.3

Scattergram of plasma copper concentrations in housebound and healthy elderly subjects and young controls. Horizontal lines show the mean values of the data sets

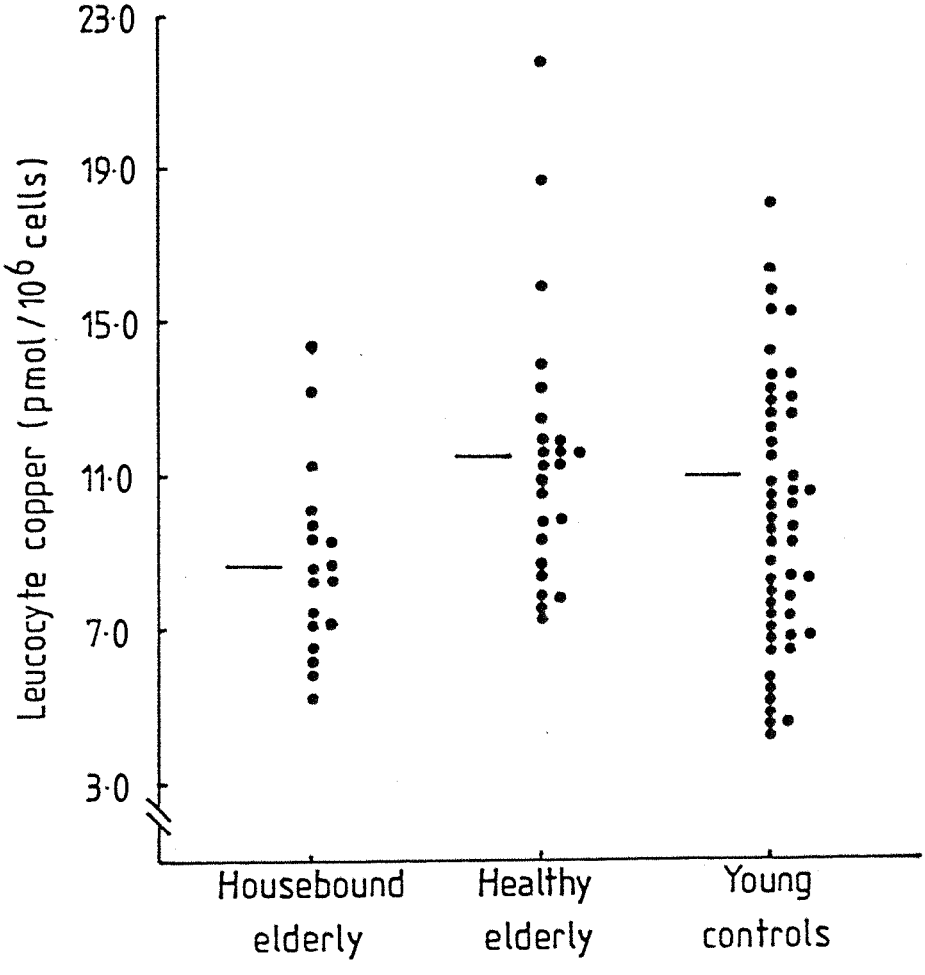


Figure 7.4

Scattergram of leucocyte copper concentrations in housebound and healthy elderly subjects and young controls. Horizontal lines show the mean values of the data sets

Comparison of healthy elderly subjects and young controls

The zinc and copper concentrations in leucocytes and plasma for the healthy elderly subjects and young controls are summarised in Table 7.III. A significant difference ($p < 0.001$) between the two groups was only observed for concentrations of zinc and copper in plasma. Plasma albumin and caeruloplasmin concentrations were similar in the healthy elderly subjects (44 ± 3 g l^{-1} and 330 ± 80 mg l^{-1} respectively) and the young controls (46 ± 3 g l^{-1} and 310 ± 55 mg l^{-1} respectively). There was a significant correlation between plasma copper and caeruloplasmin concentrations for both the healthy elderly subjects ($p < 0.001$, $r = 0.82$) and young controls ($p < 0.001$, $r = 0.89$). No significant relationship was observed between plasma zinc and albumin levels for either of the groups. There were no signifcant correlations between zinc or copper concentrations in plasma and leucocytes for the healthy elderly subjects.

Comparison of housebound and healthy elderly subjects

Table 7.IV shows a comparison of the mean values for zinc and copper in leucocytes and plasma for the housebound and healthy elderly subjects. A significant decrease ($p < 0.01$) in both leucocyte zinc and copper concentrations was observed in the housebound subjects. Plasma caeruloplasmin concentrations in the housebound subjects (382 ± 101 mg l^{-1}) were not significantly different from the healthy elderly subjects (330 ± 80 mg l^{-1}). There was a significant correlation between plasma copper and caeruloplasmin concentrations ($p < 0.001$, $r = 0.93$) for the housebound subjects. Plasma albumin levels were significantly decreased ($p < 0.05$) in the housebound (41 ± 3 g l^{-1}) compared to the healthy elderly subjects (44 ± 3 g l^{-1}). There were no significant correlations between plasma zinc and albumin levels, plasma and leucocyte zinc concentrations, or plasma and leucocyte copper concentrations for the housebound elderly subjects.

Comparison of leucocyte concentrations with data from balance studies for the healthy and housebound elderly subjects

The balance data obtained for zinc and copper are shown in Table 7.V for the healthy elderly subjects and Table 7.VI for the housebound elderly subjects. Both daily zinc and copper intakes were significantly lower ($p < 0.01$) in the housebound compared to the healthy elderly subjects. The overall zinc and copper retention of 1

TABLE 7.III

ZINC AND COPPER CONCENTRATIONS IN LEUCOCYTES AND PLASMA FOR HEALTHY ELDERLY SUBJECTS AND YOUNG CONTROLS

Analysis	Healthy elderly subjects n = 24	Young Controls n = 50	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	116 (100 - 129)$^{\Omega}$	105 (87 - 120)$^{\Omega}$	1.10 (a)	0.98 to 1.23 (a)
Plasma Zn µmol l^{-1}	11.1 ± 1.2^{+++}	13.0 ± 1.8	-1.92 (b)	-2.73 to -1.11 (b)
Leucocyte Cu pmol/10^6 cells	11.5 ± 3.6	10.9 ± 3.3	0.57 (b)	-1.12 to 2.26 (b)
Plasma Cu µmol l^{-1}	19.4 ± 3.4^{+++}	16.4 ± 2.4	3.00 (b)	1.63 to 4.37 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+++}$ $p < 0.001$

TABLE 7.IV

ZINC AND COPPER CONCENTRATIONS IN LEUCOCYTES AND PLASMA FOR HOUSEBOUND AND HEALTHY ELDERLY SUBJECTS

Analysis	Housebound elderly subjects n = 18	Healthy elderly subjects n = 24	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	96 (81 - 107)$^{\Omega++}$	116 (100 - 129)$^{\Omega}$	0.83 (a)	0.73 to 0.93 (a)
Plasma Zn μmol l^{-1}	11.2 ± 1.6	11.1 ± 1.2	0.13 (b)	-0.74 to 1.00 (b)
Leucocyte Cu pmol/10^6 cells	8.7 ± 2.4^{++}	11.5 ± 3.6	-2.73 (b)	-4.67 to -0.79 (b)
Plasma Cu μmol l^{-1}	21.8 ± 4.9	19.4 ± 3.4	2.43 (b)	-0.12 to 4.98 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{++}$ $p < 0.01$

TABLE 7.V

BALANCE DATA FOR 24 HEALTHY ELDERLY PEOPLE

(Data supplied by Dr V. Bunker)

	Zinc µmol day^{-1}	Copper µmol day^{-1}
Intake	137 *	18.7(20.1)+
	120 to 153	16.0 to 21.9
	46 - 210	10.1 - 47.8
Total excretion	136 *	19.4(20.9)+
	120 to 153	16.4 to 22.9
	43 - 211	7.8 - 47.4
Net retention	1 *	-0.8 *
	-2 to 13	-2.2 to 0.6
	-47 - 31	-8.8 - -6.3

* arithmetic mean
95% CI
observed range

\+ geometric mean (arithmetic mean)
95% CI
observed range

TABLE 7.VI

BALANCE DATA FOR 20 HOUSEBOUND ELDERLY PEOPLE

(Data supplied by Dr V. Bunker)

	Zinc μmol day^{-1}	Copper μmol day^{-1}
Intake	90 *	13.4(15.0)+
	80 to 101	10.8 to 16.6
	51 - 149	6.9 - 44.7
Total excretion	160 *	15.2(16.8)+
	94 to 118	12.3 to 18.6
	63 - 180	8.3 - 43.1
Net retention	-16 *	-1.8 *
	-24 to -6	-2.9 to 0.7
	-49 - 21	-10.9 - 4.9

* arithmetic mean
95% CI
observed range

\+ geometric mean (arithmetic mean)
95% CI
observed range

and -0.8 μmol day^{-1} respectively for the healthy elderly subjects was not significantly different from zero retention (i.e. equilibrium), whilst the overall zinc and copper balance of -16 and -1.8 μmol day^{-1} respectively for the housebound elderly subjects significantly differed from equilibrium ($p < 0.01$ and $p < 0.05$ respectively).

There was no significant correlation between leucocyte zinc concentrations and zinc intake, absorption or retention for the healthy elderly subjects. A significant correlation ($p < 0.01$, $r_s = 0.61$) was noted between the concentration of zinc in leucocytes and zinc retention (Figure 7.5), but not intake or absorption for the housebound elderly subjects. No significant relationship was noted between leucocyte copper concentrations and copper intake, absorption or retention for the healthy or housebound elderly subjects.

7.4 DISCUSSION

Leucocyte zinc concentrations were similar in the healthy elderly and younger subjects, and the mean zinc retention of 1 μmol day^{-1} for the healthy elderly subjects did not statistically differ from zero. The fact that no correlation was found between zinc concentration in leucocytes and zinc retention is not surprising as these data represent a scatter of values within a normal range. Lower plasma zinc levels were observed in the healthy elderly subjects as compared with the younger subjects and this finding is in agreement with other workers (Lindeman et al, 1971; Busher et al, 1982). It has been suggested that this is due to lower albumin levels in the elderly (Lindeman et al, 1971; Vir and Love, 1979b), although in the present study, plasma albumin levels were not significantly lower in the healthy elderly people than in the younger subjects, and there was no significant relationship between plasma zinc and albumin concentrations for either group. The lack of any significant correlation between plasma zinc and albumin values in healthy subjects, aged 18-62 years, has previously been reported (Foote and Delves, 1984).

No significant difference was observed between the leucocyte copper concentrations for the healthy elderly and younger subjects, and although the mean daily retention of copper was slightly negative, this figure did not significantly differ from equilibrium. The concentration of plasma copper in the healthy elderly people was higher than in the younger controls, an age-related finding that has

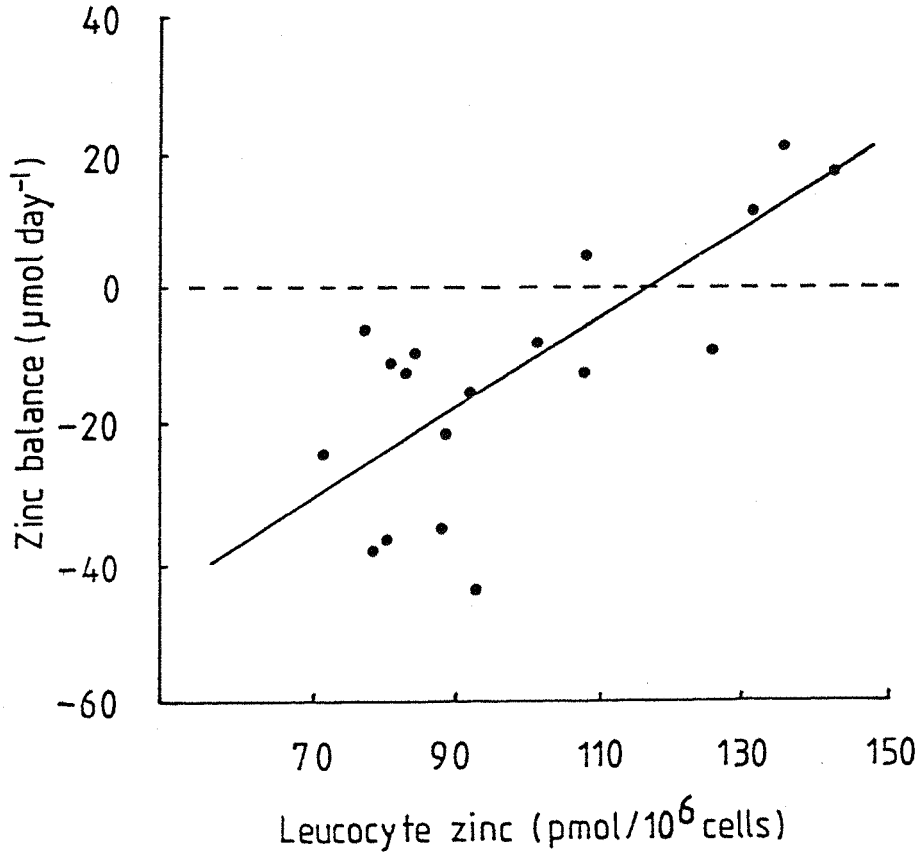


Figure 7.5

Relationship between leucocyte zinc concentration and daily zinc retention in 18 housebound elderly people

previously been reported (Harman, 1965; Yunice et al, 1974). Increased levels of caeruloplasmin were not responsible for the higher copper concentrations found in the present study, as concentrations of caeruloplasmin were similar for both groups. This lack of age-related change in caeruloplasmin levels has been reported previously (Yunice et al, 1974). It has been proposed that the raised plasma copper often found in the elderly may have a catalytic effect on lipid peroxidation, resulting in the production of free radicals, which may accelerate the development of atherosclerosis (Harman, 1965).

Comparison of the zinc and copper data obtained for the healthy elderly subjects with the data obtained from younger controls indicated a satisfactory body status of these trace elements in these elderly people. It is interesting to note that mean intakes of 137 μmol day^{-1} of zinc and 20.1 μmol day^{-1} of copper were compatible with good health in these elderly subjects, although these intakes are well below the existing recommendations. The existing recommended dietary allowance for zinc of 230 μmol (15 mg) day^{-1} and the safe and adequate dietary intake for copper of 31.5-47.2 μmol (2.0-3.0 mg) day^{-1} for people aged 51 years or more are extrapolations based on studies in younger adults (National Research Council, 1980). Metabolic balance studies carried out in specialised metabolic units, where a considerable proportion of the total daily intake of zinc was in the form of zinc sulphate, showed a negative zinc balance for subjects aged 56-83 years with daily intakes of 119 and 356 μmol (Burke et al, 1981), and conversely, a satisfactory zinc balance in subjects aged 65-74 years with daily intakes of 236 μmol (Turnlund et al, 1981). These two studies also reported that for copper, equilibrium was achieved with mean intakes of 36.7 μmol day^{-1} (Burke et al, 1981) and 51.3 μmol day^{-1} (Turnland et al, 1981). Much of the dietary copper intake was in the form of copper sulphate in both studies.

Significant reductions in the concentrations of both zinc and copper in leucocytes were observed in the housebound compared with the healthy elderly subjects. Mean daily intakes of zinc and copper were also significantly lower in the housebound subjects, and the negative retention values for these trace elements significantly differed from equilibrium. Significantly decreased leucocyte zinc concentrations have been reported in a group of geriatric patients in long-stay wards compared with age- and sex-matched persons, active and living in their own homes (Stafford et al, 1987). Unfortunately, it was not possible

to compare the leucocyte zinc values from this study with the values obtained in the present study as their units of measurement for leucocyte zinc were µg/g dry matter. It is interesting to note that in the present study there was a significant relationship between zinc retention and the concentration of zinc in the leucocytes in the group of housebound elderly subjects. These results indicate a suboptimal zinc status in some of the housebound elderly subjects. Although there was no direct relationship between copper balance and leucocyte copper concentration, the low levels found may also reflect a deficiency state of copper in this group.

There was no significant difference for plasma zinc, copper or caeruloplasmin levels between the housebound and healthy elderly subjects. Plasma albumin concentrations were significantly lower in the housebound elderly people, possibly reflecting long-term suboptimal nutrition, although the effect of underlying disease cannot be excluded.

The reason for the apparent lowered zinc and copper status of the housebound elderly subjects in the present study is not clear. Decreased dietary intake, inactivity and reduction in muscle mass, underlying disease and medication may all be contributing factors. The dietary requirements of the elderly are poorly understood, particularly with regard to trace elements. Further subdivision of the population 51 years of age and older would probably be useful for a more accurate description of the trace element needs of the elderly. Approximately 8% of the elderly in the United Kingdom are housebound, amounting to some 750,000 people (Beaumont and James, 1985), and it is important to be aware that this population group in particular appear to be at risk of zinc and copper deficiency. Since zinc and copper are both essential elements, continued negative balance with depletion of body stores will be detrimental to the health and degree of recovery of the housebound individual.

CHAPTER 8

INVESTIGATION OF ZINC, COPPER AND SELENIUM STATUS OF PATIENTS WITH GASTROINTESTINAL DISORDERS

8.1 INTRODUCTION

A range of nutritional disorders, including trace element deficiency, has been associated with gastrointestinal disorders such as Crohn's disease (Dawson, 1972; McClain et al, 1980) and coeliac desease (Elmes et al, 1976; Solomons et al, 1976). Deficiencies have also been observed in patients receiving total parental nutrition (Principi et al, 1979; Brown et al, 1986).

Crohn's disease is a chronic, destructive, inflammatory disease of unknown cause, affecting colon and small bowel (Best et al, 1976). When involvement of the small intestine is extensive, significant malabsorption may occur and associated malnutrition may be severe (Sandstead, 1982). Several clinical reports have described patients with severe Crohn's disease who demonstrated a significant clinical improvement in response to zinc supplementation (McClain et al, 1980; Sandstead, 1982).

Coeliac disease has been defined as 'a condition characterised by a lesion of the small intestinal mucosa related to gluten ingestion, associated with malabsorption and persisting throughout life in the presence of gluten' (Stewart, 1974). Most people who develop coeliac disease have increased concentrations of antibody to alpha-gliadin, a purified wheat protein, and respond clinically and histologically to treatment with a gluten-free diet (O'Farrelly et al, 1983). In contrast, patients with enteropathy associated T cell lymphoma do not have raised levels of alpha-gliadin antibodies and rarely respond to the conventional treatment for coeliac disease (O'Farrelly et al, 1986).

Patients receiving total parenteral nutrition may be at risk of trace element deficiency through inadequate supplementation of the intravenous solution with the appropriate trace element. This is of particular importance for patients who are unable to utilise their digestive tract for prolonged periods and are thus reliant on total parenteral nutrition long-term to meet their nutritional needs.

The objective of the studies described in this chapter was to investigate the zinc, copper and selenium status of patients with coeliac disease receiving a gluten-free diet, patients with mild Crohn's disease and patients receiving long-term total parenteral nutrition. In addition, the body status of these trace elements was assessed in newly diagnosed patients with coeliac disease, before and after withdrawal of gluten from the diet.

8.2 ASSESSMENT OF ZINC, COPPER AND SELENIUM STATUS OF PATIENTS WITH MILD CROHN'S DISEASE AND COELIAC DISEASE, AND OF TWO PATIENTS RECEIVING LONG-TERM TOTAL PARENTERAL NUTRITION

8.2.1 PATIENTS AND METHODS

Patients with Crohn's disease

Twenty ambulatory patients with Crohn's disease, 16 women and 4 men, aged 21-54 (mean 40.2) years were studied. Diagnosis had been made by established radiographic and clinical criteria. The clinical details of these patients are given in Table 8.I. Ten patients had disease of the small bowel only and 10 had involvement of both small and large bowel. None of them had been hospitalised in the previous 12 months, although 2 patients had undergone previous bowel resections. Chronic inflammatory bowel disease was the only pathology identified. Nine patients had quiescent disease and of these 4 were receiving prednisolone. Eleven patients complained of some diarrhoea (see Table 8.I) and these included 5 who were receiving prednisolone. None of the female patients were taking oral contraceptives. Normal serum B_{12} and plasma and red cell folate levels had been reported on all patients within 3 months prior to the study. The mean body mass index was 21.5 (range 15.5 - 30.1). By this criterion 6 of the patients were below their normal weight (Knight, 1984) and of these, 4 suffered from diarrhoea, frequency of bowel movements in these patients varying between 2 and 6 times a day. Six patients complained of a poor appetite, and of these 5 had a low body mass index.

Patients with treated coeliac disease

Sixteen patients with treated coeliac disease, 9 women and 7 men, aged 23-71 (mean 49.6) years were studied. These patients were seen as outpatients and were classed as well by the clinicians. The

TABLE 8.I

CLINICAL DETAILS OF PATIENTS WITH CROHN'S DISEASE

No.	Sex	Age (yrs)	Smoker	Area of bowel involvement	Frequency of diarrhoea over 24 hr	BMI kg/M^2	Patient's perception of appetite	Medication
1	F	54	No	Small and large	2	15.5*	Poor	None
2	F	36	No	Small and large+	None	17.7*	Good	Prednisolone sodium phosphate, metronidazole, sulphasalazine
3	F	52	Yes	Small	None	21.9	Good	None
4	F	22	No	Small	2	19.8*	Poor	Prednisolone sodium phosphate
5	F	32	No	Small	2	22.2	Satisfactory	Sulphasalazine
6	F	46	Yes	Small and large	3	22.2	Satisfactory	Metronidazole, prednisolone sodium phosphate
7	F	43	Yes	Small++	4	20.6	Good	Azathioprine, prednisolone sodium phosphate
8	F	54	No	Small	5	30.1	Good	Sulphasalazine
9	F	51	No	Small	3	18.1*	Poor	None

BMI, body mass index; * Underweight, BMI = 20.0 or less

+ Partial resection of the small and large bowel; ++ Partial resection of the small bowel

TABLE 8.I continued

No.	Sex	Age (yrs)	Smoker	Area of bowel involvement	Frequency of diarrhoea over 24 hr	BMI kg/M^2	Patient's perception of appetite	Medication
10	M	52	Yes	Small and large	None	22.8	Good	None
11	F	50	Yes	Small	3	24.7	Good	None
12	F	21	No	Small	None	18.0*	Poor	None
13	F	35	No	Small and large	3	20.6	Very good	Ibuprofen, prednisolone sodium phosphate
14	F	53	Yes	Small and large	2	16.3*	Poor	Diphenoxylate hydrochloride BP + atropine sulphate (Lomotil, Gold Cross)
15	F	37	No	Small and large	6	22.4	Good	Prednisolone sodium phosphate
16	M	20	No	Small and large	None	22.5	Very good	Sulphasalazine, prednisolone sodium phosphate
17	M	37	No	Small and large	None	29.7	Very good	Sulphasalazine
18	F	41	Yes	Small	None	21.6	Good	Sulphasalazine
19	F	31	No	Small and large	None	22.3	Poor	Prednisolone sodium phosphate
20	M	36	Yes	Small	None	20.4	Satisfactory	Prednisolone sodium phosphate, sulphasalazine

BMI, body mass index; * Underweight, BMI = 20.0 or less

clinical details are given in Table 8.II. None of the women were taking oral contraceptive agents. The mean duration of disease from diagnosis was 7.7 (range 2-19) years. All of the patients were clinically well and receiving gluten-free diets although 2 of them admitted to having difficulty in observing the diet. The mean body mass index was 22.3 (range 17.7 - 27.8) and by this criterion 4 of the patients were below their normal weight. None of the patients complained of diarrhoea.

Patients receiving total parenteral nutrition

The 2 patients investigated were both men, and were seen as outpatients. Patient A, aged 52 years, had undergone removal of the small bowel as a result of thrombosis of the superior mesenteric artery following an operation on a leg ulcer. This patient had been receiving total parenteral nutrition (TPN) for 2 years and reported good health and an increase in weight of 4.5 kg since therapy began. Patient B, aged 57 years, had undergone a colectomy after diagnosis of cancer of the bowel and had been receiving TPN for 18 months. This patient also reported good health and an increase in weight of 14.5 kg since the start of TPN. These patients had been maintained daily on the following TPN solutions: amino acids + glucose + electrolytes (Vamin 9 Glucose, KabiVitrum Ltd.); essential fatty acids + linoleic acid and linolenic acids (Intralipid, KabiVitrum Ltd.); glucose + trace elements (zinc) + electrolytes (Glucoplex 1000, Geistlich Ltd.). Preparations added to the TPN solutions were: water - soluble vitamins (Solvito, KabiVitrum Ltd.); fat-soluble vitamins (Vitlipid, KabiVitrum Ltd.); trace elements (iron, zinc, manganese, copper, fluoride, iodine) + electrolytes (Addamel, KabiVitrum Ltd.).

Healthy control subjects

The data obtained for the healthy control subjects, aged 18-64 years, previously investigated as described in Chapter 6, was used for the purpose of comparison with the data obtained from the present study. Several of the patients in the present study and in subsequent studies were aged over 65 years and additional blood samples were obtained from 8 healthy control subjects, all men, aged 70-82 (mean 75.5) years.

TABLE 8.II

CLINICAL DETAILS OF PATIENTS WITH COELIAC DISEASE

No.	Sex	Age (yrs)	Smoker	Duration of disease (yrs)	Strict adherence to diet	BMI kg/M^2	Medication
1	F	48	Yes	5	Yes	18.4*	Calcium gluconate + calciferol (Chocovite, Medo Ltd)
2	F	41	No	15	Yes	24.6	None
3	F	53	No	19	Yes	24.6	None
4	M	46	No	7	No	21.3	Phenytoin Sodium
5	M	66	Yes	10	Yes	24.5	Diazepam
6	M	67	Yes	3	Yes	23.4	None
7	M	69	No	4	No	19.8*	Atenol + chlorthalidone (Tenoretic, Stuart Ltd)
8	F	23	No	3	Yes	17.7*	None
9	F	46	No	9	Yes	22.1	None
10	M	39	Yes	3	Yes	23.7	Ibuprofen
11	F	37	No	15	Yes	23.0	None
12	F	35	Yes	2	Yes	17.9*	None
13	M	71	No	4	Yes	22.7	None
14	F	34	No	11	Yes	25.3	Atenol
15	M	69	No	6	Yes	27.8	None
16	F	50	Yes	7	Yes	20.3	None

BMI, body mass index

* Underweight, BMI = 20.0 or less

Samples

Blood samples were collected and subdivided as described in Chapter 6.

Analyses

Separation of white cells and analysis of zinc, copper and selenium in leucocytes, plasma and whole blood, cell counting and packed cell volume (PCV) measurements, were performed by the techniques given in Chapters 4 and 5. The concentrations of zinc, copper and selenium in erythrocytes (pmol/10^6 cells) were obtained by the following calculation:-

1. Contribution from plasma in 1 litre of whole blood =

$$\frac{\text{Concentration of trace element in plasma (pmol } l^{-1}) \times (100 - \text{PCV})}{100} = A$$

2. Contribution from leucocytes in 1 litre of whole blood =

$$\frac{\text{Concentration of trace element in leucocytes (pmol/}10^6 \text{ cells)} \times \text{Number of white cells in whole blood } (10^9\ l^{-1})}{10^6} = B$$

3. Contribution from erythrocytes in 1 litre of whole blood =

$$\text{Concentration of trace element in whole blood (pmol } l^{-1}) - (A + B) = C$$

Concentration of trace element in red cells (pmol/10^6 cells)

$$= \frac{C}{\text{Number of red cells in whole blood } (10^{12}\ l^{-1})} \times 10^6$$

Plasma albumin and caeruloplasmin concentrations were determined as described in Chapter 7. The selenium concentration in the TPN solutions and in the preparations added to these solutions was investigated by the method given in Chapter 5.

Statistical procedures

The statistical procedures used in this project are given in Chapter 5. Each patient was matched for age, sex, and smoking habit in men, with 2 control subjects. For each pair of control subjects, the results for each parameter were meaned, and statistical analysis was performed using the mean value. The age range for the 40 adult healthy controls who were matched with the Crohn's patients was 21-59 (mean 40.0) years, and for the 32 adult healthy controls who were matched with the coeliac patients was 21-74 (mean 47.1) years.

8.2.2 RESULTS

Patients with Crohn's disease

The individual results for the patients with Crohn's disease are given in Appendix 5. The white cell counts, red cell counts and PVC values for the whole blood samples were $7.38 \pm 2.44 \times 10^9\ l^{-1}$, $4.79 \pm 0.57 \times 10^{12}\ l^{-1}$ and 42.4 ± 3.7 respectively.

To investigate the effect of steroids on zinc, copper and selenium concentrations in leucocytes, erythrocytes and plasma, patients taking steroids (n = 9) were matched for sex, smoking habit in men, and presence or absence of diarrhoea, with those patients not taking steroids. Comparison of these two groups demonstrated significantly lower concentrations of plasma zinc ($p < 0.05$), and significantly higher concentrations of plasma copper ($p < 0.05$) in patients taking steroids (Table 8.III). There were no significant differences between these two groups for any of the other parameters shown in Table 8.III.

The zinc and copper results for the patients not taking steroids (n = 11) and the matched controls (n = 22) are shown in Figures 8.1 and 8.2 respectively. There was no significant difference for the concentration of zinc or copper in leucocytes, erythrocytes or plasma (Table 8.IV).

Figure 8.3 shows the selenium results for the total number of patients (n = 20) and the matched controls (n = 40). Patients with a

TABLE 8.III

CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN THE STEROID AND NON-STEROID TREATED GROUPS OF CROHN'S PATIENTS

Analysis	Patients taking steroids n = 9	Matched patients not taking steroids n = 9	Ratio of means (a) or mean of individual patient - patient differences (b)	95% CI for the ratio of means(a) or the mean of individual patient - patient differences(b)
Leucocyte Zn pmol/10^6 cells	101(88-97)$^{\Omega}$	101(89-104)$^{\Omega}$	0.99 (a)	0.89 to 1.11 (a)
Erythrocyte Zn pmol/10^6 cells	15.9 ± 2.7	16.1 ± 2.4	-0.23 (b)	-2.46 to 2.00 (b)
Plasma Zn µmol l^{-1}	10.1 ± 1.5^{+}	12.7 ± 2.0	-2.53 (b)	-4.71 to -0.35 (b)
Leucocyte Cu pmol/10^6 cells	10.1 ± 4.9	9.3 ± 4.9	0.81 (b)	-3.86 to 5.48 (b)
Erythrocyte Cu pmol/10^6 cells	1.06 ± 0.23	1.09 ± 0.16	-0.04 (b)	-0.27 to 0.19 (b)
Plasma Cu µmol l^{-1}	23.8 ± 6.1^{+}	17.8 ± 3.5	6.00 (b)	0.93 to 11.07 (b)
Leucocyte Se pmol/10^6 cells	1.59 ± 0.41	1.65 ± 0.59	-0.05 (b)	-0.31 to 0.41 (b)
Erythrocyte Se pmol/10^6 cells	0.144 ± 0.043	0.153 ± 0.052	-0.009 (b)	-0.060 to 0.042 (b)
Plasma Se µmol l^{-1}	1.25 ± 0.25	1.32 ± 0.21	-0.07 (b)	-0.32 to 0.18 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+}$ $p < 0.05$

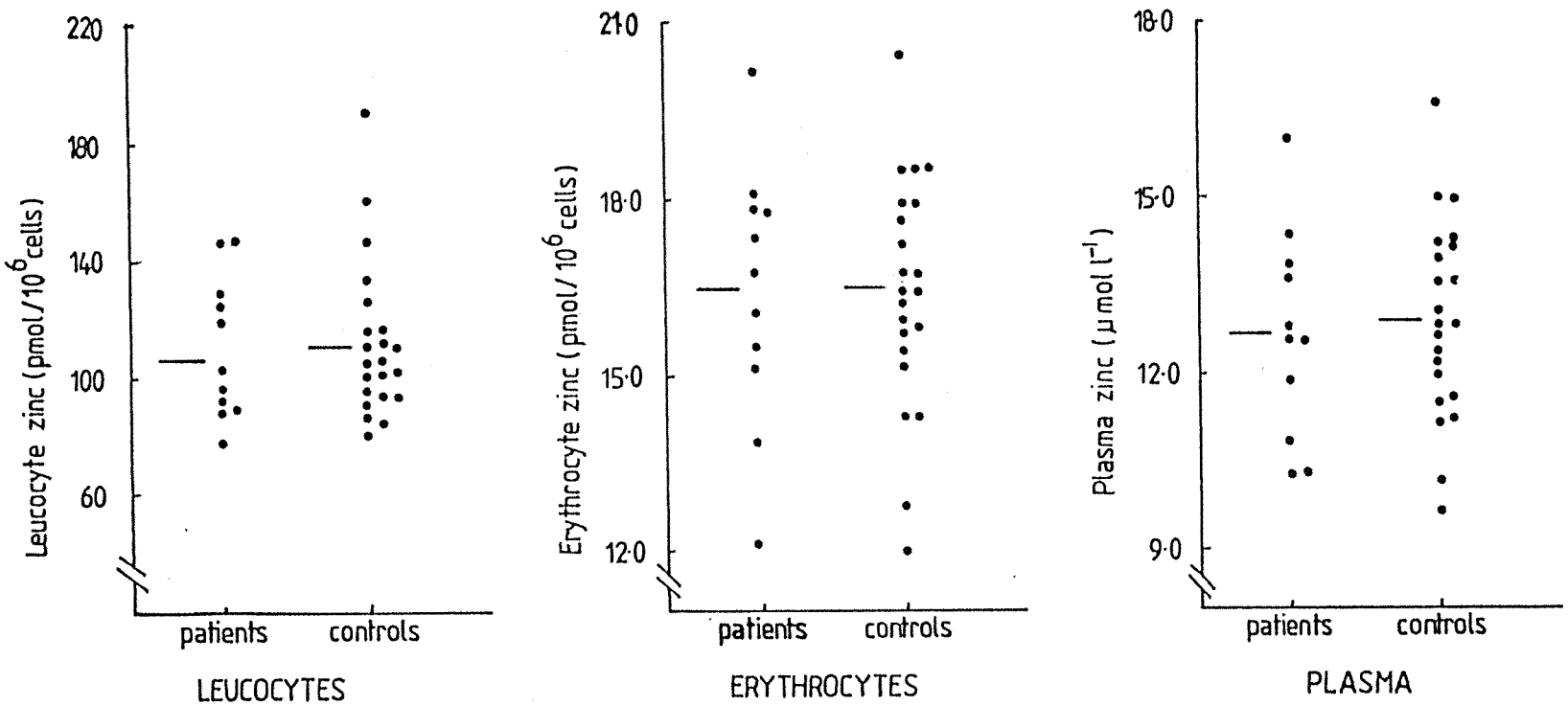


Figure 8.1

Scattergram of zinc concentrations in leucocytes, erythrocytes and plasma, in patients with Crohn's disease not taking steroids and matched controls. Horizontal lines show the mean values (geometric mean for leucocyte zinc values) of the data sets.

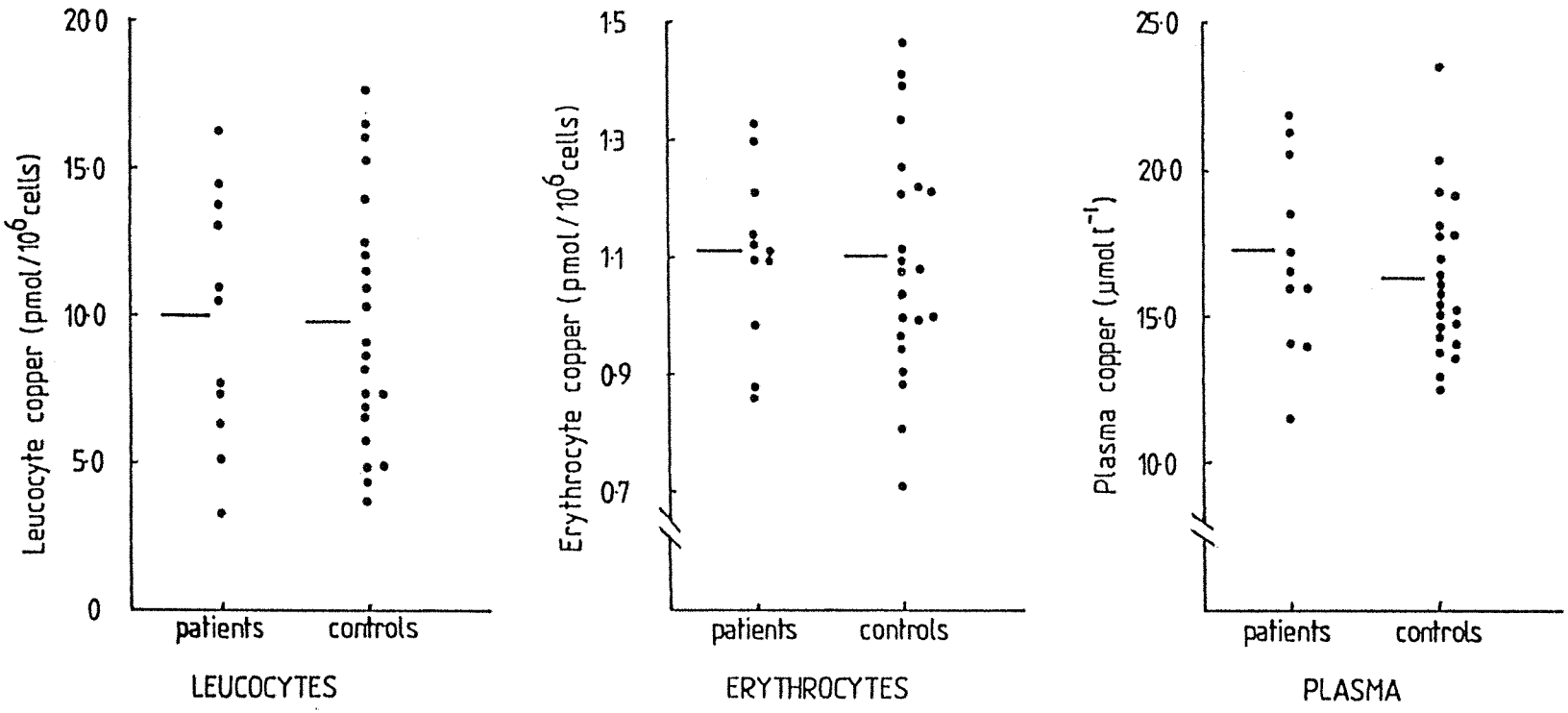


Figure 8.2

Scattergram of copper concentrations in leucocytes, erythrocytes and plasma, in patients with Crohn's disease not taking steroids and matched controls. Horizontal lines show the mean values of the data sets

TABLE 8.IV

CONCENTRATIONS OF ZINC AND COPPER IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN THE CROHN'S PATIENTS NOT TAKING STEROIDS AND MATCHED CONTROLS

Analysis	Non-steroid treated group n = 11	Matched controls n = 22	Ratio of means (a) or mean of individual patient - control differences (b)	95% CI for the ratio of means (a) or the mean of individual patient - control differences (b)
Leucocyte Zn pmol/10^6 cells	107(90-127)$^{\Omega}$	111(96-121)$^{\Omega}$	0.96 (a)	0.89 to 1.04 (a)
Erythrocyte Zn pmol/10^6 cells	16.5 ± 2.3	16.5 ± 2.0	-0.06 (b)	-1.91 to 1.79 (b)
Plasma Zn µmol l^{-1}	12.7 ± 1.8	12.9 ± 1.7	-0.25 (b)	-1.31 to 0.81 (b)
Leucocyte Cu pmol/10^6 cells	10.0 ± 4.3	9.8 ± 4.2	0.15 (b)	-3.37 to 3.67 (b)
Erythrocyte Cu pmol/10^6 cells	1.11 ± 0.15	1.10 ± 0.20	-0.01 (b)	-0.16 to 0.15 (b)
Plasma Cu µmol l^{-1}	17.3 ± 3.3	16.3 ± 2.6	0.92 (b)	-1.55 to 3.39 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

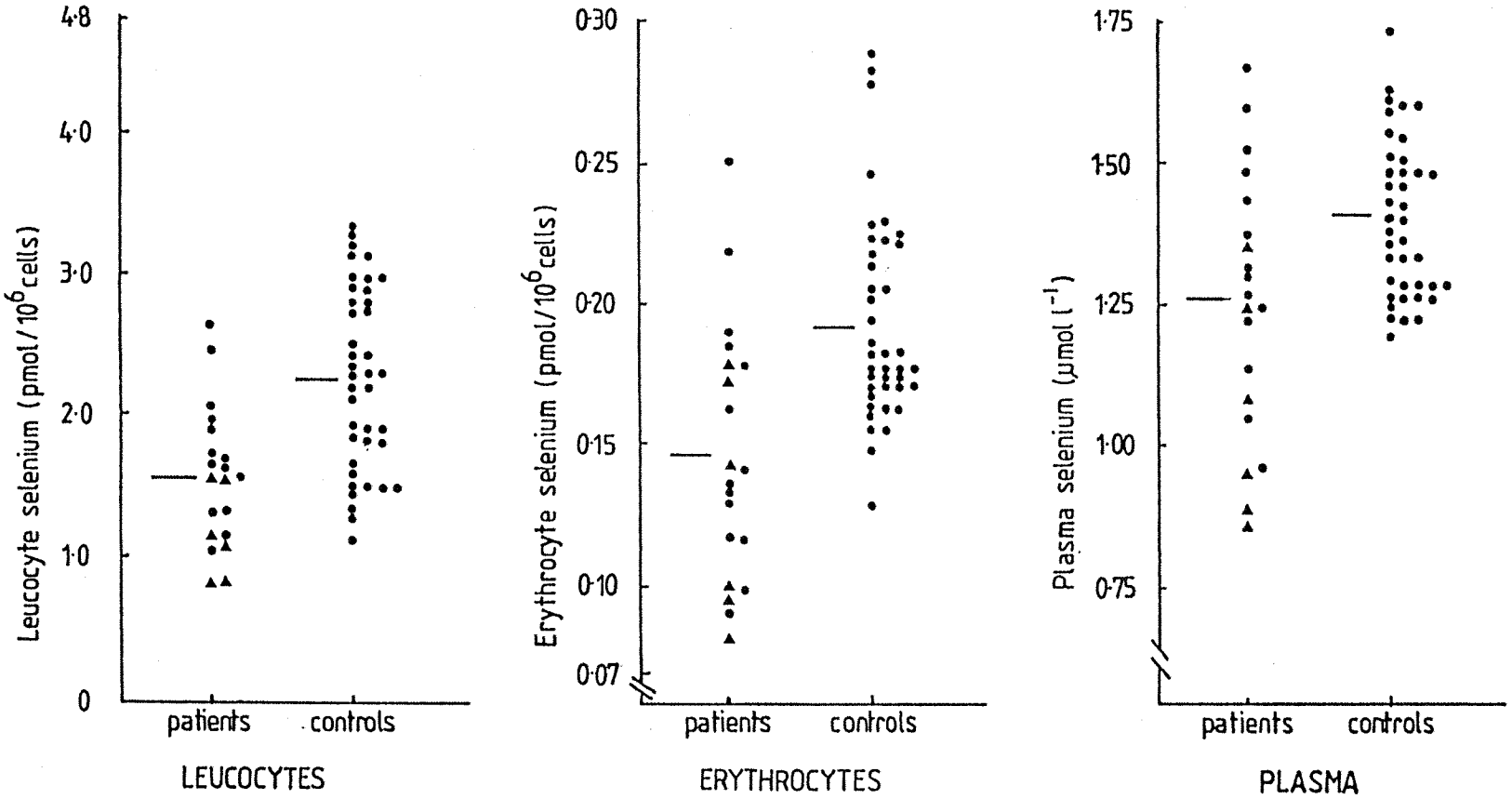


Figure 8.3

Scattergram of selenium concentrations in leucocytes, erythrocytes and plasma, in patients with Crohn's disease and matched controls. Horizontal lines show the mean values of the data sets; ▲ denotes patients with a low body mass index

TABLE 8.V

CONCENTRATIONS OF SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN CROHN'S PATIENTS AND MATCHED CONTROLS

Analysis	Crohn's patients n = 20	Matched controls n = 40	Mean of individual patient - control differences	95% CI for the mean of individual patient - control differences
Leucocyte Se pmol/10^6 cells	$1.57 \pm 0.49^{+++}$	2.26 ± 0.64	-0.65	-0.96 to -0.34
Erythrocyte Se pmol/10^6 cells	$0.146 \pm 0.046^{+++}$	0.193 ± 0.037	-0.047	-0.068 to -0.026
Plasma Se μmol l^{-1}	$1.26 \pm 0.23^{++}$	1.41 ± 0.14	-0.15	-0.27 to -0.04

$^{++}$ $p < 0.01$

$^{+++}$ $p < 0.001$

low body mass index are also indicated in Figure 8.3. A significant decrease in the concentration of selenium in leucocytes and erythrocytes ($p < 0.001$) and plasma ($p < 0.01$) was found in the patients with Crohn's disease when compared with the control subjects (Table 8.V).

There were significant correlations between body mass index and selenium concentrations in leucocytes ($p < 0.01$, $r = 0.60$) and plasma ($p < 0.01$, $r = 0.59$), but not in erythrocytes, in the Crohn's patients. It is also interesting to note that in the patients studied there were no significant correlations between the presence or absence of diarrhoea and body mass index, or the presence or absence of diarrhoea and selenium concentrations in leucocytes, erythrocytes or plasma.

A significant correlation was demonstrated between concentrations of selenium in leucocytes and plasma ($p < 0.001$, $r = 0.73$), leucocytes and erythrocytes ($p < 0.001$, $r = 0.69$) and erythrocytes and plasma ($p < 0.001$, $r = 0.72$) in the patient group. No similar relationship was noted for zinc or copper. Plasma albumin concentrations were similar in patients (45 ± 4 g l^{-1}) and matched controls (46 ± 3 g l^{-1}), and there was no significant correlation between plasma zinc and albumin or plasma selenium and albumin concentrations in either of the groups.

Patients with treated coeliac disease

The individual results for the patients with treated coeliac disease are given in Appendix 6. The white cell counts, red cell counts and PCV values for the whole blood samples were $5.63 \pm 1.12 \times 10^9$ l^{-1}, $4.95 \pm 0.42 \times 10^{12}$ l^{-1} and 43.9 ± 3.0 respectively.

The zinc, copper and selenium concentrations in leucocytes, erythrocytes and plasma for the patient and control groups are summarised in Table 8.VI. There were no significant differences in the concentrations of zinc and copper between the coeliac and control groups, although the 95% confidence intervals for the difference between, or ratio of, population means show that leucocyte and plasma zinc concentrations are more likely to be lower in patients with coeliac disease. Scattergrams of zinc and copper results for the patients and matched controls are presented in Figures 8.4 and 8.5 respectively. A significant decrease in the concentration of selenium in leucocytes ($p < 0.001$), erythrocytes ($p < 0.05$) and plasma ($p < 0.01$) was found in the patients with coeliac disease when compared to

TABLE 8.VI

CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN PATIENTS WITH COELIAC DISEASE AND MATCHED CONTROLS

Analysis	Coeliac patients n = 16	Matched controls n = 32	Ratio of means (a) or mean of individual patient - control differences (b)	95% CI for the ratio of means (a) or the mean of individual patient - control differences (b)
Leucocyte Zn pmol/10^6 cells	106(93-110)$^{\Omega}$	116(103-130)$^{\Omega}$	0.91 (a)	0.81 to 1.03 (a)
Erythrocyte Zn pmol/10^6 cells	16.3 ± 2.2	16.4 ± 1.9	-0.16 (b)	-1.75 to 1.44 (b)
Plasma Zn µmol l^{-1}	11.5 ± 1.5	12.4 ± 1.5	-0.92 (b)	-1.96 to 0.12 (b)
Leucocyte Cu pmol/10^6 cells	9.7 ± 3.8	9.9 ± 4.3	-0.14 (b)	-2.83 to 2.55 (b)
Erythrocyte Cu pmol/10^6 cells	1.06 ± 0.18	1.09 ± 0.17	-0.03 (b)	-0.14 to 0.08 (b)
Plasma Cu µmol l^{-1}	17.0 ± 4.9	16.1 ± 2.4	0.94 (b)	-1.77 to 3.68 (b)
Leucocyte Se pmol/10^6 cells	1.39 ± 0.42^{+++}	2.11 ± 0.59	-0.72 (b)	-1.07 to -0.37 (b)
Erythrocyte Se pmol/10^6 cells	0.146 ± 0.052^{+}	0.182 ± 0.037	-0.037 (b)	-0.069 to -0.005(b)
Plasma Se µmol l^{-1}	1.11 ± 0.25^{++}	1.43 ± 0.13	-0.32 (b)	-0.48 to -0.16 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+}$ $p < 0.05$

$^{++}$ $p < 0.01$

$^{+++}$ $p < 0.001$

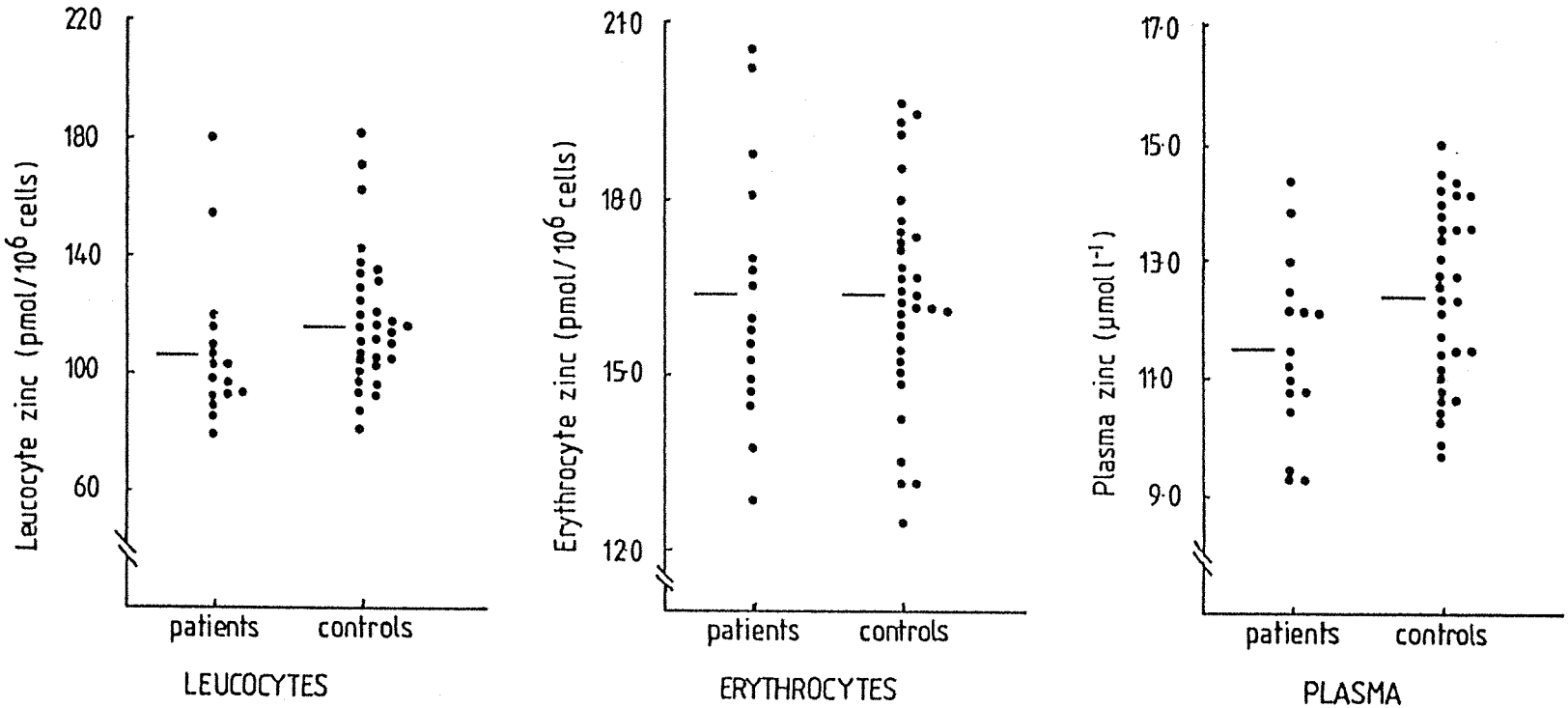


Figure 8.4

Scattergram of zinc concentrations in leucocytes, erythrocytes and plasma, in patients with coeliac disease and matched controls. Horizontal lines show the mean values (geometric mean for leucocyte zinc values) of the data sets

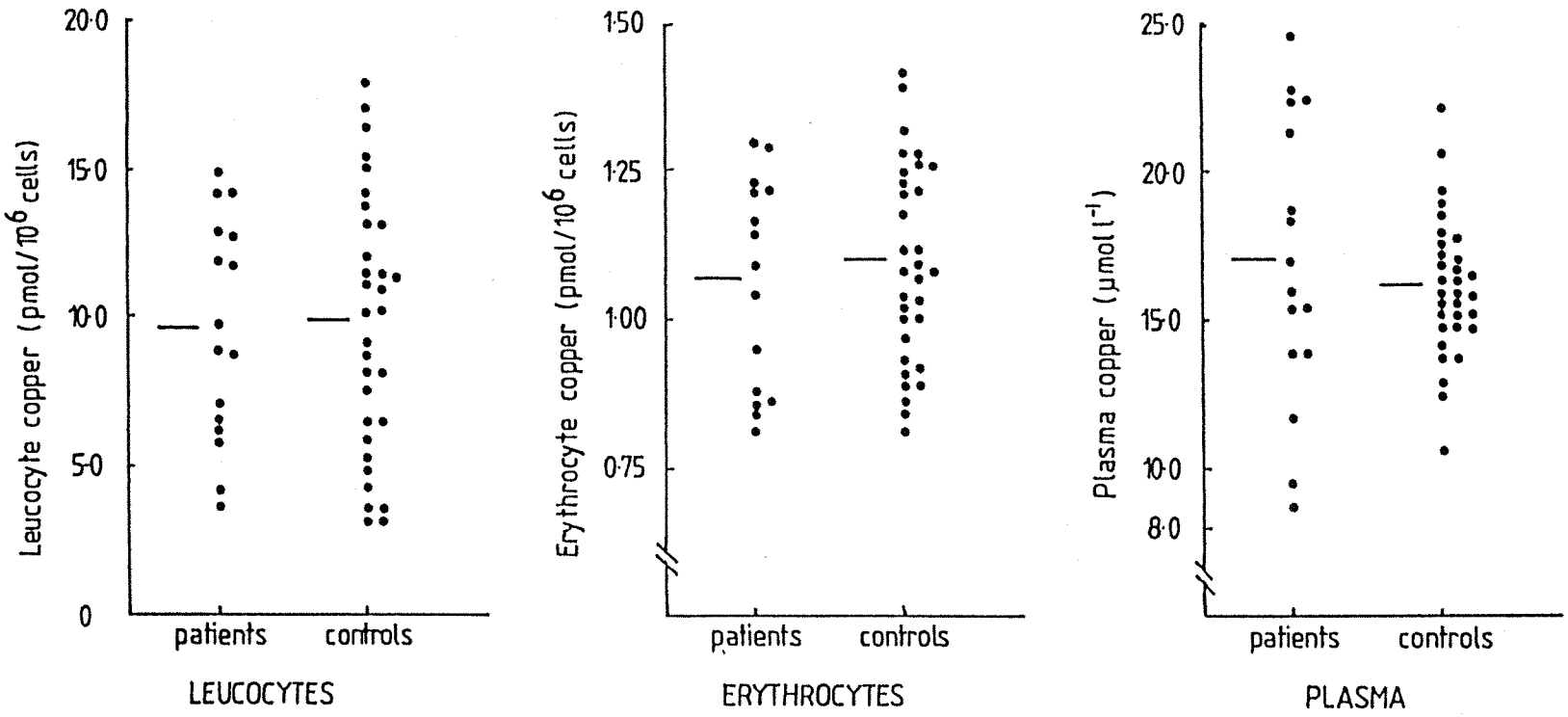


Figure 8.5

Scattergram of copper concentrations in leucocytes, erythrocytes and plasma, in patients with coeliac disease and matched controls. Horizontal lines show the mean values of the data sets

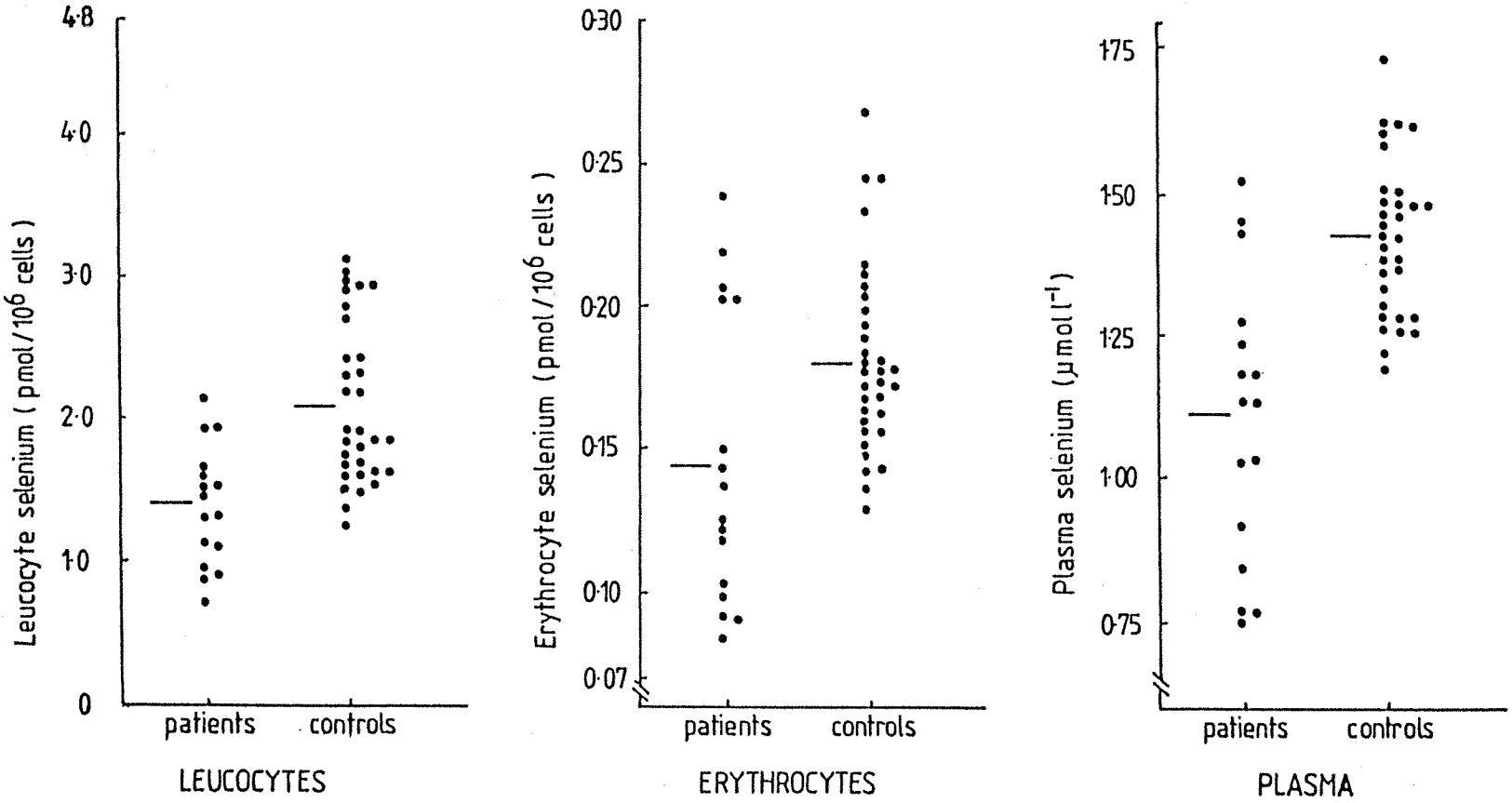


Figure 8.6

Scattergram of selenium concentrations in leucocytes, erythrocytes and plasma, in patients with coeliac disease and matched controls. Horizontal lines show the mean values of the data sets

the control subjects. Figure 8.6 presents a scattergram of the selenium results for patients and controls.

In the patients studied, there were no significant correlations between body mass index and selenium concentrations in leucocytes, erythrocytes or plasma. There was no significant difference between plasma albumin concentrations in patients (45 ± 4 g l^{-1}) and matched controls (44 ± 3 g l^{-1}), and there was no significant correlation between plasma zinc and albumin or plasma selenium and albumin concentrations in either of the groups. There was a significant correlation between selenium concentrations in leucocytes and plasma ($p < 0.001$, $r = 0.75$), leucocytes and erythrocytes ($p < 0.01$, $r = 0.66$), and erythrocytes and plasma ($p < 0.05$, $r = 0.57$) in the patient group. No similar relationship was noted for zinc. A significant correlation was observed for copper concentrations in erythrocytes and plasma ($p < 0.05$, $r = 0.58$), but not in leucocytes and plasma or leucocytes and erythrocytes.

Patients receiving total parenteral nutrition (TPN)

The zinc, copper and selenium results for the 2 patients receiving TPN are shown in Table 8.VII. Both patients demonstrated zinc and copper concentrations in leucocytes, erythrocytes and plasma within the reference ranges as given in Chapter 6. In contrast, the selenium concentrations in leucocytes, erythrocytes and plasma were extremely low, and well below the reference ranges for all measurements. There was no detectable selenium in the TPN solutions which had been administered to these patients, or in the preparations added to these solutions.

8.3 ZINC, COPPER AND SELENIUM STATUS OF NEWLY DIAGNOSED PATIENTS WITH COELIAC DISEASE, BEFORE AND AFTER WITHDRAWAL OF GLUTEN FROM THE DIET

8.3.1 PATIENTS AND METHODS

Patients

Four newly diagnosed adult patients with coeliac disease, 3 women, aged 25, 45 and 48 years (A, B and C respectively), and 1 man, aged 39 years (D), were studied. At initial presentation, the untreated patients had partial villous atrophy as shown by jejunal

TABLE 8.VII

ZINC, COPPER AND SELENIUM RESULTS FOR PATIENTS RECEIVING TOTAL PARENTERAL NUTRITION

		Patient A	Patient B
ZINC	Leucocytes pmol/10^6 cells	135	193
	Erythrocytes pmol/10^6 cells	16.9	14.2
	Plasma µmol l^{-1}	11.2	9.4
COPPER	Leucocytes pmol/10^6 cells	7.8	15.4
	Erythrocytes pmol/10^6 cells	1.31	1.27
	Plasma µmol l^{-1}	12.3	17.2
SELENIUM	Leucocytes pmol/10^6 cells	0.37 *	0.33 *
	Erythrocytes pmol/10^6 cells	0.051*	0.054*
	Plasma µmol l^{-1}	0.38 *	0.29 *

* Values below the reference ranges

biopsy. This group subsequently demonstrated a clinical response to a gluten-free diet, and all biopsy samples which were obtained approximately 10 months after withdrawal of gluten from the diet showed histological improvement in response to treatment.

Blood samples were taken prior to gluten-withdrawal and at 2-monthly intervals after start of treatment, over a period of 10 months. Two control subjects, both women, aged 35 and 49 years (E and F respectively) were also studied at similar time intervals.

Analyses

Separation of white cells and analysis of zinc, copper and selenium in leucocytes and plasma were performed by the methods given in Chapters 4 and 5.

Statistical procedures

As the number of subjects studied was small, there was no statistical analysis of the data.

8.3.2 RESULTS

The zinc results for the coeliac patients and control subjects are presented in Figure 8.7. Table 8.VIII gives the mean leucocyte and plasma zinc values for each subject, and the percentage variation about the mean values over the period studied. Patients A, B and C demonstrated similar percentage variations about the mean leucocyte and plasma zinc values to those for the control subjects, E and F. Patient D showed a much larger variation for these parameters. The reference ranges for zinc, copper and selenium in leucocytes, plasma and whole blood are given in Chapter 6. Although patients B and D demonstrated leucocyte and plasma zinc concentrations below the reference ranges before diet enforcement, these values increased to within the reference ranges after gluten was withdrawn from the diet (Table 8.IX). Patients A and C showed a satisfactory body zinc status at all times during the study.

Figure 8.8 shows the copper results for the patients and control subjects. Over the period of study, all of the patients showed similar percentage variations about the mean leucocyte and plasma copper values to those for the control subjects (Table 8.X). None of the individual values for leucocyte and plasma copper in the coeliac

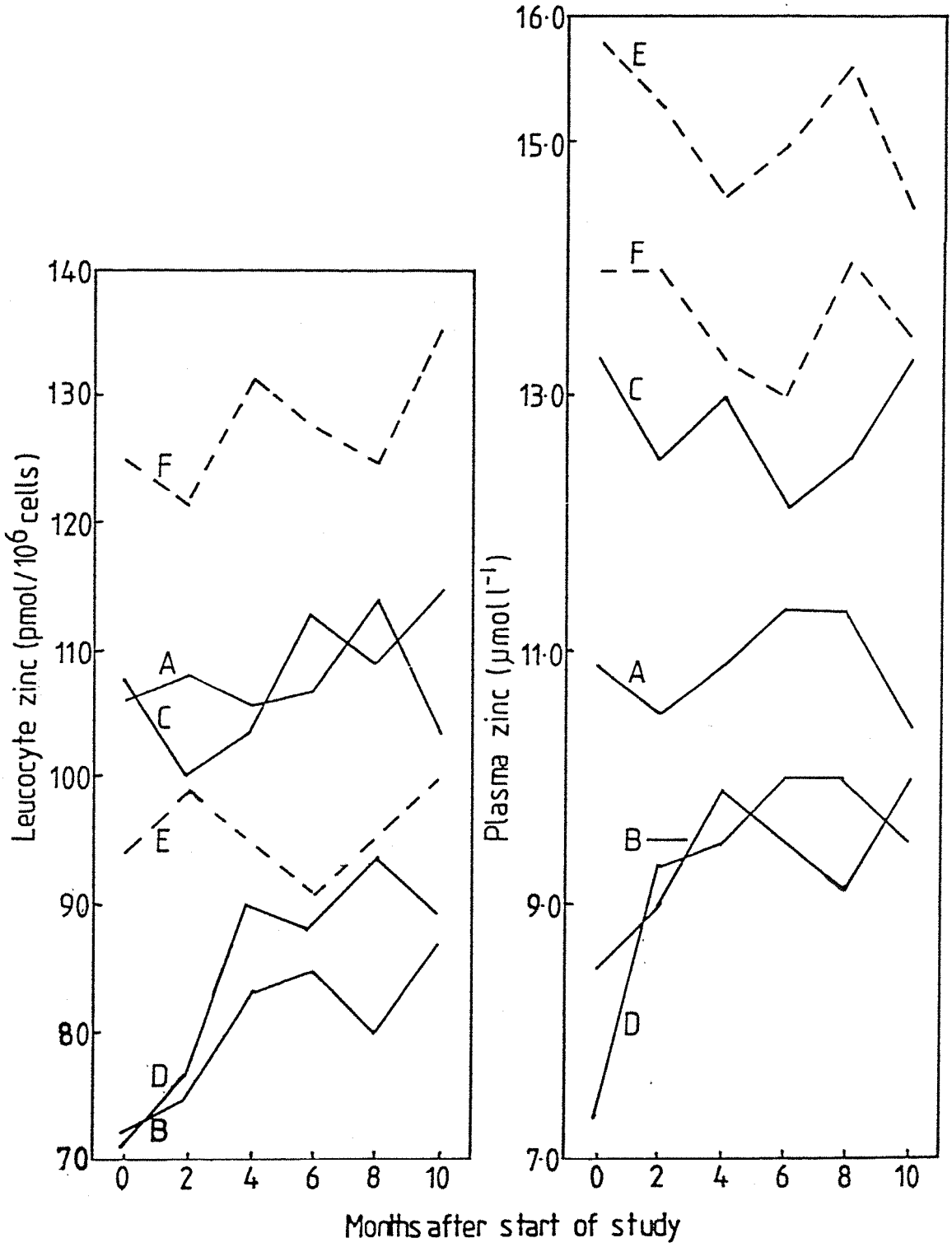


Figure 8.7

Zinc concentrations in leucocytes and plasma, in coeliac patients (A,B,C,D ——) before (0 months) and after (2,4,6,8,10 months) withdrawal of gluten from the diet, and in control subjects (E,F---) who were studied at similar time intervals

TABLE 8.VIII

MEAN LEUCOCYTE AND PLASMA ZINC VALUES IN COELIAC PATIENTS AND CONTROLS, AND PERCENTAGE VARIATION ABOUT THE MEAN OVER THE PERIOD STUDIED

	LEUCOCYTE ZINC		PLASMA ZINC	
	mean ($pmol/10^6$ cells)	% variation about the mean	mean ($\mu mol\ l^{-1}$)	% variation about the mean
Patient A	107.3	96 - 106	10.9	95 - 104
Patient B	80.3	90 - 108	9.3	91 - 108
Patient C	108.2	92 - 106	12.8	95 - 104
Patient D	86.3	81 - 115	9.3	78 - 108
Control E	95.7	95 - 104	15.1	96 - 105
Control F	127.7	96 - 106	13.7	95 - 103

TABLE 8.IX

INDIVIDUAL VALUES FOR LEUCOCYTE AND PLASMA ZINC IN COELIAC PATIENTS BEFORE AND AFTER GLUTEN-WITHDRAWAL, AND IN CONTROL SUBJECTS

Analysis	Period after start of study (months)	Patients A	B	C	D	Controls E	F
Leucocyte zinc $pmol/10^6$ cells	0	106	72*	108	70*	94	125
	2	108	75*	100	77*	99	122
	4	106	83	104	90	95	131
	6	107	85	113	88	91	128
	8	114	80	109	94	95	125
	10	103	87	115	99	100	135
Plasma zinc $\mu mol\ l^{-1}$	0	10.9	8.5*	13.3	7.3*	15.8	14.0
	2	10.5	9.0	12.5	9.3	15.3	14.0
	4	10.9	9.9	13.0	9.5	14.6	13.3
	6	11.3	9.5	12.1	10.0	15.0	13.0
	8	11.3	9.1	12.5	10.0	15.6	14.1
	10	10.4	10.0	13.3	9.5	14.5	13.5

* Values below the reference ranges

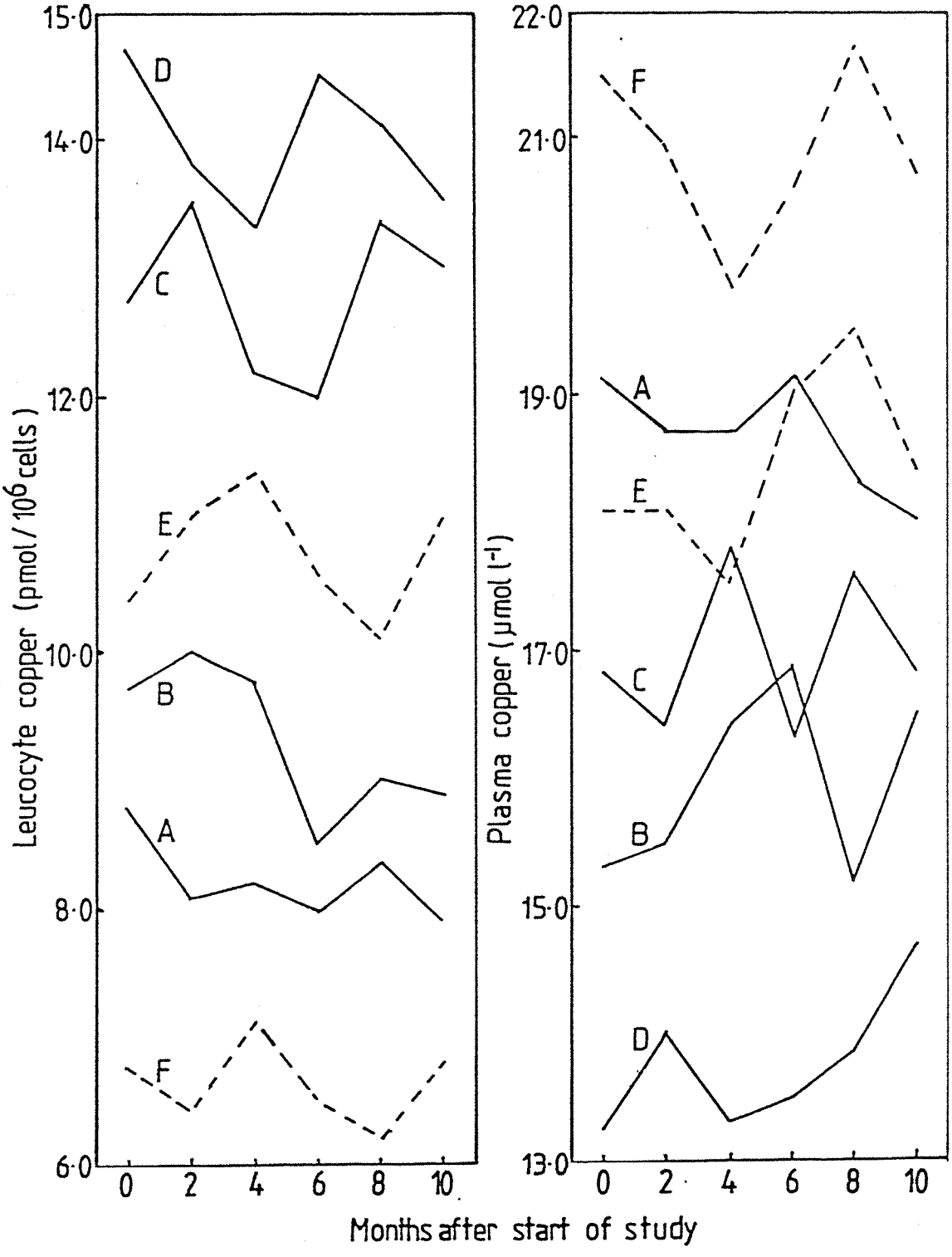


Figure 8.8

Copper concentrations in leucocytes and plasma, in coeliac patients (A,B,C,D ——) before (0 months) and after (2,4,6,8,10 months) withdrawal of gluten from the diet, and in control subjects (E,F---) who were studied at similar time intervals

TABLE 8.X

MEAN LEUCOCYTE AND PLASMA COPPER VALUES IN COELIAC PATIENTS AND CONTROLS, AND PERCENTAGE VARIATION ABOUT THE MEAN OVER THE PERIOD STUDIED

	LEUCOCYTE COPPER		PLASMA COPPER	
	mean ($pmol/10^6$ cells)	% variation about the mean	mean ($\mu mol\ l^{-1}$)	% variation about the mean
Patient A	8.2	96 - 107	18.7	96 - 102
Patient B	9.4	90 - 106	15.9	96 - 103
Patient C	12.8	94 - 105	16.9	96 - 105
Patient D	14.0	95 - 105	13.8	96 - 107
Control E	10.8	94 - 106	18.4	95 - 106
Control F	6.6	94 - 108	20.9	95 - 104

TABLE 8.XI

INDIVIDUAL VALUES FOR LEUCOCYTE AND PLASMA COPPER IN COELIAC PATIENTS BEFORE AND AFTER GLUTEN-WITHDRAWAL, AND IN CONTROL SUBJECTS

Analysis	Period after start of study (months)	Patients A	B	C	D	Controls E	F
Leucocyte copper $pmol/10^6$ cells	0	8.8	9.7	12.7	14.7	10.4	6.8
	2	8.1	10.0	13.5	13.7	11.1	6.4
	4	8.2	9.7	12.2	13.3	11.4	7.1
	6	8.0	8.5	12.0	14.5	10.6	6.5
	8	8.3	9.0	13.4	14.1	10.1	6.2
	10	7.9	9.4	13.0	13.5	11.0	6.8
Plasma copper $\mu mol\ l^{-1}$	0	19.1	15.3	16.8	13.2	18.1	21.5
	2	18.7	15.5	16.4	14.0	18.1	20.9
	4	18.7	16.4	17.8	13.3	17.5	19.8
	6	19.1	16.8	16.3	13.5	19.0	20.6
	8	18.3	15.2	17.3	13.9	19.5	21.7
	10	18.0	16.0	16.8	14.7	18.4	20.7

patients were below the reference ranges before or after gluten withdrawal (Table 8.XI).

Selenium concentrations in leucocytes and plasma for the patients and controls are shown in Figure 8.9. Patients A and C demonstrated a satisfactory selenium status before the start of treatment, although concentrations in leucocytes and plasma decreased to below the reference ranges during the 10 month period following diet enforcement (Table 8.XII). Patient B demonstrated a lowered selenium status before withdrawal of gluten from the diet, but also showed a further decrease in selenium concentrations in leucocytes and plasma during the period of study (Table 8.XII). The decrease in selenium concentrations after withdrawal of gluten from the diet in patients A, B and C was reflected by the larger percentage variations about the mean values for these patients when compared to the control subjects (Table 8.XIII). Although patient D demonstrated a lowered selenium status both before and after gluten-withdrawal (Table 8.XII), the percentage variations about the mean selenium values for leucocytes and plasma were similar to those for the control subjects (Table 8.XIII), and there was no noticeable trend towards a decrease in values.

8.4 DISCUSSION

PATIENTS WITH CROHN'S DISEASE

In the group of Crohn's patients investigated in the present study, treatment with steroids appeared to have a significant effect on the plasma zinc and copper concentrations. The effect of steroids on plasma zinc and copper levels has been well documented (Lifschitz and Henkin, 1971; Yunice et al, 1981) and discussed in Chapter 2. Some studies however have failed to find a significant association between plasma zinc concentrations and the use of steroids in patients with severe Crohn's disease although an overall decrease in plasma zinc levels was demonstrated (Solomons et al, 1977; Fleming et al, 1981). These patients had more severe disease than the patients in the present study, and it is possible that the severity of the disease was overriding the less potent effect of the steroids. In the present study, there was no significant difference in leucocyte zinc or copper concentrations in the Crohn's patients taking steroids compared to those who were not. These findings emphasize the problems involved

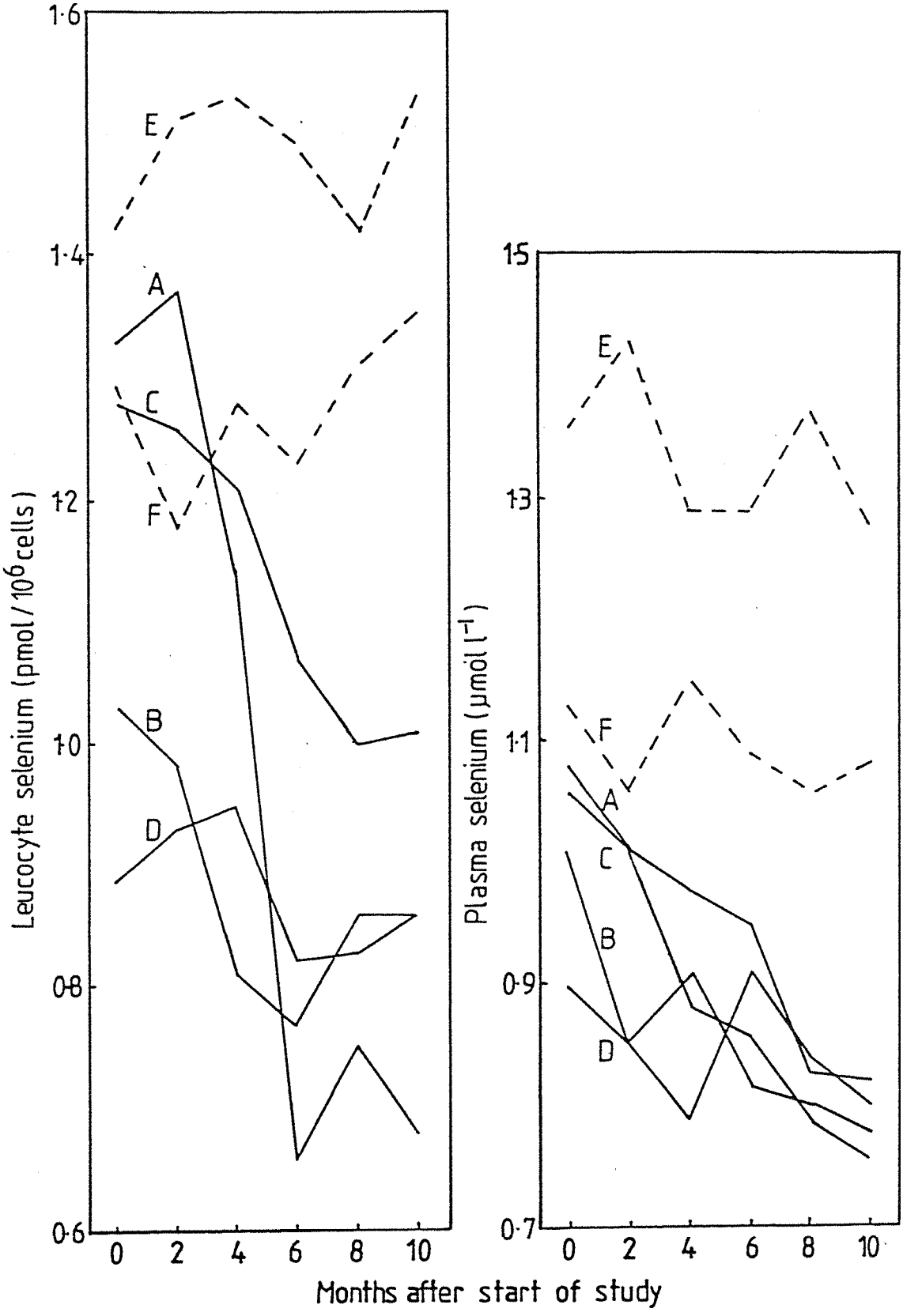


Figure 8.9

Selenium concentrations in leucocytes and plasma, in coeliac patients (A,B,C,D ——) before (0 months) and after (2,4,6,8,10 months) withdrawal of gluten from the diet, and in control subjects (E,F---) who were studied at similar time intervals

TABLE 8.XII

INDIVIDUAL VALUES FOR LEUCOCYTE AND PLASMA SELENIUM IN COELIAC PATIENTS BEFORE AND AFTER GLUTEN-WITHDRAWAL, AND IN CONTROL SUBJECTS

Analysis	Period after start of study (months)	Patients A	B	C	D	Controls E	F
Leucocyte	0	1.33	1.03*	1.28	0.89*	1.42	1.29
selenium	2	1.37	0.98*	1.26	0.93*	1.51	1.18
pmol/10^6 cells	4	1.14	0.81*	1.21	0.95*	1.53	1.28
	6	0.66*	0.77*	1.07	0.82*	1.49	1.23
	8	0.75*	0.86*	1.00*	0.83*	1.42	1.31
	10	0.68*	0.86*	1.01*	0.86*	1.53	1.35
	0	1.06	1.01*	1.08	0.90*	1.36	1.13
Plasma	2	1.01*	0.85*	1.01*	0.85*	1.43	1.06
selenium	4	0.88*	0.79*	0.98*	0.91*	1.29	1.15
µmol l^{-1}	6	0.86*	0.91*	0.95*	0.82*	1.29	1.09
	8	0.79*	0.84*	0.83*	0.80*	1.37	1.06
	10	0.76*	0.80*	0.82*	0.77*	1.28	1.08

* Values below the reference ranges

TABLE 8.XIII

MEAN LEUCOCYTE AND PLASMA SELENIUM VALUES IN COELIAC PATIENTS AND CONTROLS, AND PERCENTAGE VARIATION ABOUT THE MEAN OVER THE PERIOD STUDIED

	LEUCOCYTE SELENIUM		PLASMA SELENIUM	
	mean (pmol/10^6 cells)	% variation about the mean	mean (µmol l^{-1})	% variation about the mean
Patient A	0.99	67 - 138	0.89	85 - 119
Patient B	0.89	87 - 118	0.87	92 - 116
Patient C	1.14	88 - 112	0.95	86 - 114
Patient D	0.88	94 - 108	0.84	92 - 107
Control E	1.48	96 - 103	1.34	96 - 107
Control F	1.27	93 - 106	1.08	94 - 106

when relying on plasma concentrations alone to assess trace element status.

The zinc and copper results for the Crohn's patients not receiving steroids in the present study were not significantly different compared with those of the control subjects, indicating a satisfactory body status of these trace elements. Several studies have reported decreased levels of serum or plasma zinc concentrations in patients with severe Crohn's disease (Solomons et al, 1977; Sturniolo et al, 1980; Main et al, 1982). The depression in serum zinc concentrations observed in Crohn's disease, as suggested by Solomons et al (1977) could be due to reduced intake, impaired absorption, increased excretion, hypoalbuminaemia, or to a redistribution of zinc due to release of leucocyte endogenous mediator (LEM). Decreased plasma albumin concentrations, as well as decreased plasma zinc concentrations, have commonly been reported in Crohn's disease, and a significant relationship between these two parameters has been demonstrated (Solomons et al, 1977; McClain et al, 1980; Sturniolo et al, 1980; Main et al, 1982). It has been suggested that the commonest cause of hypoalbuminaemia in Crohn's disease is loss of the protein from the inflamed bowel into the lumen, with inability of the liver to resynthesise albumin sufficiently to maintain normal levels (Dawson, 1972). Sturniolo et al (1980) proposed that the low serum zinc values observed in patients with Crohn's disease who also had decreased serum albumin levels, reflected a reduction in serum albumin concentrations rather than a deficiency of this trace element. Increased plasma LEM activity in association with a significant decrease in plasma zinc concentrations has also been reported in patients with active Crohn's disease, but not in outpatients with clincially quiescent disease (Solomons et al, 1978). These authors concluded that acute inflammation of the bowel itself, through mediation of LEM, had contributed to the depression of circulating zinc levels, and would also invalidate the use of measurements of plasma copper in the assessment of copper status in patients with acute inflammation. The lack of any significant findings for copper, zinc or albumin values in the Crohn's patients not taking steroids in the present study may well reflect the fact that these patients were outpatients with reasonably stable disease.

Although other investigations of outpatients with Crohn's disease have also failed to find any significant difference for mean serum

zinc concentrations in the patient groups when compared to control groups, depressed concentrations of serum zinc were observed in a small proportion of the patients studied (Fleming et al, 1981; Lanfranchi et al, 1982). Interestingly, one of these studies demonstrated significantly reduced concentrations of zinc in patients with ileal resection in comparison with those with ileal or colonic involvement or colonic resection (Lanfranchi et al, 1982), whereas the other study reported no association between serum zinc values and large bowel resections or length of small bowel resected (Fleming et al, 1981). McClain et al (1980) reported an approximate equal frequency of depressed serum zinc concentrations in patients with small bowel involvement as those with terminal ileum involvement. All of the patients in the present study had involvement of the small or small and large bowel, and the two patients who had undergone partial resection of the small or small and large bowel, and who were also taking steroids, did not demonstrate decreased leucocyte zinc concentrations. Chronic hypozincaemia has been reported in 45% of patients with Crohn's disease who were followed longitudinally on an outpatient basis and depressed levels of serum zinc after oral administration of pharmacologic doses of zinc were also demonstrated in four of these patients (McClain et al, 1980). Studies with isotopic zinc have also reported impaired zinc absorption in Crohn's patients with both active disease and in remission (Sturniolo et al, 1980). Malabsorption of zinc, with resulting zinc deficiency, may play an aetiologic role in certain complications of Crohn's disease. McClain et al (1980) described two patients with Crohn's disease who developed classic symptoms of acrodermatitis enteropathica which responded rapidly to zinc supplementation. Nevertheless, it is apparent from reports in the literature that the use of indices of zinc status other than measurment of plasma zinc are necessary to evaluate the zinc status of patients with Crohn's disease. The use of taste acuity as an index of zinc status in Crohn's disease (Solomons et al, 1977; McClain et al, 1980) is questionable, and although individuals with low hair zinc content have been observed amongst a group of patients with Crohn's disease (Solomons et al, 1977), this measurement, as discussed in Chapter 2, has proved unreliable in the assessment of zinc status.

In the present study, reduced concentrations of selenium were observed in leucocytes, erythrocytes and plasma in the group of Crohn's patients, whether or not receiving steroids. Although plasma

and erythrocyte selenium concentrations below the reference values have also been reported in patients with Crohn's disease, these were inpatients with severe disease about to undergo preoperative total parenteral nutrition (Jacobson and Plantin, 1985). The reason for the lowered selenium levels observed in the group of patients in the present study is not clear. Although patients with Crohn's disease, particularly severe disease, are at risk of malabsorption, the patients in the present study had relatively stable disease and were also shown to have a satisfactory zinc and copper status. Furthermore, the selenium status of these patients was apparently unrelated to the frequency of diarrhoea. Unfortunately, it was not possible to assess any relationship between selenium concentrations and the Crohn's Disease Activity Index (CDAI) (Best et al, 1976) as there was insufficient data to calculate the CDAI. Nevertheless, it is interesting to note that there was a significant relationship between body mass index and selenium concentrations in leucocytes and plasma.

A significant association between body mass index and serum selenium concentrations has been reported in a prospective study of a large group of Finnish men who were being investigated for the relationship between serum selenium levels and cardiovascular disease (Virtamo et al, 1985). These authors postulated that the relationship between serum selenium concentrations and body mass index was indirect, and reflected the association of high serum selenium levels with general health and an above average intake of various nutrients. It is generally accepted that blood selenium concentration depends to a large extent on dietary intake, and decreased serum selenium concentrations in surgical patients who complained of lack of appetite and loss of weight have been reported (Robinson et al, 1979). In the present study, five of the six patients with a low body mass index also reported a poor appetite, and a reduced intake of selenium may therefore be in part responsible for the low selenium status observed in the Crohn's patients in the present study.

PATIENTS WITH COELIAC DISEASE

In the group of treated coeliac patients in the present study who were considered to be clinically well, reduced concentrations of selenium were observed in leucocytes, plasma and whole blood. There

was no significant relationship between any of these parameters and body mass index. Concentrations of copper and zinc were not significantly different in these coeliac patients compared with the control subjects, although a trend towards lowered leucocyte and plasma zinc concentrations was demonstrated in the patient group.

Essential trace elements have been poorly investigated in gluten-sensitive enteropathy. Strict adherence to a gluten-free diet is more difficult to implement in children. Clayton (1980) reported plasma zinc and copper concentrations in a group of children with coeliac disease and showed plasma zinc values to be toward or below the lower end of the reference range; plasma copper concentrations were not depressed. Significantly decreased serum zinc concentrations have also been demonstrated in a group of children with acute coeliac disease when compared to a control group, although not in a group of children who had been receiving a gluten-free diet for 3 to 24 months (Naveh et al, 1983). An investigation of adult patients with coeliac sprue found significantly lower concentrations of plasma zinc and albumin in the patient group when compared to the control group although only half of their patients were clinically well on gluten-free diets at the time of study (Solomons et al, 1976). These authors also reported a poor correlation between the plasma zinc and albumin values in the patient group. In the same study, there was no statistical difference for plasma copper concentrations between the patient and control groups. Decreased concentrations of plasma zinc have also been reported in patients with non-responsive coeliac syndrome, in which there is malabsorption and a flat jejunal mucosa but which there is failure to regenerate villi after at least five years of a strict gluten-free diet (Jones and Peters, 1981). It has been estimated that this group of patients comprises between 10 and 20% of adult coeliacs and in some, the malabsorption may be severe enough to cause death (Booth, 1970). A brief report by Elmes et al (1976) decribed a small group of adults with unresponsive coeliac disease whilst on a gluten-free diet who were severely ill despite administration of steroids and nutritional supplements, and who demonstrated decreased serum zinc values. Intravenous, followed by oral, adminstration of zinc supplements to these patients led to an immediate improvement in intestinal absorptive function and nutritional state. Although decreased plasma zinc concentrations were also demonstrated in a small group of patients with mild non-

responsive coeliac syndrome, oral zinc supplementation failed to provide an improvement in jejunal morphology or in the clinical status of the patients (Jones and Peters, 1981). These patients however were not so severely ill and had less marked malabsorption than the patients described by Elmes et al (1976).

There are few reports in the literature regarding the selenium status of patients with coeliac disease. Collins et al (1984) reported no significant difference in blood glutathione peroxidase activity between a group of adult patients with coeliac disease maintained on a gluten-free diet for at least one year, and a group of control subjects; blood selenium concentrations were not reported. Investigation of children with coeliac disease, whether or not they were on a gluten-free diet, also demonstrated blood glutathione peroxidase activity within the reference range (Ward et al, 1984). These authors however also reported reduced concentrations of blood selenium in both the untreated group of children and those following a gluten-free diet.

In the small group of newly diagnosed coeliac patients investigated in the present study before and after withdrawal of gluten from the diet, the lowered concentrations of both zinc and selenium that were demonstrated before commencement of treatment can most probably be attributed to the intestinal malabsorption associated with the untreated condition of this disease. After the start of diet enforcement the concentration of zinc in leucocytes and plasma increased to within the reference ranges. Crofton et al (1983b) used a zinc tolerance test to demonstrate an impaired intestinal absorption of zinc in a group of patients with untreated coeliac disease when compared to a control group, and also to demonstrate improvement in zinc absorption in the same patients after the commencement of a strict gluten-free dietary regime. It is interesting to note that three of the four patients investigated in the present study showed a decrease in selenium concentrations in leucocytes, plasma and whole blood following dietary treatment, whether or not they had a satisfactory selenium status at presentation. Although selenium concentrations did not appear to decrease in the fourth patient, a lowered selenium status was demonstrated both before and after gluten withdrawal. These observations suggest that the gluten-free diet may contain a reduced amount of selenium compared with a normal diet. In 1976, approximately half of the average intake of selenium in Britain

was reported to be derived from cereals and cereal products (Thorn et al, 1978b) and a strict gluten-free diet excludes foods which contain any form of wheat, rye, barley and oats.

Although the treated coeliac patients who had been on a gluten-free diet for an average of 7.7 years in the present study showed a tendency towards lowered levels of zinc in leucocytes and plasma, the diet regime does not exclude foods with a high zinc content and therefore a reduced intake of zinc in these patients is unlikely. There may be a risk of malabsorption of zinc, even when the patient is clinically well. Many products which are listed as gluten-free may however contain minute amounts of gluten (H. Warwick, personal communication). As it is likely that coeliac disease is due to an immunological reaction directed against gluten or one of its components (Marsh, 1983), perhaps traces of gluten which are present in most of the diets are sufficient to damage the mechanism for the absorption of this element.

In addition to malignant intestinal lymphoma, an increased incidence of gastrointestinal carcinoma has been reported in patients with adult coeliac disease (Harris et al, 1967; Brandt et al, 1978; Collins et al, 1978). Although the majority of patients show a dramatic improvement when placed on a gluten-free diet, there is no convincing evidence that the treatment is effective in preventing malignant complications (Holmes et al, 1976; Selby and Gallagher, 1979). It is interesting to note that epidemiological studies in man have reported an association between a low prediagnostic serum selenium concentration and the risk of cancer, especially gastointestinal cancers (Willett et al, 1983; Salonen et al, 1984). Unlike studies based on the comparison of serum selenium concentrations between cancer patients and controls free of cancer, prospective studies in which serum selenium levels are measured in persons free of cancer prior to follow-up do not suffer from the possibility of bias in which low selenium levels may be a consequence of already existing cancer. Although decreased plasma selenium concentrations were demonstrated in a group of patients with cancer compared with a group of normal subjects, the plasma selenium concentrations were no lower than a group of surgical patients without cancer (Robinson et al, 1979). These authors concluded that the low selenium status of the patients with cancer was more likely to be a consequence of their illness than the cause of the cancer. Nevertheless, the reported

association between a low prediagnostic selenium concentration and the risk of gastrointestinal cancer, and the role of selenium in the prevention of cancer as discussed in Chapter 2, suggests that a low selenium status may be a contributing factor to the increased risk of malignancy demonstrated in adult patients with coeliac disease.

PATIENTS RECEIVING TOTAL PARENTERAL NUTRITION

The two patients receiving long-term total parenteral nutrition (TPN) in the present study demonstrated a satisfactory zinc and copper status, but very low concentrations of selenium in leucocytes, erythrocytes and plasma.

Numerous studies have reported a depletion of zinc and copper in patients receiving their total nutritional need from intravenous solutions that were not supplemented with these trace elements at their optimum concentrations (Vilter et al, 1974; Fleming et al, 1976). Severe skin lesions similar to those seen in acrodermatitis enteropathica were observed in zinc deficiency induced by TPN and were reversed by oral adminstration of zinc sulphate (Principi et al, 1979). Neutropenia due to copper deficiency, observed in an adult receiving long-term home TPN, was corrected by intravenous administration of copper (Sriram et al, 1986). Intravenous trace element requirements may differ significantly from patient to patient. In patients with gastrointestinal disease requiring TPN, zinc and copper requirements vary with losses from the gastrointestinal tract either as a result of diarrhoea or fistulous discharge or both (Wolman et al, 1979; Shike et al, 1981). The requirements for zinc in adult patients receiving TPN for the treatment of gastointestinal disease has been determined (Wolman et al, 1979). Of particular importance was the observation that in the absence of significant diarrhoea an intravenous supplement of 46 µmol (3mg) zinc daily was sufficient to maintain zinc balance. Another study on similar patients found that 4.7 µmol (0.3mg) copper daily was sufficient to maintain copper balance in the absence of diarrhoea (Shike et al, 1981).

The requirements of selenium in TPN are less well defined. Metabolic balance studies for selenium were carried out for 7 to 12 days on 6 patients receiving postoperative TPN (van Rij et al, 1979). This study reported very low concentrations of selenium in all of the solutions infused, providing an intake of selenium of less than 7.6 to

10.1 nmol (0.6 to 0.8µg) daily during the balance periods. As a result of the combined losses of selenium of 11µg per day, of which 31% was accounted for from losses via the gastrointestinal tract and wounds and 69% from losses in the urine, there was a marked negative selenium balance in these six patients. Decreased concentrations of selenium in plasma and erythrocytes in adult patients receiving long-term home TPN have been reported (Lane et al, 1981). Patients with malabsorption may be especially at risk of developing selenium deficiency during TPN. A group of patients with Crohn's disease demonstrated a reduced selenium status at the time that TPN was introduced and showed a further decrease in the plasma and erythrocyte selenium concentrations over a three week period of intravenous infusion of solutions providing only about 5µg of selenium per 24 hours (Jacobson and Plantin, 1985).

The clinical consequences of decreasing selenium levels in patients receiving TPN is unclear. An association of parenteral nutrition, selenium deficiency, and fatal cardiomyopathy has been reported (Johnson et al, 1981b). A female patient developed muscular pain thirty days after commencement of TPN, which disappeared when selenomethionine was added to her intravenous nutrition (van Rij et al, 1979). Muscular pain and tenderness, which developed in a child receiving TPN for 18 months, was associated with very low serum selenium concentrations (Kien and Ganther, 1983). These abnormal findings reverted to normal after intravenous supplementation of elemental selenium. More recently, a woman receiving home TPN for four years after loss of her small bowel developed muscle weakness, and demonstrated extremely low concentrations of selenium in plasma and erythrocytes and decreased platelet and erythrocyte glutathione peroxidase activities (Brown et al, 1986). Treatment with intravenous selenous acid reversed the symptoms and biochemical abnormalities. Both the patients in the present study reported good health despite the low concentrations of selenium in leucocytes, erythrocytes and plasma.

Although over-enthusiastic selenium supplementation may produce toxicity, there may be a need for selenium supplementation in patients receiving TPN, particularly in those receiving long-term and home TPN (Lane et al, 1987). The chemical form and amount of selenium most suitable for supplementation in TPN has yet to be determined (Levander and Burk, 1986). A comparison of intravenous administration of

selenomethionine and sodium selenite in adult patients receiving TPN indicated that selenium retention was greater with selenomethionine (van Rij et al, 1981). These authors concluded however that further observations were necessary for the more long-term use of both these compounds by the intravenous route.

CHAPTER 9

ASSESSMENT OF ZINC, COPPER AND SELENIUM STATUS OF A POPULATION OF ALCOHOLIC SUBJECTS

9.1 INTRODUCTION

Alcoholism is a complex condition, and its definition has been and continues to be a subject of controversy. A brief definition of this disorder has been given as 'the repeated consumption of alcohol leading to dependence, physical disease or other types of harm', and daily intakes of greater than 80g of ethanol have been considered as excessive (Paton and Saunders, 1981). One of the most sensitive biochemical markers of excess chronic alcohol intake is the serum concentration of the enzyme gamma-glutamyltranspeptidase, although the activity rapidly returns to normal with abstention from alcohol, and hepatocellular damage will also contribute to enzyme elevation (Lewis and Paton, 1981). The activity of the serum enzymes, aspartate aminotransferase and alkaline phosphatase, may also be elevated as a consquence of hepatocellular injury, and although these enzymes are substantially less sensitive than gamma-glutamyltranspeptidase for alcoholism identification, their determination may assist in the identification of alcoholic liver disease (Rosalki, 1984). Reduction in serum albumin levels is uncommon in alcoholism in the absence of cirrhosis (Pollak and Buckell, 1973).

Clinical and biochemical evidence of malnutrition is frequently found in chronic alcoholics, and the mechanisms underlying nutritional deficiency involve decreased intake, poor absorption and increased requirements (Ryle and Thomson, 1984). The gastrointestinal tract is exposed to much higher concentrations of alcohol than any other tissue in the body and direct structural damage to the mucosal lining of the intestine is not uncommon in this condition (Lake-Bakaar, 1984). Damage to the small intestine, including shortened villi and decreased numbers of villous cells, has been reported in rats after chronic ingestion of ethanol (Baraona et al, 1974).

Chronic alcohol consumption has been shown to induce hepatic damage which includes fatty liver, alcoholic hepatitis and liver cirrhosis (Lieber and Salaspuro, 1985). It is now generally accepted

that malnutrition per se is not the cause of liver disease in the alcoholic subject (Morgan and McIntyre, 1985). Although the pathogenesis is still unclear, it has been proposed that hepatic injury from ethanol might be a result of enhanced lipid peroxidation (Di Luzio, 1966). Hepatic lipoperoxide levels, expressed in terms of malondialdehyde concentration/g liver, were increased in heavy drinkers with deposition of fat in the liver compared with non-alcoholic subjects with histologically normal liver (Suemastsu et al, 1981). The same study also showed a significant correlation between liver lipoperoxide levels and serum aspartate aminotransferase activity, both on admission to the hospital wards and after two weeks abstinence from ethanol. The reasons for ethanol stimulation of lipid peroxidation are however still not well defined. It has been proposed that ethanol starts lipid peroxidation due to the intermediate production of oxygen free radical species formed during its metabolism by the smooth endoplasmic reticulum system (Dianzani, 1987). Although the main pathway for ethanol metabolism in the hepatocyte is via the alcohol dehydrogenase pathway of the cytosol, there is an adaptive increase in the microsomal ethanol oxidising system, located in the endoplasmic reticulum, during chronic alcohol consumption (Lieber and Salaspuro, 1985).

This chapter describes the investigation of the zinc, copper and selenium status of alcoholics with and without established liver disease, to determine if these subjects were at risk of a deficiency of these trace elements.

9.2 PATIENTS AND METHODS

Patients

Eighteen patients with established alcoholic liver disease, all men, aged 36-62 (mean 48.4) years were identified either as inpatients or on attending gastroenterology outpatient clinics. Clincial details of these patients are given in Table 9.I. Fourteen of them had biopsy-proven alcoholic liver disease, and the other 4 patients had established liver disease on the basis of clinical examination, biochemical abnormalities, and ultrasound examination. All but 4 of the patients were cigarette smokers. The mean body mass index was 22.9 (range 17.3-30.2) and by this criterion 6 of the patients were below their normal weight. Although accurate details of drinking

TABLE 9.I

CLINICAL DETAILS OF PATIENTS WITH ALCOHOLIC LIVER DISEASE

No.	Sex	Age (yrs)	Smoking	BMI kg/M^2	Reported drinking history: daily ethanol ingestion(g) Past	Present	Medication
1+	M	54	Yes	20.7	275	None	Paracetamol + codeine phosphate (Paracodol, Fisons plc); vitamins B and C (Parentrovite, Bencard)
2	M	62	Yes	19.3*	140	32	Lactulose
3	M	43	Yes	19.3*	104	104	Ferrous sulphate; spironolactone BP
4+	M	62	Yes	17.3*	112	None	Paracetamol + dihydrocodeine tartrate (Paramol, Duncan, Flockhart and Co. Ltd)
5	M	57	Yes	22.4	140	18	Chlormezanone
6	M	44	No	23.3	140	140	None
7+	M	37	Yes	18.7*	240	240	Rantidine hydrochloride; lactulose; mixture of ant-acids (Nulacin, Bencard)
8+	M	50	Yes	17.8*	128	128	None

BMI, body mass index * Underweight, BMI = 20.0 or less + Biopsy - proven liver disease

TABLE 9.I continued

No.	Sex	Age (yrs)	Smoking	BMI kg/M^2	Reported drinking history: daily ethanol ingestion(g) Past	Present	Medication
9+	M	56	Yes	24.7	275	None	Paracetamol + dihydrocodeine tartrate (Paramol, Duncan, Flockhart and Co. Ltd)
10+	M	37	Yes	21.9	140	None	Dapsone + pyrimethamine (Maloprim, Wellcome)
11+	M	36	No	26.2	160	64	Spironolactone BP
12+	M	46	Yes	24.6	100	100	Levodopa + carbidopa (Sinemet, Merck Sharp and Dohme Ltd)
13+	M	55	Yes	28.9	140	None	Imipramine hydrochloride BP; chlordiazepoxide; a preparation of fibre (Proctofibe, Cassenne Ltd)
14+	M	46	Yes	25.6	130	64	Oxprenolol hydrochloride BP
15+	M	48	Yes	19.5*	275	64	Spironolactone BP
16+	M	41	Yes	30.2	275	None	Paracetamol + codeine phosphate (Paracodol, Fisons plc)
17+	M	48	Yes	27.9	160	32	Rantidine hydrochloride; chlordiazepoxide; mixture of ant-acids (Gaviscon, Reckitt and Colman)
18+	M	49	Yes	24.7	140	None	None

BMI, body mass index * Underweight, BMI = 20.0 or less + Biopsy - proven liver disease

history were difficult to obtain, all of the patients admitted to heavy drinking in the past, with an intake of 100-280g of ethanol per day. Six of the patients claimed to have stopped drinking alcohol, 7 reported to have reduced their ethanol intake to 18-64g per day, and 5 admitted to current heavy daily drinking. Liver function tests, as measured on the day of study, are shown in Table 9.II. The activity of the serum enzymes, gamma-glutamyltranspeptidase and aspartate aminotransferase were elevated in all of the patients.

Thirteen male alcoholics, aged 28-63 (mean 45.6) years were identified through the St. Dismas alcoholic community centre. No clinical evidence of liver disease was detected in these subjects. Clinical details of the subjects are given in Table 9.III. All of them were cigarette smokers. The mean body mass index was 22.7 (range 17.3-30.2) and by this criterion 2 of the subjects were below their normal weight. All of the subjects admitted to having ingested between 110 and 280g of ethanol per day in the past. Two of them admitted to current heavy daily drinking, and the remainder claimed abstention from alcohol for a period of 1-8 months. Increased serum gamma-glutamyltranspeptidase activity was noted in only two of the subjects; other liver function tests at the time of study were normal (Table 9.IV).

The amount of alcohol intake in terms of grams (g) of absolute alcohol was calculated by a nomogram (Mellor, 1970).

Healthy subjects

The data obtained for the healthy control subjects previously investigated as described in Chapter 6 were used for the purpose of comparison in the present study.

Samples

Blood samples were collected and aliquoted as described in Chapter 6. In addition a 10ml sample of venous blood was taken for routine liver function tests.

Analyses

Separation of white cells, analysis of zinc, copper and selenium in leucocytes and plasma, and cell counting were performed by the techniques given in Chapters 4 and 5. Plasma albumin and caeruloplasmin concentrations were determined as described in Chapter 7.

TABLE 9.II

LIVER FUNCTION TESTS IN PATIENTS WITH ALCOHOLIC LIVER DISEASE AS MEASURED ON DAY OF STUDY

No.	Serum AST $U\ l^{-1}$	Serum ALP $U\ l^{-1}$	Serum TBili $\mu mol\ l^{-1}$	Serum GGTP $U\ l^{-1}$
1	120	206	37	107
2	48	158	30	58
3	84	445	37	189
4	61	371	61	200
5	110	354	77	67
6	134	338	157	65
7	46	322	45	95
8	67	230	76	192
9	98	129	20	129
10	78	140	25	113
11	61	177	18	163
12	84	323	21	296
13	59	205	19	71
14	52	245	18	196
15	63	255	20	57
16	70	264	23	49
17	58	278	17	78
18	140	192	19	48

Abbreviations: AST, aspartate aminotransferase;
ALP, alkaline phosphatase;
TBili, total bilirubin;
GGTP, gamma-glutamyltranspeptidase

Reference Ranges: serum AST = 5 - 42 $U\ l^{-1}$ 37°C
serum ALP = 100 - 300 $U\ l^{-1}$
serum TBili = 0 - 17 $\mu mol\ l^{-1}$
serum GGTP = 10 - 46 $U\ l^{-1}$ 37°C

TABLE 9.III

CLINICAL DETAILS OF ALCOHOLICS IN THE COMMUNITY

No.	Sex	Age (yrs)	Smoking	BMI kg/M^2	Reported drinking history: daily ethanol ingestion (g)		Medication
					Past	Present	
1	M	42	Yes	20.8	110	None	None
2	M	55	Yes	16.9*	160	None	None
3	M	54	Yes	24.7	120	None	Diazepam
4	M	38	Yes	22.2	240	None	Aminophylline
5	M	63	Yes	23.5	120	None	None
6	M	46	Yes	30.5	275	None	None
7	M	33	Yes	17.7*	200	200	None
8	M	36	Yes	25.5	170	None	None
9	M	47	Yes	23.1	110	None	None
10	M	51	Yes	20.8	185	None	Fluphenazine hydrochloride
11	M	48	Yes	24.0	120	None	None
12	M	52	Yes	20.7	275	275	None
13	M	28	Yes	24.6	170	None	None

BMI, Body mass index

* Underweight, BMI = 20.0 or less

TABLE 9.IV

LIVER FUNCTION TESTS IN ALCOHOLICS IN THE COMMUNITY AS MEASURED ON DAY OF STUDY

No.	Serum AST $U\ l^{-1}$	Serum ALP $U\ l^{-1}$	Serum TBili $\mu mol\ l^{-1}$	Serum GGTP $U\ l^{-1}$
1	16	191	10	32
2	29	160	7	18
3	34	191	8	8
4	39	197	8	20
5	16	163	11	13
6	22	142	8	22
7	38	116	9	58
8	13	186	16	14
9	20	148	7	14
10	23	194	8	26
11	27	194	7	23
12	37	160	7	110
13	30	134	4	14

Abbreviations: AST, aspartate aminotransferase;
ALP, alkaline phosphatase;
TBili, total bilirubin;
GGTP, gamma-glutamyltranspeptidase

Reference ranges: serum AST = 5 - 42 $U\ l^{-1}$
serum ALP = 100 - 300 $U\ l^{-1}$
serum TBili = 0 - 17 $\mu mol\ l^{-1}$
serum GGTP = 10 - 46 $U\ l^{-1}$ $37^{o}C$

Statistical procedures

The statistical procedures used in this project are given in Chapter 5. A group of male control subjects (n = 25), all cigarette smokers, aged 23-64 (mean 44.9) years, were selected for comparison with the group of patients with established alcoholic liver disease, and the group of alcoholics in the community. Although 4 of the control subjects were daily drinkers, none of them ingested greater than 50g of ethanol per day.

9.3 RESULTS

The individual results for the alcoholics with established liver disease, and alcoholics in the community are given in Appendices 7 and 8 respectively.

A comparison of the zinc, copper and selenium results between the patients with established liver disease and the controls is shown in Table 9.V, and between the alcoholics in the community and the controls, in Table 9.VI. Significantly decreased concentrations of leucocyte and plasma zinc ($p < 0.01$) were observed in the group of patients with established liver disease, but not in the group of alcoholics in the community, compared with the control group. However, 95% confidence intervals for the ratio of population means (Table 9.VI) demonstrate that leucocyte zinc values are much more likely to be low in an alcoholic population with no established liver disease, compared with non-alcoholic subjects. Scattergrams of the plasma and leucocyte zinc values are shown in Figures 9.1 and 9.2 respectively for the population under study and the control group. Significantly lower ($p < 0.001$) concentrations of plasma albumin were observed in the patients with established liver disease (38 ± 6 g l^{-1}) compared to the control group (46 ± 3 g l^{-1}), and there was a significant correlation between plasma zinc and albumin concentrations ($p < 0.01$, $r = 0.66$) in this patient group. Plasma albumin concentrations were similar in the group of alcoholics in the community (46 ± 3 g l^{-1}) and the control group (46 ± 3 g l^{-1}) and there was no significant correlation between plasma zinc and albumin values in either of these groups.

A significant increase ($p < 0.01$) in plasma copper concentration was observed in the group of patients with established liver disease compared with the control group (Table 9.V). No similar increase was

TABLE 9.V

COMPARISON OF ZINC, COPPER AND SELENIUM CONCENTRATIONS IN LEUCOCYTES AND PLASMA BETWEEN PATIENTS WITH ESTABLISHED LIVER DISEASE AND THE CONTROL SUBJECTS

Analysis	Patients with established liver disease n = 18	Controls n = 25	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	85(69-105)$^{\Omega++}$	111(94-124)$^{\Omega}$	0.79 (a)	0.70 to 0.84 (a)
Plasma Zn µmol l^{-1}	10.6 ± 1.9^{++}	12.3 ± 1.8	-1.67 (b)	-2.80 to -0.54 (b)
Leucocyte Cu pmol/10^6 cells	10.9 ± 4.1	10.4 ± 3.9	0.46 (b)	-2.03 to 2.95 (b)
Plasma Cu µmol l^{-1}	18.9 ± 3.0^{++}	15.9 ± 2.6	3.05 (b)	1.31 to 4.79 (b)
Leucocyte Se pmol/10^6 cells	1.23 ± 0.29^{+++}	2.01 ± 0.44	-0.78 (b)	-1.02 to -0.54 (b)
Plasma Se µmol l^{-1}	0.92 ± 0.24^{+++}	1.39 ± 0.15	-0.47 (b)	-0.59 to -0.35 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{++}$ $p < 0.01$

$^{+++}$ $p < 0.001$

TABLE 9.VI

COMPARISON OF ZINC, COPPER AND SELENIUM CONCENTRATIONS IN LEUCOCYTES AND PLASMA BETWEEN ALCOHOLICS IN THE COMMUNITY AND THE CONTROL SUBJECTS

Analysis	Alcoholics in the community n = 13	Control subjects n = 25	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	97(80-118)$^{\Omega}$	111(94-124)$^{\Omega}$	0.90 (a)	0.79 to 1.02 (a)
Plasma Zn µmol l^{-1}	12.4 ± 1.7	12.3 ± 1.8	0.08 (b)	-1.12 to 1.28 (b)
Leucocyte Cu pmol/10^6 cells	10.7 ± 3.9	10.4 ± 3.9	0.29 (b)	-2.42 to 3.00 (b)
Plasma Cu µmol l^{-1}	16.2 ± 1.7	15.9 ± 2.6	0.36 (b)	-1.26 to 1.98 (b)
Leucocyte Se pmol/10^6 cells	1.42 ± 0.26^{+++}	2.01 ± 0.44	-0.60 (b)	-0.86 to -0.33 (b)
Plasma Se µmol l^{-1}	1.09 ± 0.15^{+++}	1.39 ± 0.15	-0.30 (b)	-0.40 to -0.20 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

$^{+++}$ $p < 0.001$

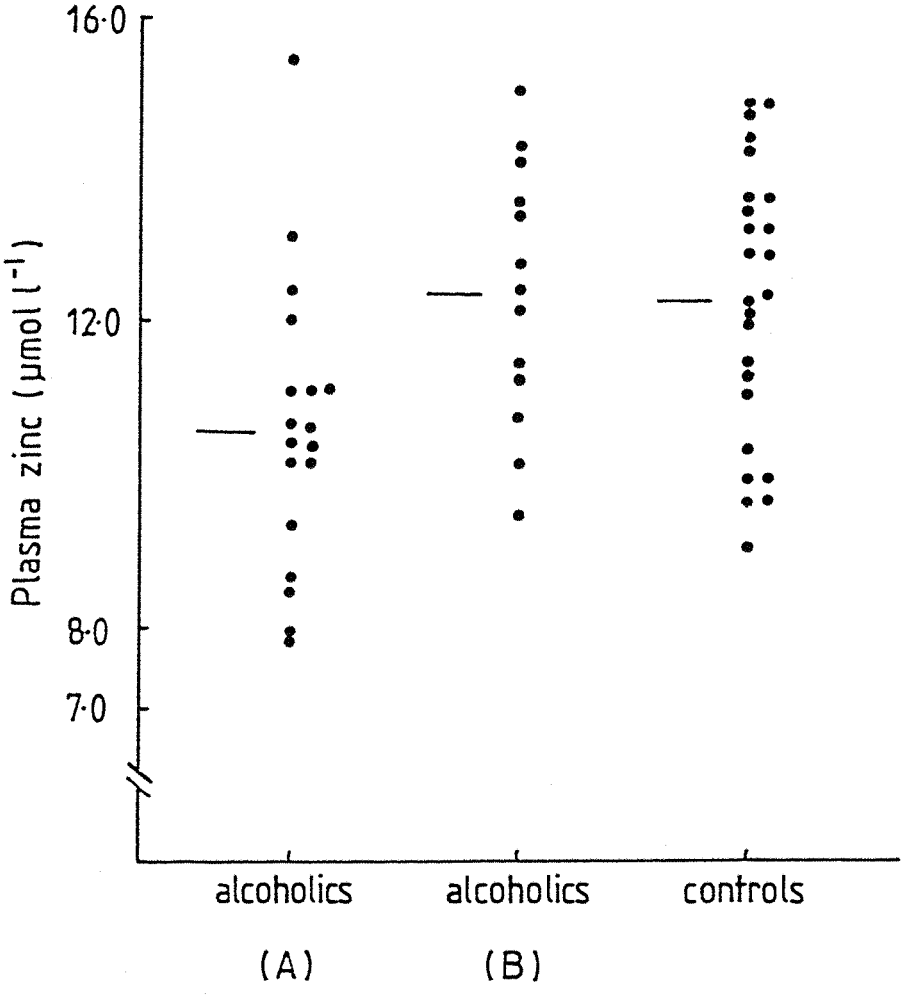


Figure 9.1

Scattergram of plasma zinc concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B), and controls. Horizontal lines show the mean values of the data sets

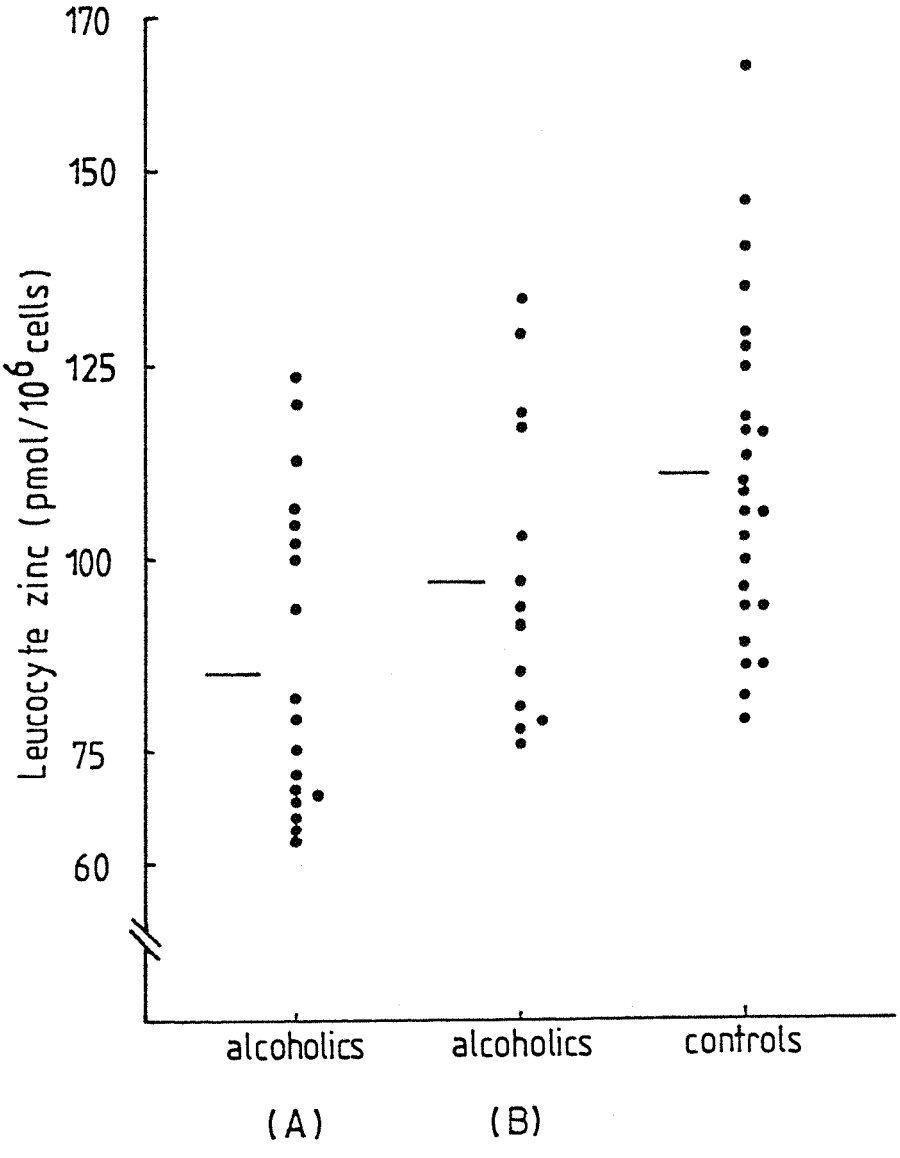


Figure 9.2

Scattergram of leucocyte zinc concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B), and controls. Horizontal lines show the geometric mean values of the data sets

found for leucocyte copper concentration. There was no significant difference in plasma or leucocyte copper concentrations between the group of alcoholics in the community and the control group (Table 9.VI). Scattergrams of the plasma and leucocyte copper values are shown in Figures 9.3 and 9.4 respectively for the study population and the control group. Significantly higher ($p < 0.001$) concentrations of plasma caeruloplasmin were demonstrated in the patients with established liver disease (407 ± 67 mg l^{-1}) compared with the controls (308 ± 61 mg l^{-1}), and there was a significant correlation between plasma copper and caeruloplasmin concentrations in both patients ($p < 0.001$, $r = 0.82$) and controls ($p < 0.001$, $r = 0.86$). Plasma caeruloplasmin concentrations were similar in the group of alcoholics in the community (304 ± 44 mg l^{-1}) and the control group (308 ± 61 mg l^{-1}), and there was also a significant correlation between plasma copper and caeruloplasmin values in the alcoholics in the community ($p < 0.001$, $r = 0.83$).

The concentration of selenium in leucocytes and plasma was significantly decreased ($p < 0.001$) in the patients with established liver disease (Table 9.V) and in the alcoholics in the community (Table 9.VI) compared with the control group. It is interesting to note that the patients with established liver disease had significantly lower ($p < 0.05$) plasma selenium concentrations than the alcoholics in the community. Although leucocyte selenium values were not significantly lower, the 95% confidence intervals (-0.40 to 0.02 pmol/10^6 cells) for the difference between population means (-0.19 pmol/10^6 cells) demonstrate that the concentration of selenium in leucocytes is much more likely to be lower in patients with established alcoholic liver disease than in alcoholics with no established liver disease. Scattergrams of the plasma and leucocyte selenium values are presented in Figures 9.5 and 9.6 respectively for the study population and the control group. Alcoholic subjects with a low body mass index are indicated in these figures. Interestingly, there was a significant correlation between plasma selenium and albumin concentrations ($p < 0.05$, $r = 0.48$) in the patients with established liver disease, but not in the alcoholics in the community or in the control group.

Significant correlations were observed in the group of patients with established liver disease, and alcoholics in the community, between leucocyte selenium concentrations and body mass index ($p <$

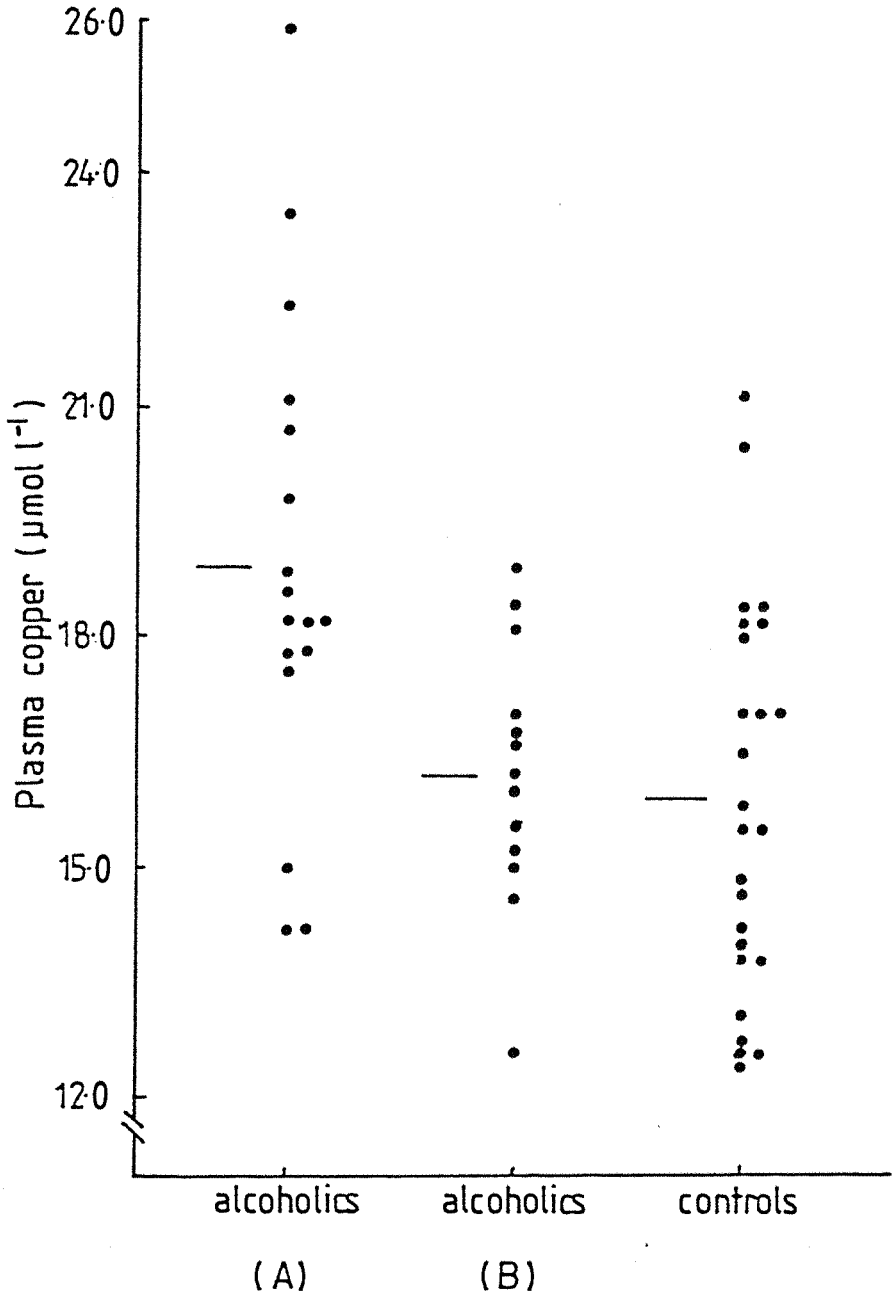


Figure 9.3

Scattergram of plasma copper concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B), and controls. Horizontal lines show the mean values of the data sets

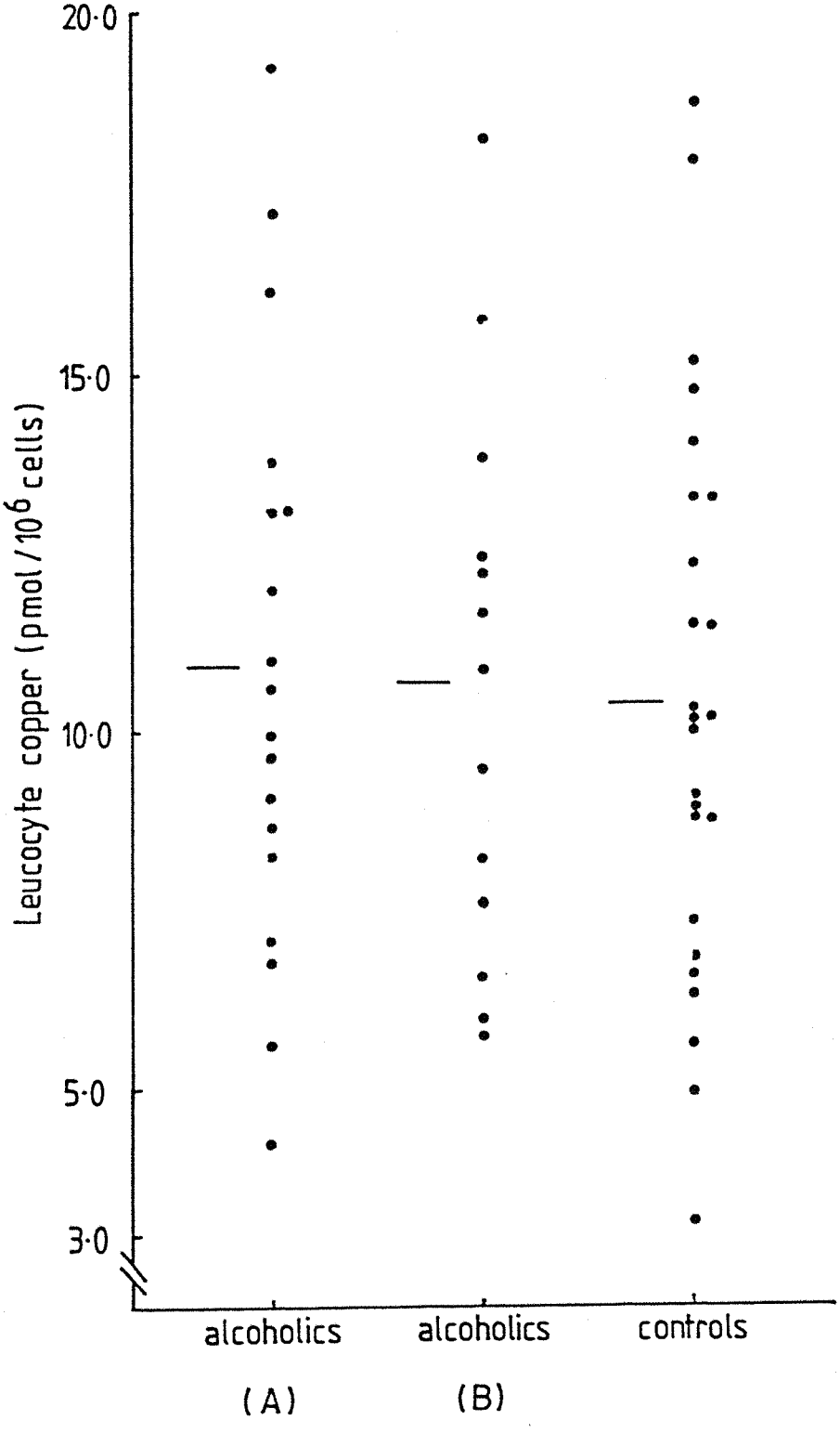


Figure 9.4

Scattergram of leucocyte copper concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B), and controls. Horizontal lines show the mean values of the data sets

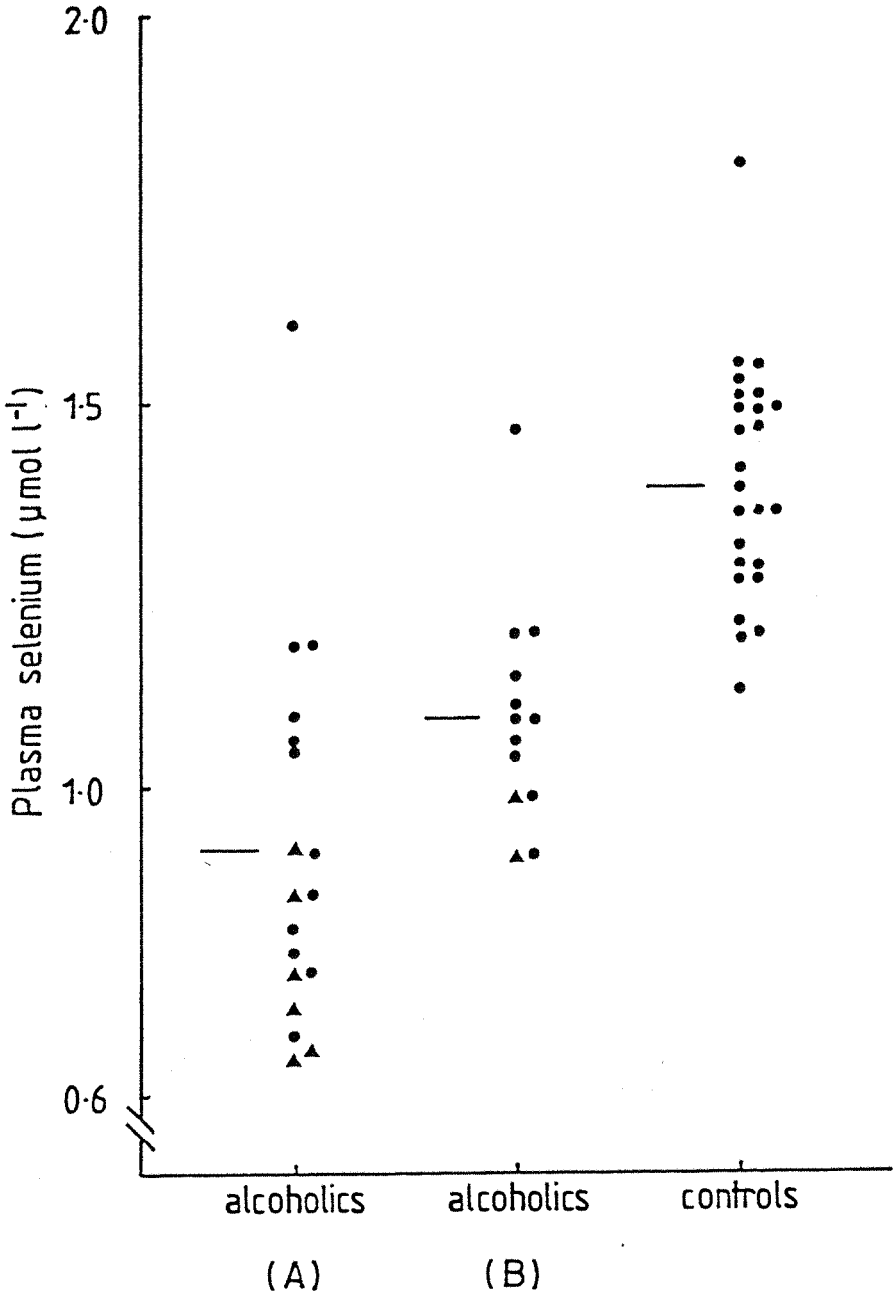


Figure 9.5

Scattergram of plasma selenium concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B),and controls. Horizontal lines show the mean values of the data sets; ▲ denotes subjects from the alcoholic population with a low body mass index

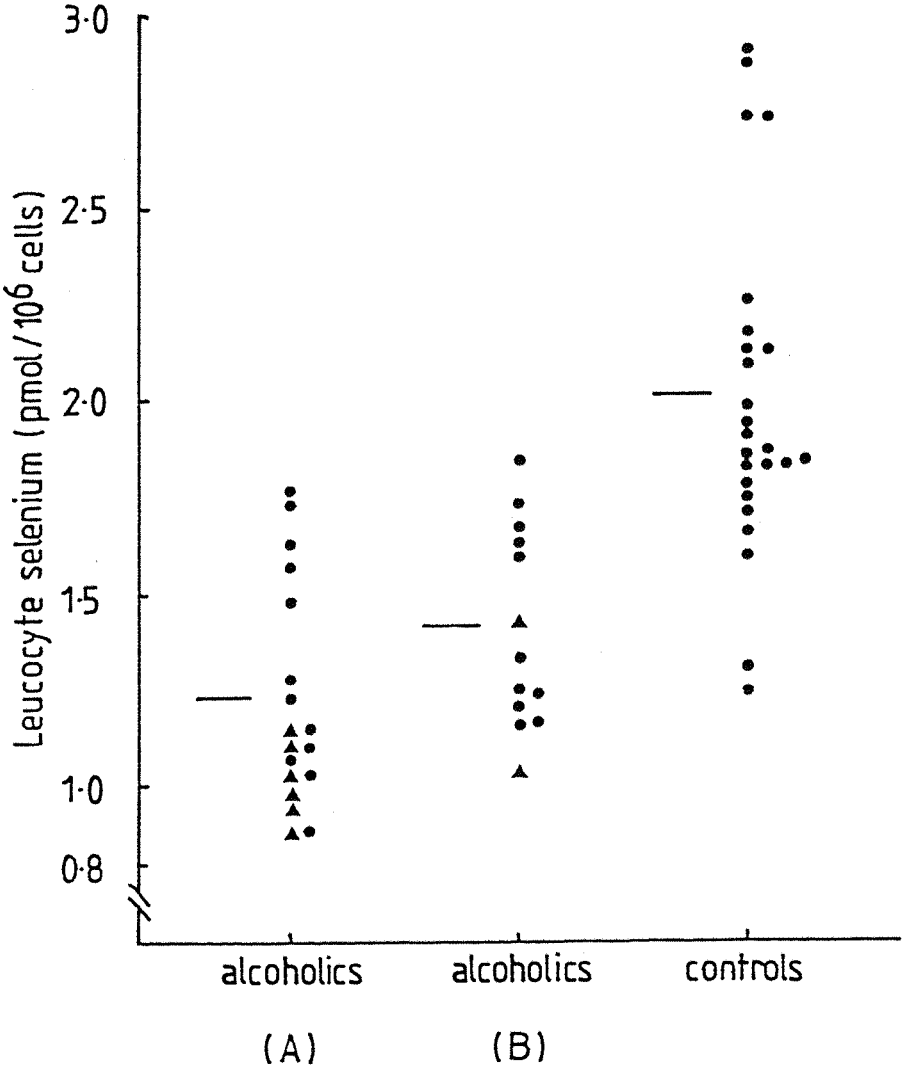


Figure 9.6

Scattergram of leucocyte selenium concentrations in alcoholic patients with established liver disease (A), alcoholics in the community (B), and controls. Horizontal lines show the mean values of the data sets; ▲ denotes subjects from the alcoholic population with a low body mass index

0.001, r = 0.82; $p < 0.05$, r = 0.59 respectively), plasma selenium concentrations and body mass index ($p < 0.001$, r = 0.75; $p < 0.001$, r = 0.88 respectively) and leucocyte and plasma selenium concentrations ($p < 0.001$, r = 0.87; $p < 0.05$, r = 0.59 respectively). No similar relationships were noted for zinc or copper.

9.4 DISCUSSION

Hypozincaemia has frequently been reported in patients with alcoholic liver disease (Halsted and Smith 1970; Smith et al 1975; Mills et al, 1983; Dinsmore et al, 1985a). Significantly reduced plasma zinc concentrations in alcoholics without liver disease have also been reported, although it is interesting to note that the alcoholics without liver disease had significantly higher plasma zinc concentrations than patients with liver disease (Dinsmore et al, 1985a). In the present study, a significant decrease was noted in plasma zinc and albumin concentrations in the alcoholics with established liver disease compared with the control group, although no similar findings were demonstrated in the group of alcoholics in the community. It has been suggested that the hypoalbuminaemia observed in patients with alcoholic liver disease may be partly due to a primary defect in secretion rather than in the biosynthesis of albumin (Tavill, 1985). Hypoalbuminaemia may also result from inadequate nutrition, and albumin synthesis has been reported to fall following a reduction in protein intake (Price and Alberti, 1985). A decrease in albumin-bound serum zinc has been demonstrated in patients with alcoholic liver disease (Kiilerich et al, 1980b; Mills et al, 1983) and non-alcoholic liver disease (Kiilerich et al, 1980b).

Reduced leucocyte zinc concentrations in patients with cirrhosis were first demonstrated by Fredericks et al, (1960). In the same study, red cell zinc concentrations were normal or increased, the latter finding possibly being a reflection of slightly increased red cell volume. A more recent study demonstrated a reduction in leucocyte zinc concentration in patients with liver disease, including alcoholic cirrhosis, but reported no change in erythrocyte zinc concentration (Keeling et al, 1980). In the present study, significantly decreased concentrations of leucocyte zinc were demonstrated only in the patients with established alcoholic liver

disease although there was a trend towards lowered levels in the group of alcoholics in the community.

The mechanism for the development of zinc deficiency in alcoholism is not well defined. Information obtained from careful dietary histories revealed that approximately 90% of alcoholics with and without liver disease had an inadequate dietary intake of zinc (McClain et al, 1979). Several investigators have reported increased urinary excretion of zinc in alcoholics with (Vallee et al, 1957; Mills et al, 1983) and without (Sullivan and Lankford, 1965; Giovetti and Russell, 1979) liver disease. Hyperzincuria has been reported in normal subjects during administration of alcoholic beverages compared with non-alcoholic ones (McDonald and Margen, 1980). It has been proposed that a possible mechanism for the ethanol effect on zinc excretion may be due in part to a shift of zinc from albumin to other ligands such as amino acids, resulting in a more diffusible zinc which is then lost at the kidney (Russell, 1980). Absorption of zinc may be impaired in the chronic alcoholic, and chronic ingestion of ethanol in rats significantly decreased zinc absorption in the ileum (Antonson and Vanderhoof, 1983). Several workers have provided evidence to support the concept of zinc malabsorption occurring during ethanol ingestion in humans. Subjects with alcoholic cirrhosis given an oral zinc tolerance test had a decreased area under the curve, reflecting either impaired absorption or increased clearance of zinc (Sullivan et al, 1979a). Decreased Zn^{65} absorption in alcoholic subjects with (Dinsmore et al, 1985b; Valberg et al, 1985b) and without (Dinsmore et al, 1985b) alcoholic liver disease has been reported. The degree of abnormality in Zn^{65} absorption in patients with alcoholic cirrhosis was related to the extent of the liver damage and low leucocyte zinc concentrations were observed in the more severely ill patients (Valberg et al, 1985b). In the same study, Zn^{65} absorption was not significantly different in patients with non-alcoholic cirrhosis compared with healthy controls, and these authors concluded that decreased intestinal absorption of zinc was related to the chronic ingestion of alcohol rather than to the liver damage per se. While the mechanism by which alcohol interferes with zinc absorption is not well defined, it has been proposed that long-term exposure to ethanol may affect intestinal transport by altering the physical properties of the intestinal membrane or by inhibiting the activities of membrane-bound enzymes, and that intestinal absorption of substances that

require a carrier-mediated process is more likely to be decreased in contrast to substances that are predominantly transported by diffusion (Wilson and Hoyumpa, 1979).

A decrease in hepatic zinc concentration in patients with alcoholic liver disease has also been reported (Kiilerich et al, 1980b; Mills et al, 1983). In order to correct for the amount of fibrous connective tissue present, these studies expressed the liver zinc concentration in relation to the amount of hepatocytes (Kiilerich et al, 1980b) or in terms of protein or magnesium as markers of intracellular content (Mills et al, 1983). However, hepatic zinc concentration alone does not allow for the estimation of whole-liver zinc content, and it is important to note that many livers in patients with alcoholic liver disease are enlarged.

Although some workers were unable to show that supplemental zinc was of therapeutic value in alcoholic cirrhosis (Sullivan et al, 1979b), others have reported beneficial effects of zinc supplementation on some liver parameters and an improvement in taste function (Weismann et al, 1979). Ilchyshyn and Mendelsohn (1982) described a patient with alcoholic cirrhosis and malnutrition who developed lesions similar to those seen in acrodermatitis enteropathica, and who showed a clinical response to oral zinc therapy.

Significantly increased plasma copper and caeruloplasmin concentrations were demonstrated in the patients with established alcoholic liver disease in the present study, although not in the group of alcoholics in the community. Leucocyte copper concentrations did not differ significantly in either of the groups of alcoholics compared with the control group. Increased plasma copper concentrations have previously been reported in alcoholics with and without liver disease (Dinsmore et al, 1985a). Although high liver copper concentrations were demonstrated in patients with Wilson's disease and long-standing biliary cirrhosis , the liver copper concentrations were within normal limits for patients with alcoholic liver disease (Smallwood et al, 1968). The increased plasma copper concentrations observed in the patients with established liver disease in the present study can presumably be attributed to the inflammatory process involved. There is no evidence available in the literature to suggest that alcoholics with or without liver disease are at risk of copper deficiency.

Significantly reduced concentrations of leucocyte and plasma selenium in alcoholics with established liver disease and alcoholics in the community were observed in the present study. The occurrence of low serum selenium concentrations in alcoholics with (Aaseth et al, 1982; Valimaki et al, 1983), and without (Dutta et al, 1983; Valimaki et al, 1983), liver disease has previously been reported. Furthermore, serum selenium concentrations were shown to be lower in cirrhotic alcoholics compared with non-cirrhotic alcoholics (Valimaki et al, 1983). In the present study, alcoholics with established liver disease had significantly lower plasma selenium concentrations than the alcoholics in the community and there was also a trend towards lowered leucocyte selenium concentrations in the former group. Reduced concentrations of liver selenium have been reported in alcoholic cirrhosis (Aaseth et al, 1982).

The mechanisms leading to a lowered selenium status in alcoholism are unclear. Insufficient dietary intake of selenium could provide one explanation. Alcoholic beverages are a poor source of selenium (Dutta et al, 1983). In the present study, it is of interest that there was a significant correlation between body mass index and the concentration of selenium in both leucocytes and plasma in the alcoholics with and without established liver disease. The difference in selenium concentrations between these two groups may possibly reflect differences in nutritional status. There was a higher proportion of subjects who were underweight in the group of alcoholics with liver disease compared to the group of alcoholics in the community. Selenium deficiency in alcoholic subjects has been related primarily to poor nutrition (Dutta et al, 1983). These authors described a group of acutely inebriated alcoholic subjects who demonstrated significantly decreased plasma selenium concentrations and urinary excretion of selenium on admittance to an alcoholic detoxification unit. Selenium intake during the 4-week period before hospitalisation was estimated to be low in the majority of study subjects. In the same study, cessation of ethanol consumption and adequate dietary intake of selenium over a period of one week was associated with an increase in plasma selenium concentrations and urinary excretion of selenium. In contrast to these findings, a Finnish study found that abstinence from ethanol for two weeks had no effect on the serum concentrations of selenium in non-cirrhotic alcoholics (Valimaki et al, 1983). These authors suggested that the

two week period may not have been sufficient time to make up the depleted stores, particularly in Finland where the diet is not rich in selenium.

In the present study, there was a significant correlation between plasma selenium and albumin concentrations in the group of alcoholics with established liver disease, and this observation confirms earlier findings (Aaseth et al, 1982). However, plasma albumin and selenium concentrations in the alcoholics in the community were similar to those of the control group, and there was no significant correlation between these two parameters. Hypoalbuminaemia of non-hepatic origin is not related to low serum selenium concentrations (Valimaki et al, 1983) and it is unlikely that the decreased concentrations of plasma selenium in the alcoholics with established liver disease in the present study can be attributed to the decreased plasma albumin concentrations. Although intestinal absorption of selenium may be decreased as a result of chronic ethanol ingestion, there do not appear to be any reports in the literature concerning the effect of ethanol on selenium absorption.

In a separate study to the present one, the selenium and antioxidant status of an alcoholic population (alcoholics with liver disease, n = 32; alcoholics in the community, n = 19), which included the subjects in the present study, was investigated (Tanner et al, 1986). In this study, selenium concentrations in serum and whole blood were measured by myself, whilst serum vitamin E levels, erythrocyte glutathione peroxidase activity and serum lipid peroxides were determined by others. Despite lowered selenium levels in the subjects studied, erythrocyte glutathione peroxidase activity was not significantly decreased. Interestingly, vitamin E levels were significantly reduced in both alcoholics with established liver disease and alcoholics in the community. Increased serum lipid peroxides were also reported in the alcoholics with liver disease compared with the alcoholic population without liver disease. Lipid peroxides were measured by the colorimetric method as described by Satoh (1978), which measures the thiobarbituric acid reactants. This method however is prone to several interfering substances, notably bilirubin (Gutteridge and Tickner, 1978; Ohkawa et al, 1979), and therefore may not be reliable for the determination of lipid peroxides in serum. Nevertheless, it is apparent from the findings of the present study and from reports in the literature that alcoholic

subjects, particularly those with liver disease, are at risk of a lowered zinc, selenium and vitamin E status, which may lead to an important lack of protection of cell membranes against damage by free radicals. This is of particular interest considering the proposed intermediate production of oxygen free radical species formed during the metabolism of ethanol by the liver (Dianzani, 1987), which may result in hepatic damage due to enhanced lipid peroxidation (Di Luzio, 1966).

It is also noteworthy that a significant correlation, independent of smoking, exists between daily alcohol consumption and the frequency of cancers of the upper digestive tract (Wynder and Bross, 1961; Schmidt and de Lint, 1972). Considering the reported role of selenium in the prevention of cancer, a deficiency of selenium in chronic alcoholics may be of particular relevance to the increase in upper gastrointestinal tract cancers due to daily alcohol consumption.

CHAPTER 10

ASSESSMENT OF ZINC, COPPER AND SELENIUM STATUS OF PATIENTS WITH DERMATOLOGICAL DISEASES

10.1 INTRODUCTION

Psoriasis and eczema are common dermatoses. Malabsorption of fat has been reported in patients with eczema and psoriasis (Shuster and Marks, 1965) and an association between severe psoriasis and changes in the mucosal architecture of the small bowel has been described (Barry et al, 1971). Eczema is an inflammatory disorder of the skin, predominantly involving the epidermis (Shuster, 1978). Psoriasis is characterised by a much increased epidermal turnover with abnormal keritinisation, and at the same time there is evidence of a pronounced inflammatory reaction (Michaelsson, 1984). An increased epidermal cell turnover has been suggested as a possible source of significant loss of both zinc and copper in extensive psoriasis (Molin and Wester, 1973).

Although the exact role of selenium in skin function is not well defined, topical use of selenium-containing preparations for the treatment of seborrhoeic scalp disorders is well established. Studies in Sweden have reported decreased erythrocyte glutathione peroxidase activity in patients with various skin disorders, and have suggested that this indicated a possible selenium deficiency although selenium levels were not directly measured (Juhlin et al, 1982; Michaelsson and Edqvist, 1984).

The purpose of the study described in this chapter was to investigate the zinc, copper and selenium status of patients with eczema and psoriasis to determine if this group of patients had a deficiency of these trace elements.

10.2 PATIENTS AND METHODS

Patients with eczema

Twenty four patients with eczema, 12 women and 12 men, aged 18-80 (mean 39.0) years were studied. The clinical details of these patients are shown in Table 10.I. The surface area involved in skin

TABLE 10.I

CLINICAL DETAILS OF PATIENTS WITH ECZEMA

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
1	F	40	Yes	<10%	3	22.3	Hydrocortisone cream, betamethasone valerate cream	Mebhydrolin
2	F	63	No	<10%	5	21.7	Hydrocortisone cream	Trimeprazine tartrate
3	F	23	No	<10%	9	18.6*	Hydrocortisone cream, betamethasone valerate cream	Promethazine hydrochloride, levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
4	M	56	Yes	<10%	32	26.4	Clobetasone butyrate cream	Trimeprazine tartrate
5	F	24	Yes	<10%	2	20.5	Beclomethasone dipropionate cream	Levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
6	M	48	No	<10%	1	23.1	Beclomethasone dipropionate cream, arachis oil BP cream	None
7	F	20	No	<10%	20	21.3	Hydrocortisone cream, betamethasone valerate cream	Trimeprazine tartrate, levonorgestrel + Ethinyloestradiol (Ovranette, Wyeth)
8	M	58	No	<10%	5	22.8	Hydrocortisone cream	None

BMI, body mass index * Underweight, BMI = 20.0 or less

TABLE 10.I continued

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
9	F	30	Yes	<10%	15	28.0	Betamethasone valerate cream, arachis oil BP cream	None
10	M	58	Yes	<10%	3	28.7	Clobetasone butyrate ointment	Trimeprazine tartrate
11	M	42	Yes	<10%	17	22.8	Hydrocortisone cream, betamethasone valerate cream	None
12	F	19	No	>10%	19	22.2	Hydrocortisone cream, beclomethasone dipropionate cream	Promethazine hydrochloride, levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
13	M	75	No	>10%	16	23.0	Hydrocortisone cream, betamethasone valerate cream	None
14	F	21	Yes	>10%	18	20.0*	Hydrocortisone cream, beclomethasone dipropionate cream	Chlorpheniramine maleate, levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
15	M	19	No	>10%	2	26.2	Hydrocortisone cream, betamethasone valerate cream	None
16	M	38	No	>10%	38	19.0*	Beclomethasone dipropionate cream	None

BMI, body mass index * Underweight, BMI = 20.0 or less

TABLE 10.I continued

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
17	F	24	No	>10%	24	21.7	Hydrocortisone cream, betamethasone valerate cream	Levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
18	F	18	No	>10%	2	21.0	Hydrocortisone cream, beclomethasone dipropionate cream	Terfenadine, levonorgestrel + Ethinyloestradiol (Ovranette, Wyeth)
19	M	23	Yes	>10%	20	23.9	Hydrocortisone cream, betamethasone valerate cream, arachis oil BP cream	None
20	F	26	No	>10%	10	23.3	Hydrocortisone cream, betamethasone valerate cream	Trimeprazine tartrate, levonorgestrel + Ethinyloestradiol (Microgynon, Schering)
21	M	80	No	>10%	27	25.9	Hydrocortisone cream, betamethasone valerate cream	None
22	M	24	No	>10%	20	23.0	Hydrocortisone cream, betamethasone valerate cream	Trimeprazine tartrate
23	F	30	No	>10%	30	18.1*	Hydrocortisone cream, betamethasone valerate cream	Hydroxyzine hydrochloride
24	M	77	No	>10%	1	21.6	Hydrocortisone cream, betamethasone valerate cream	None

BMI, body mass index

* Underweight, BMI = 20.0 or less

lesions was determined by visual assessment, and was less than 10% in 11 of the patients and greater than 10% in 13 of the patients. The mean duration of disease was 14.1 (range 1-38) years. All of the patients were receiving topical steroid treatment and 8 of the women were taking oral contraceptives. No patient had a history of gastro-intestinal disease at the time of investigation and none of the patients complained of diarrhoea. The mean body mass index was 22.7 (range 18.6-28.7) and by this criterion 4 of the patients were below their normal weight.

Patients with psoriasis

Twenty three patients with psoriasis, 8 women and 15 men, aged 17-68 (mean 41.3) years were studied. The clinical details of these patients are given in Table 10.II. The surface area involvement was determined by visual assessment, and was less than 10% in 17 of the patients and greater than 10% in 6 of them. The mean duration of disease was 16.8 (1-45) years. Five of the patients were receiving topical steroid treatment and 7 were using applications containing zinc. One woman only was taking oral contraceptives. No patient had a history of gatrointestinal disease at the time of study and none complained of diarrhoea. The mean body mass index was 22.9 (range 18.4-32.0) and by this criterion 3 of the patients were below their normal weight.

Healthy control subjects

The data obtained for the healthy control subjects previously investigated as described in Chapters 6 and 8 was used for the purpose of comparison in the present study.

To ensure the validity of the continued use of the control data obtained prior to the present investigation, a small group of healthy control subjects was also studied at the time of the present study. Fifteen healthy volunteers, 9 women and 6 men, aged 22-46 (mean 29.9) years were investigated. Only one of the male subjects was a cigarette smoker. Three of the women who participated were taking oral contraceptives (Levonorgestrel + Ethinyloestradiol, Microgynon, Schering).

TABLE 10.II

CLINICAL DETAILS OF PATIENTS WITH PSORIASIS

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
1	M	53	No	<10%	10	22.2	Dithranol cream	None
2	M	56	Yes	<10%	45	22.5	Zinc and salicylic acid paste	Etretinate, isosorbide mononitrate
3	M	17	No	<10%	1	18.4*	Hydrocortisone cream	Ibuprofen
4	F	17	Yes	<10%	12	19.1*	Dithranol cream	Trimeprazine tartrate
5	F	47	No	<10%	18	24.1	Zinc and coal tar paste, dithranol cream	None
6	F	43	Yes	<10%	20	24.9	Betamethasone valerate cream, dithranol cream	None
7	M	31	No	<10%	23	21.7	Dithranol cream	None
8	F	33	Yes	<10%	14	22.8	Zinc and coal tar paste	Flurbiprofen
9	M	68	No	<10%	15	21.6	Zinc and coal tar paste	None

BMI, body mass index

* Underweight, BMI = 20.0 or less

TABLE 10.II continued

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
10	F	25	Yes	<10%	13	21.8	Hydrocortisone cream, dithranol cream	Levonorgestrel + Ethinyloestradiol (Ovranette, Wyeth)
11	M	56	Yes	<10%	29	25.0	Zinc and salicylic acid paste	Trimeprazine tartrate
12	M	30	No	<10%	6	24.9	Salicylic acid ointment	None
13	F	63	Yes	<10%	40	23.2	Coal tar paste	None
14	M	35	Yes	<10%	20	25.5	Zinc and coal tar paste	None
15	M	41	No	<10%	14	24.0	Clobetasone butyrate cream	Trimeprazine tartrate
16	M	23	No	<10%	10	19.1*	Hydrocortisone 17 - butyrate cream	None
17	M	55	No	<10%	28	22.5	Dithranol cream	None
18	M	52	Yes	>10%	3	23.4	Dithranol cream	Etretinate,lorazepam
19	F	28	No	>10%	13	20.4	Coal tar paste	None

BMI, body mass index

* Underweight, BMI = 20.0 or less

TABLE 10.II continued

No.	Sex	Age (yrs)	Smoker	Surface area involvement	Duration of disease (yrs)	BMI kg/M^2	Topical Applications	Oral Medication
20	M	53	No	>10%	21	22.9	Dithranol cream	None
21	F	32	No	>10%	1	21.3	Dithranol cream	None
22	M	55	Yes	>10%	1	23.6	Zinc and coal tar paste	None
23	M	38	Yes	>10%	29	32.0	Salicylic acid ointment	Trimeprazine tartrate

BMI, body mass index

* Underweight, BMI = 20.0 or less

Samples

Blood samples were collected and aliquoted as described in Chapter 6.

Analyses

Separation of white cells and analysis of zinc, copper and selenium in leucocytes, plasma and whole blood, cell counting and packed cell volume (PCV) measurements were performed by the techniques given in Chapters 4 and 5. The concentrations of zinc, copper and selenium in erythrocytes for the patients under investigation were obtained by calculation as described in Chapter 8. Plasma albumin and caeruloplasmin concentrations were determined as described in Chapter 7.

Statistical procedures

The statistical procedures used in this project are given in Chapter 5. Each patient was matched for age, sex, smoking habit in men, and use of oral contraceptives in women, with 2 control subjects. For each pair of control subjects, the results for each parameter were meaned, and statistical analysis was performed using the mean value. The age range for the 48 adult healthy controls used for comparison with the eczema group was 18-82 (mean 38.9) years, and for the psoriasis group the age range for the 46 adult healthy controls was 18-70 (mean 40.2) years.

A similar procedure as above was used to compare the data obtained from the control subjects investigated at the time of the present study with control data previously obtained. The age range for the 30 adult healthy controls used for this comparison was 22-48 (30.5) years.

10.3 RESULTS

Healthy control subjects

The zinc, copper and selenium concentrations in whole blood for the control subjects investigated in the present chapter were 84.7 ± 8.7, 15.8 ± 2.8 and 1.68 ± 2.0 μmol l^{-1} respectively, and the white cell counts, red cell counts and PCV values for the whole blood samples were 5.69 ± 1.42 x 10^9 l^{-1}, 4.76 ± 0.51 x 10^{12} l^{-1} and 42.3 ± 3.0 respectively.

Table 10.III shows the concentrations of zinc, copper and selenium in leucocytes, erythrocytes and plasma for the two control groups. No significant differences were observed between the two groups for any of the parameters shown in Table 10.III. Furthermore, the 95% confidence intervals for the ratio of means, or means of individual subject-subject differences, showed that there were no differences that were very near to significance for any of the parameters measured. Scattergrams of the zinc, copper and selenium results for the two control groups are shown in Figures 10.1, 10.2 and 10.3 respectively.

Patients with eczema

The individual results for the patients with eczema are given in Appendix 9. The white cell counts, red cell counts and PCV values for the whole blood samples were $6.12 \pm 1.46 \times 10^9\ l^{-1}$, $4.87 \pm 0.51 \times 10^{12}\ l^{-1}$ and 43.3 ± 3.6 respectively.

As there was a similar percentage of men and women, men who smoked, women taking oral contraceptives, and subjects over 55 years in the two group of patients with less than, and greater than 10% surface area involvement, it was possible to compare the data obtained for zinc, copper and selenium for these two groups (Table 10.IV). There was no significant difference in the concentrations of zinc, copper or selenium in leucocytes, erythrocytes or plasma.

Table 10.V summarises the zinc, copper and selenium concentrations in leucocytes, erythrocytes and plasma for the patient and control groups. A significant increase in the concentration of plasma copper ($p < 0.01$) was found in the group of patients with eczema. No similar increase was noted in leucocyte or erythrocyte copper concentrations. Concentrations of zinc in leucocytes, erythrocytes and plasma did not differ significantly in the patient group compared with the control group. Scattergrams of the zinc and copper results for the patients and matched controls are presented in Figures 10.4 and 10.5 repectively. A significant decrease in the concentration of selenium in leucocytes ($p < 0.001$), erythrocytes ($p < 0.01$) and plasma ($p < 0.001$) was found in the patients with eczema compared with the controls (Table 10.V). Figure 10.6 presents a scattergram of the selenium results for patients and controls. In the patient group, there was a significant correlation between selenium in leucocytes and erythrocytes ($p < 0.01$, $r = 0.54$), leucocytes and plasma ($p < 0.001$,

TABLE 10.III

COMPARISON OF ZINC, COPPER AND SELENIUM RESULTS FOR THE CONTROL SUBJECTS INVESTIGATED IN THE PRESENT CHAPTER (A) WITH THE RESULTS OBTAINED FOR A SIMILAR GROUP OF CONTROL SUBJECTS INVESTIGATED IN CHAPTER 6 (B)

Analysis	Control subjects (A) n = 15	Control subjects (B) n = 30	Ratio of means (a) or mean of individual subject - subject differences (b)	95% CI for the ratio of means (a) or the mean of individual subject - subject differences (b)
Leucocyte Zn pmol/10^6 cells	113(106-123)$^{\Omega}$	117(103-136)$^{\Omega}$	0.97 (a)	0.83 to 1.13 (a)
Erythrocyte Zn pmol/10^6 cells	16.3 ± 1.8	16.6 ± 1.9	-0.25 (b)	-1.34 to 0.84 (b)
Plasma Zn µmol l^{-1}	12.5 ± 1.5	12.7 ± 1.5	-0.20 (b)	-1.13 to 0.73 (b)
Leucocyte Cu pmol/10^6 cells	10.8 ± 3.8	11.2 ± 4.2	-0.46 (b)	-2.95 to 2.30 (b)
Erythrocyte Cu pmol/10^6 cells	1.11 ± 0.15	1.13 ± 0.17	-0.01 (b)	-0.12 to 0.09 (b)
Plasma Cu µmol l^{-1}	17.2(15.7-17.5)$^{\Omega}$	16.9(14.8-18.6)$^{\Omega}$	1.02 (a)	0.90 to 1.15 (a)
Leucocyte Se pmol/10^6 cells	2.16 ± 0.53	2.10 ± 0.64	0.06 (b)	-0.23 to 0.35 (b)
Erythrocyte Se pmol/10^6 cells	0.181 ± 0.026	0.187 ± 0.029	-0.006 (b)	-0.025 to 0.013 (b)
Plasma Se µmol l^{-1}	1.43 ± 0.19	1.40 ± 0.16	0.03 (b)	-0.06 to 0.12 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

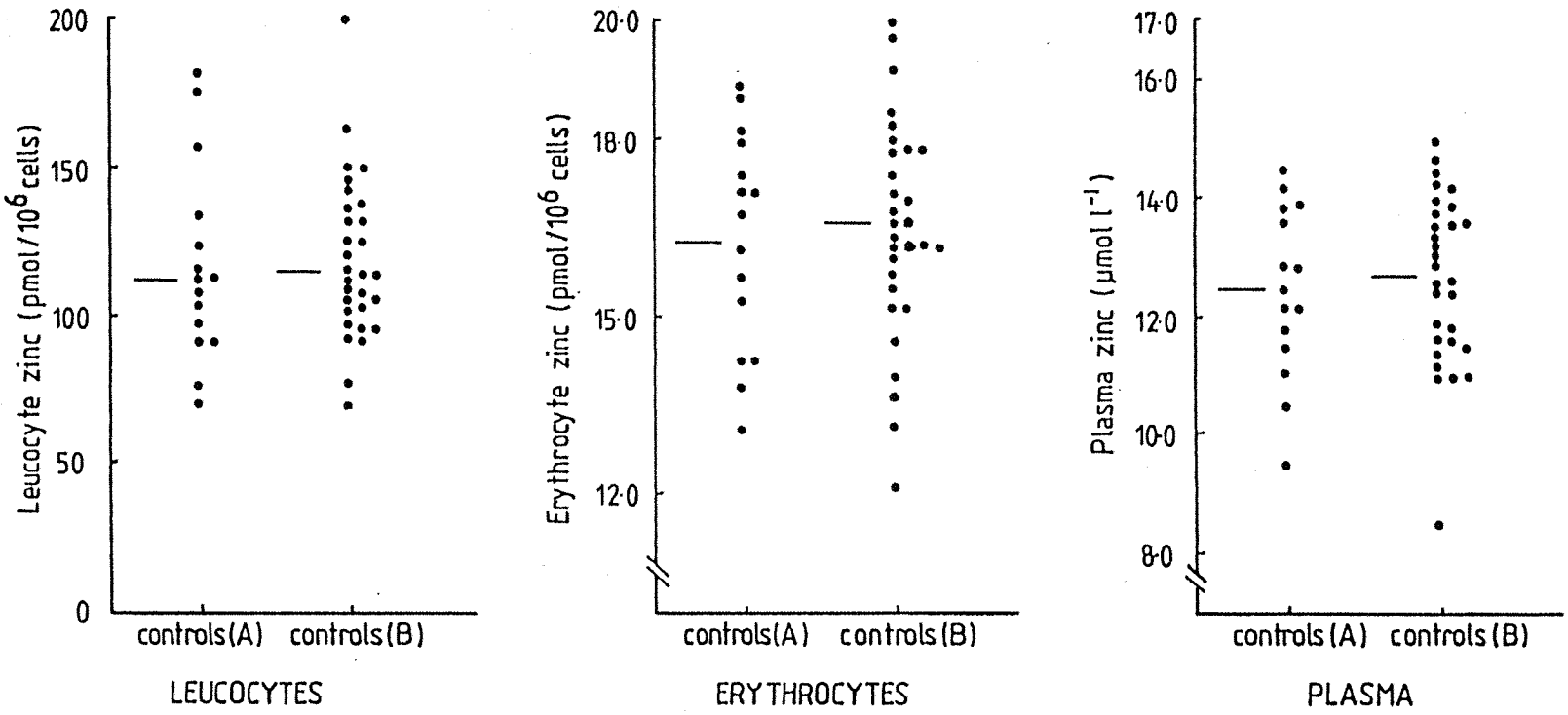


Figure 10.1

Scattergram of zinc concentrations in leucocytes, erythrocytes and plasma in the control subjects (A) investigated in the present chapter and for a similar group of control subjects (B) investigated in Chapter 6. Horizontal lines show the mean values (geometric mean for leucocyte zinc values) of the data sets

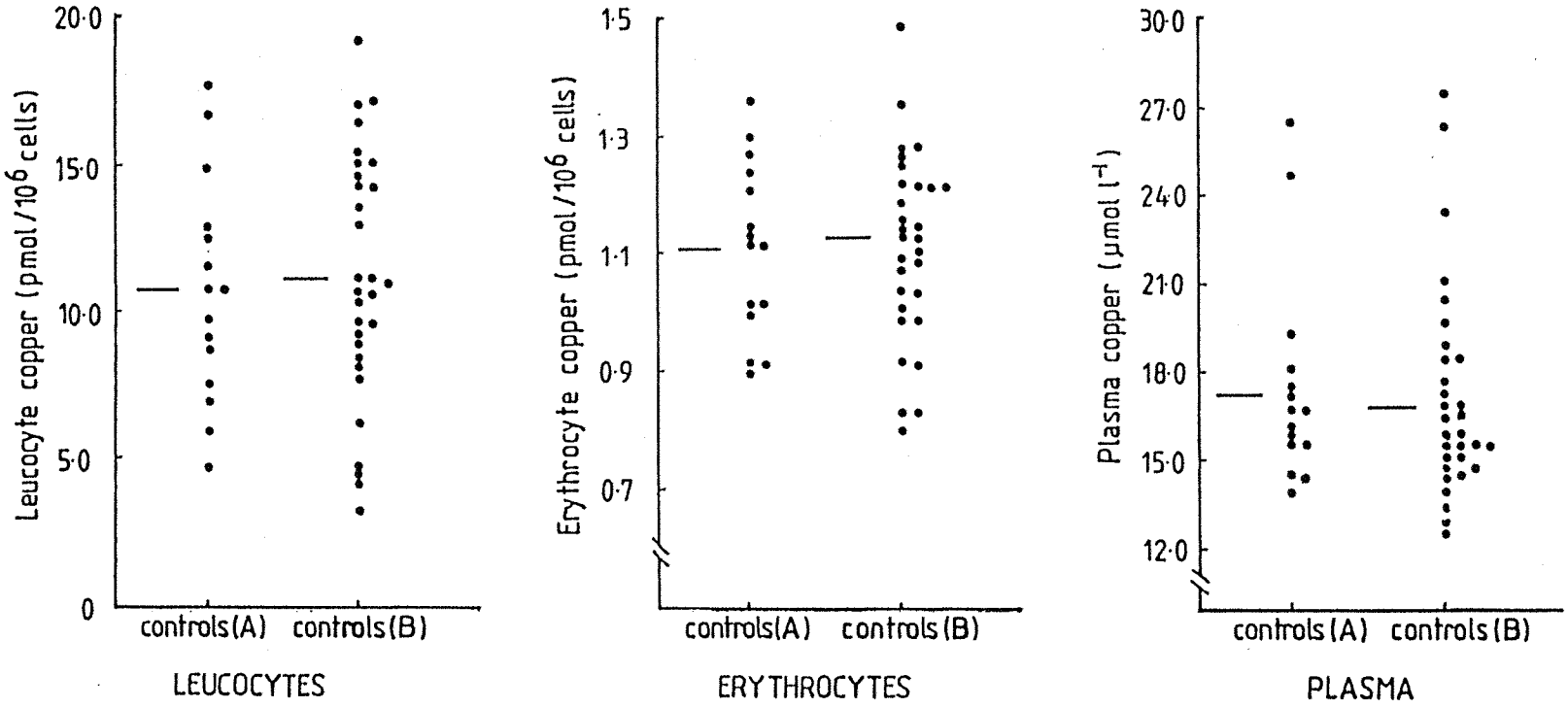


Figure 10.2

Scattergram of copper concentrations in leucocytes, erythrocytes and plasma in the control subjects (A) investigated in the present chapter and for a similar group of control subjects (B) investigated in Chapter 6. Horizontal lines show the mean values (geometric mean for plasma copper values) of the data sets

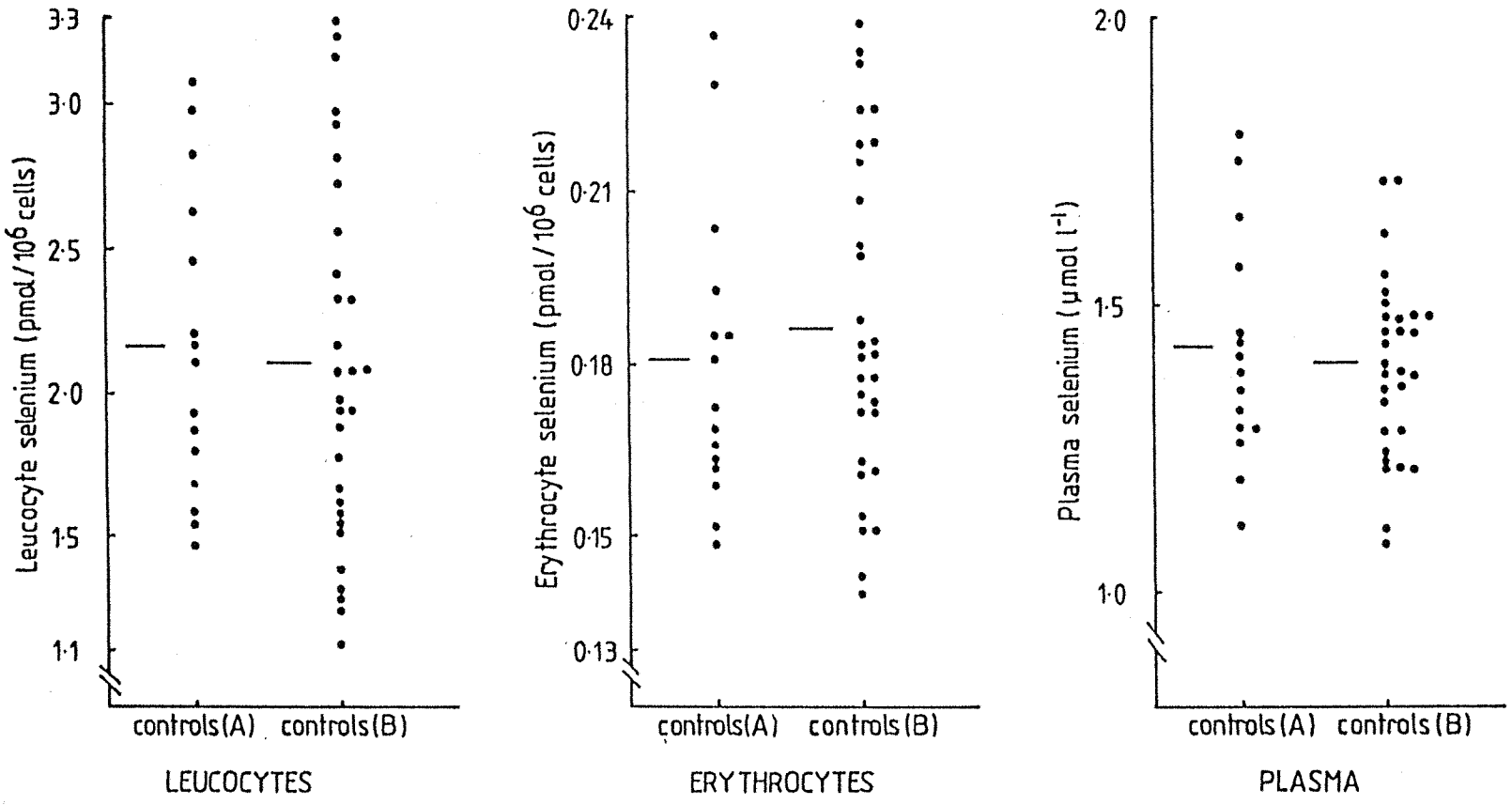


Figure 10.3

Scattergram of selenium concentrations in leucocytes, erythrocytes and plasma in the control subjects (A) investigated in the present chapter and for a similar group of control subjects (B) investigated in Chapter 6. Horizontal lines show the mean values of the data sets

TABLE 10.IV

COMPARISON OF CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA BETWEEN PATIENTS WITH LESS THAN, AND GREATER THAN 10% SURFACE AREA INVOLVEMENT

Analysis	Patients with <10% surface area involvement n = 11	Patients with >10% surface area involvement n = 13	Ratio of (a) or difference between (b) means	95% CI for the ratio of (a) or difference between (b) means
Leucocyte Zn pmol/10^6 cells	116(110-129)$^{\Omega}$	109(97-113)$^{\Omega}$	1.07 (a)	0.93 to 1.23 (a)
Erythrocyte Zn pmol/10^6 cells	16.2 ± 2.1	16.2 ± 1.7	-0.05 (b)	-1.64 to 1.54 (b)
Plasma Zn µmol l^{-1}	14.1 ± 1.22	13.1 ± 2.08	0.99 (b)	-0.49 to 2.47 (b)
Leucocyte Cu pmol/10^6 cells	10.3 ± 3.5	10.0 ± 4.8	0.27 (b)	-3.37 to 3.91 (b)
Erythrocyte Cu pmol/10^6 cells	1.13 ± 0.20	1.12 ± 0.18	0.04 (b)	-0.19 to 0.20 (b)
Plasma Cu µmol l^{-1}	22.4(20.0-23.5)$^{\Omega}$	20.4(16.5-24.8)$^{\Omega}$	1.10 (a)	0.89 to 1.35 (a)
Leucocyte Se pmol/10^6 cells	1.18 ± 0.20	1.22 ± 0.28	-0.05 (b)	-0.26 to 0.16 (b)
Erythrocyte Se pmol/10^6 cells	0.132 ± 0.42	0.140 ± 0.053	-0.008 (b)	-0.049 to 0.033 (b)
Plasma Se µmol l^{-1}	1.13 ± 0.26	1.09 ± 0.36	0.03 (b)	-0.24 to 0.30 (b)

$^{\Omega}$ Geometric mean (interquartile distance)

TABLE 10.V

CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN PATIENTS WITH ECZEMA, AND MATCHED CONTROLS

Analysis	Patients with eczema n = 24	Matched controls n = 48	Ratio of means (a) or mean of individual patient - control differences (b)	95% CI for the ratio of means (a) or the mean of individual patient - control differences (b)
Leucocyte Zn pmol/10^6 cells	111(97-129)$^{\Omega}$	111(96-128)$^{\Omega}$	1.00 (a)	0.91 to 1.10 (a)
Erythrocyte Zn pmol/10^6 cells	16.2 ± 1.8	16.2 ± 2.1	-0.05 (b)	-1.10 to 1.00 (b)
Plasma Zn µmol l^{-1}	13.5 ± 1.8	12.9 ± 1.9	0.60 (b)	-0.36 to 1.52 (b)
Leucocyte Cu pmol/10^6 cells	10.1 ± 4.2	10.2 ± 3.2	-0.06 (b)	-2.06 to 1.94 (b)
Erythrocyte Cu pmol/10^6 cells	1.13 ± 0.18	1.12 ± 0.17	0.01 (b)	-0.11 to 0.09 (b)
Plasma Cu µmol l^{-1}	21.3(17.6-24.8)$^{\Omega ++}$	18.1(15.5-21.7)$^{\Omega}$	1.18 (a)	1.05 to 1.32 (a)
Leucocyte Se pmol/10^6 cells	1.20 ± 0.24^{+++}	1.98 ± 0.49	-0.77 (b)	-0.96 to -0.58 (b)
Erythrocyte Se pmol/10^6 cells	0.136 ± 0.047^{++}	0.176 ± 0.032	-0.040 (b)	-0.063 to -0.017 (b)
Plasma Se µmol l^{-1}	1.11 ± 0.32^{+++}	1.41 ± 0.17	-0.30 (b)	-0.43 to -0.17 (b)

$^{\Omega}$ Geometric mean (interquartile distance) $^{++}$ $p < 0.01$ $^{+++}$ $p < 0.001$

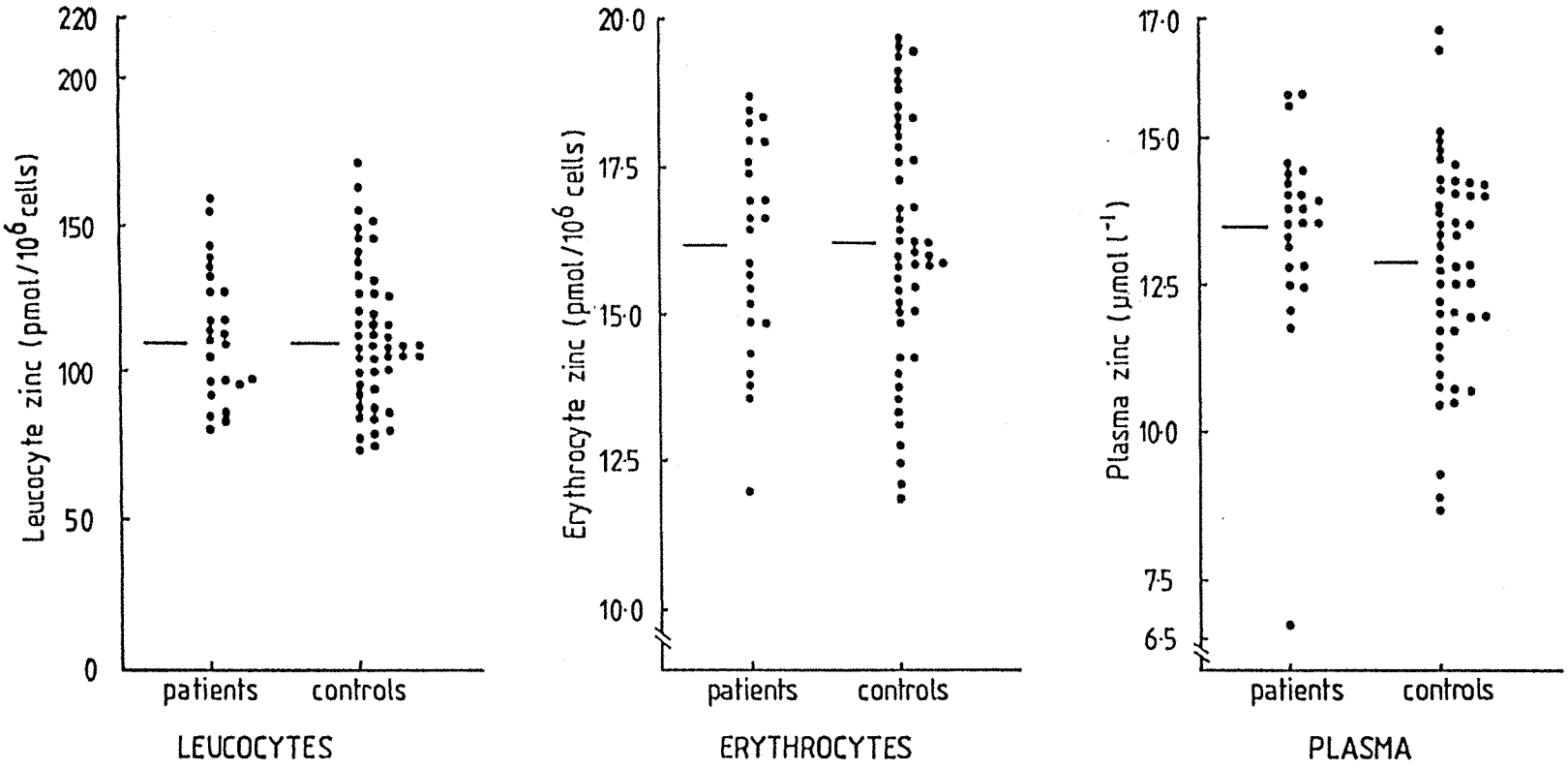


Figure 10.4

Scattergram of zinc concentrations in leucocytes, erythrocytes and plasma in patients with eczema and matched controls. Horizontal lines show the mean values (geometric mean for leucocyte zinc values) of the data sets

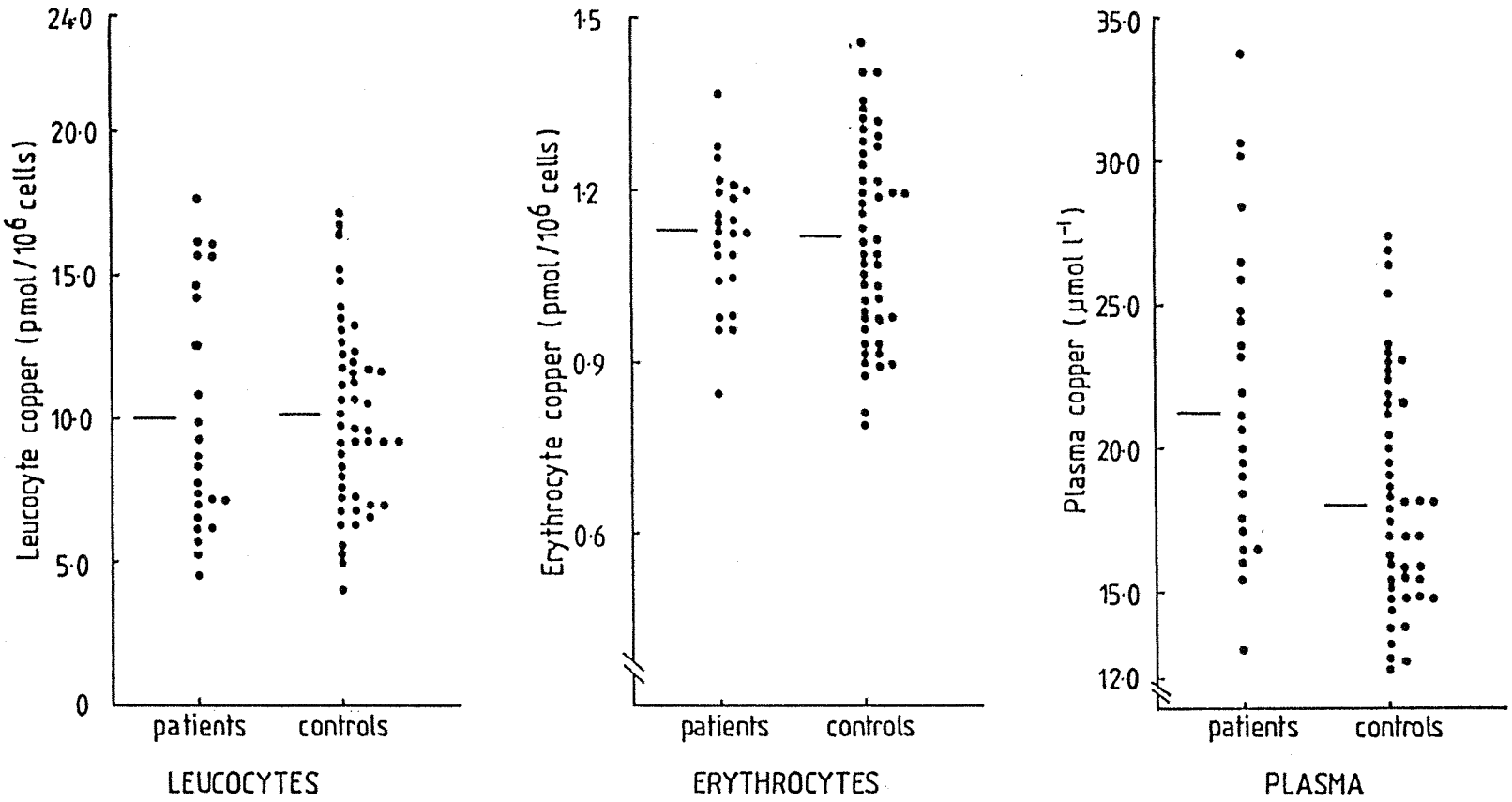


Figure 10.5

Scattergram of copper concentrations in leucocytes, erythrocytes and plasma in patients with eczema and matched controls. Horizontal lines show the mean values (geometric mean for plasma copper values) of the data sets

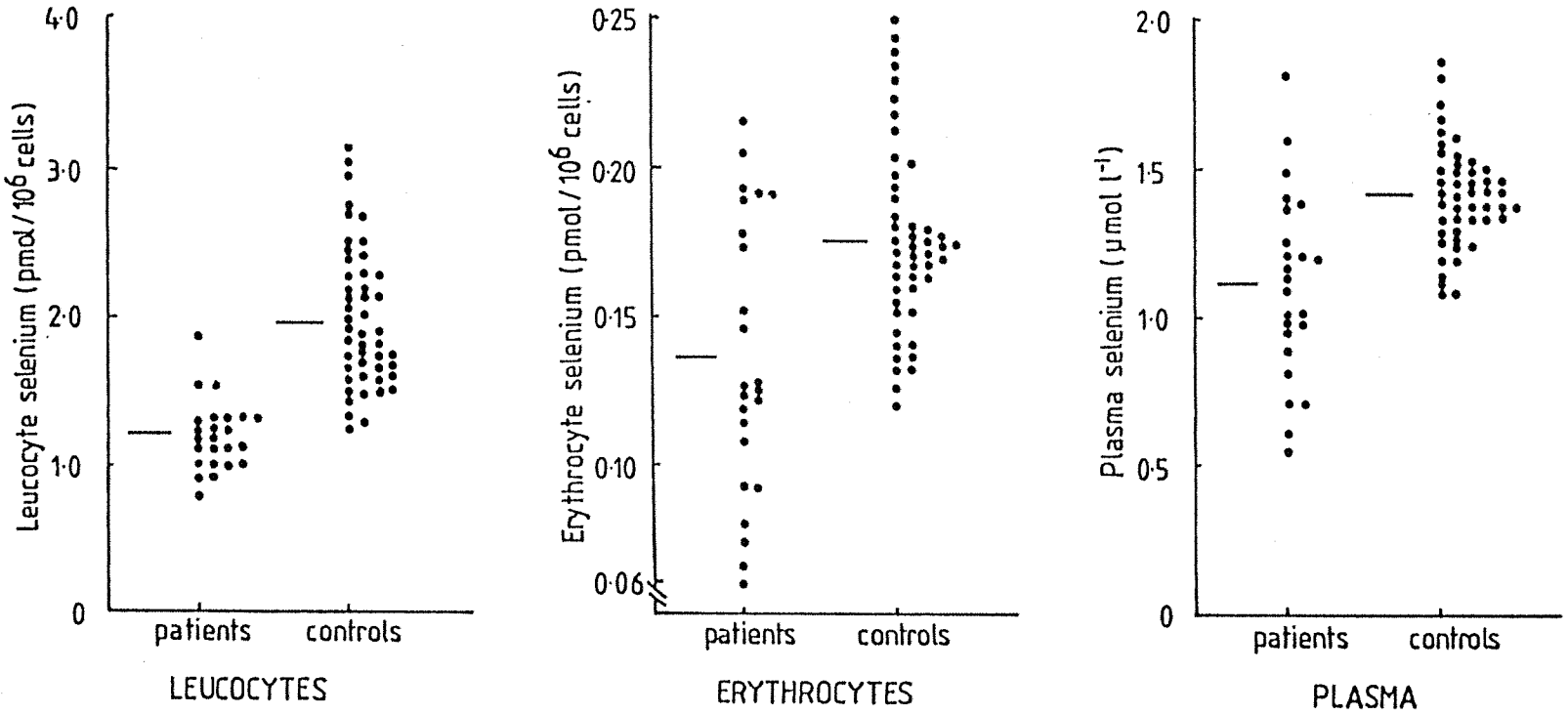


Figure 10.6

Scattergram of selenium concentrations in leucocytes, erythrocytes and plasma in patients with eczema and matched controls. Horizontal lines show the mean values of the data sets

r = 0.71) and erythrocytes and plasma ($p < 0.001$, r = 0.88). No similar relationship was noted for copper and zinc. There were no significant correlations between body mass index and selenium concentrations in leucocytes, erythrocytes or plasma in the patient group.

Plasma caeruloplasmin concentrations were significantly higher ($p < 0.01$) in the group of patients with eczema (426 ± 105 mg l^{-1}) when compared to the control group (340 ± 80 mg l^{-1}). A significant correlation was found between plasma copper and caeruloplasmin concentrations in both patients ($p < 0.002$, $r_s = 0.87$) and controls ($p < 0.001$, $r_s = 0.86$).

Plasma albumin concentrations were similar in patients (45 ± 4 g l^{-1}) and matched controls (44 ± 3 g l^{-1}), and there was no correlation between plasma zinc and albumin or plasma selenium and albumin concentrations in either of the groups.

Patients with psorasis

The individual results for the patients with psoriasis are given in Appendix 10. The white cell counts, red cell counts and PCV values for the whole blood samples were 6.24 ± 1.67 x 10^9 l^{-1}, 5.05 ± 0.45 x 10^{12} l^{-1} and 44.0 ± 3.4 respectively.

The effect of surface area involvement, use of topical steroids, and topical zinc applications were assessed, where appropriate, on the data obtained for zinc, copper and selenium in the patient group. Other independent variables i.e. sex, age and smoking, where known to influence the parameters measured, were also included in the statistical analysis. As only one of the women in the patient group was taking oral contraceptives, the results for this subject were excluded for the purpose of this analysis. Statistical analysis was performed on the logged data for leucocyte zinc. None of the variables investigated had any significant effect on the concentration of zinc, copper or selenium in leucocytes, erythrocytes or plasma.

A summary of the zinc, copper and selenium results for the patient and control groups is given in Table 10.VI. There was no significant difference in the concentration of zinc in leucocytes, erythrocytes or plasma between these two groups, although the 95% confidence intervals for the ratio of population means show that leucocyte zinc concentrations are much more likely to be lower in patients with psoriasis. A significant increase in the concentration of plasma copper ($p < 0.01$) was demonstrated in the group of patients

TABLE 10.VI

CONCENTRATIONS OF ZINC, COPPER AND SELENIUM IN LEUCOCYTES, ERYTHROCYTES AND PLASMA IN PATIENTS WITH PSORIASIS, AND MATCHED CONTROLS

Analysis	Patients with psoriasis n = 23	Matched controls n = 46	Ratio of means (a) or mean of individual patient - control differences (b)	95% CI for the ratio of means (a) or the mean of individual patient - control differences (b)
Leucocyte Zn pmol/10^6 cells	101(87-108)$^{\Omega}$	112(97-134)$^{\Omega}$	0.90 (a)	0.81 to 1.00 (a)
Erythrocyte Zn pmol/10^6 cells	16.2 ± 2.04	16.4 ± 1.9	-0.18 (b)	-1.08 to 1.44 (b)
Plasma Zn µmol l^{-1}	14.1 ± 2.2	13.0 ± 2.2	1.01 (b)	-0.26 to 2.28 (b)
Leucocyte Cu pmol/10^6 cells	9.5 ± 4.2	9.8 ± 3.3	-0.33 (b)	-2.04 to 1.38 (b)
Erythrocyte Cu pmol/10^6 cells	1.14 ± 0.18	1.14 ± 0.16	0.00 (b)	-0.10 to 0.10 (b)
Plasma Cu µmol l^{-1}	19.3 ± 4.2^{++}	17.0 ± 3.0	2.64 (b)	0.71 to 4.57 (b)
Leucocyte Se pmol/10^6 cells	1.14 ± 0.25^{+++}	2.07 ± 0.53	-0.94 (b)	-1.15 to -0.73 (b)
Erythrocyte Se pmol/10^6 cells	0.128 ± 0.028^{+++}	0.177 ± 0.037	-0.046 (b)	-0.063 to -0.029 (b)
Plasma Se µmol l^{-1}	1.05 ± 0.21^{+++}	1.38 ± 0.15	-0.34 (b)	-0.44 to -0.24 (b)

$^{\Omega}$ Geometric mean (interquartile distance) $^{++}$ $p < 0.01$ $^{+++}$ $p < 0.001$

with psoriasis when compared to the control group. No similar increase was found for leucocyte or erythrocyte copper concentrations. Scattergrams of the zinc and copper results for patients and controls are presented in Figures 10.7 and 10.8 respectively. A significant decrease in the concentration of selenium in leucocytes, erythrocytes and plasma ($p < 0.001$) was demonstrated in the patients compared with the controls (Table 10.VI). Figure 10.9 presents a scattergram of the selenium results for the patient and control groups. There was a significant correlation between selenium in leucocytes and erythrocytes ($p < 0.001$, $r = 0.68$), leucocytes and plasma ($p < 0.001$, $r = 0.74$) and erythrocytes and plasma ($p < 0.001$, $r = 0.79$) in the patient group. No similar relationship was noted for copper or zinc. There were no significant correlations betwen body mass index and selenium concentrations in leucocytes, erythrocytes or plasma.

Significantly higher concentrations ($p < 0.001$) of plasma caeruloplasmin were found in the patients with psoriasis (385 ± 86 mg l^{-1}) compared with the controls (306 ± 52 mg l^{-1}). A significant correlation was noted for plasma copper and caeruloplasmin concentrations in patients ($p < 0.001$, $r = 0.89$) and controls ($p < 0.001$, $r = 0.84$).

Plasmin albumin concentrations did not differ significantly in the patient group (45 ± 4 g l^{-1}) compared with the control group (46 ± 3 g l^{-1}), and there was no correlation between plasma zinc and albumin or plasma selenium and albumin concentrations in either group.

10.4 DISCUSSION

Comparison of two groups of control data

The clinical investigations described in this thesis were carried out in the order presented. As the use of historical control data is open to criticism, a comparison of the data obtained for the control subjects investigated in the present study was made with the data obtained for a similar group of control subjects investigated in Chapter 6. As shown in the preceding section of this chapter, there was an excellent agreement between the results for zinc, copper and selenium for these two groups which validated the continued use of the original control data.

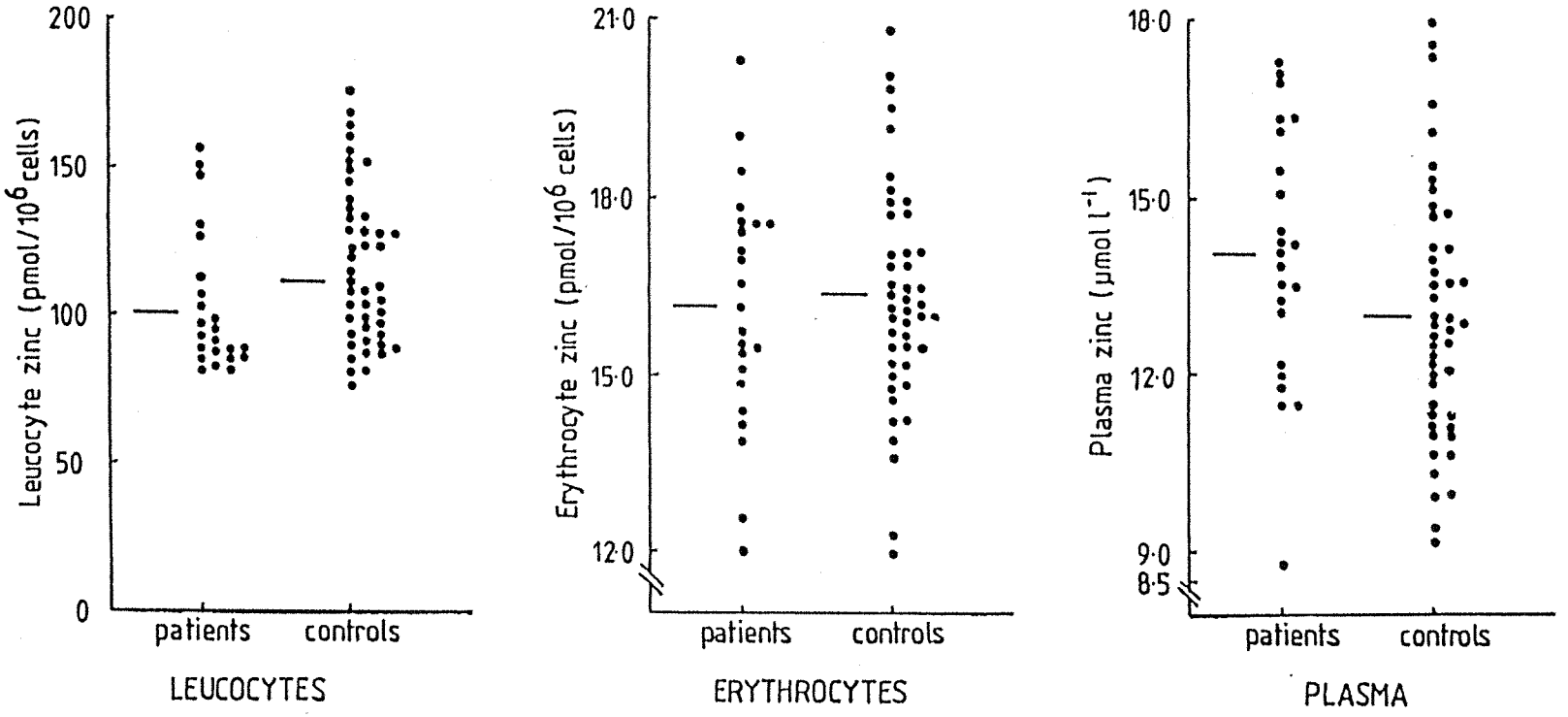


Figure 10.7

Scattergram of zinc concentrations in leucocytes, erythrocytes and plasma in patients with psoriasis and matched controls. Horizontal lines show the mean values (geometric mean for leucocyte zinc values) of the data sets

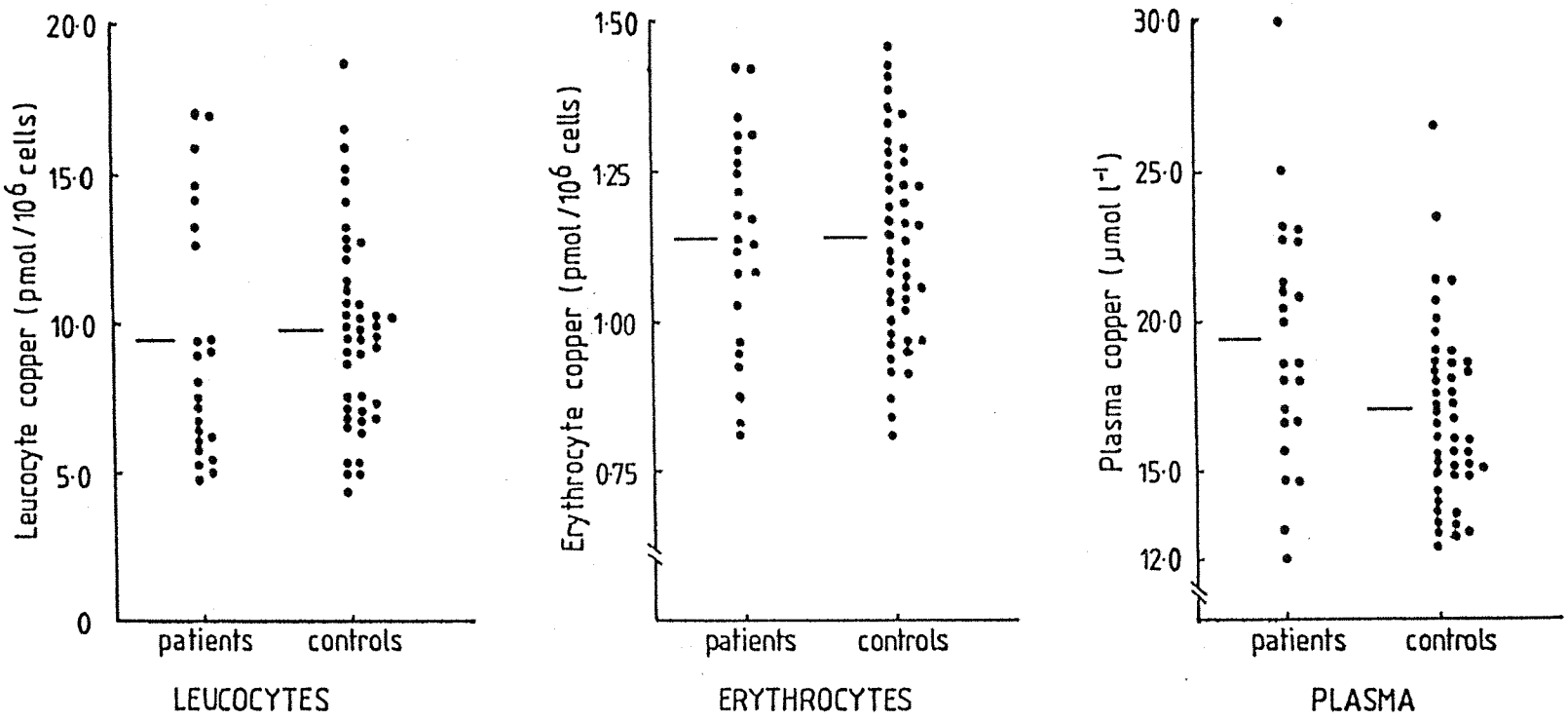


Figure 10.8

Scattergram of copper concentrations in leucocytes, erythrocytes and plasma in patients with psoriasis and matched controls. Horizontal lines show the mean values of the data sets

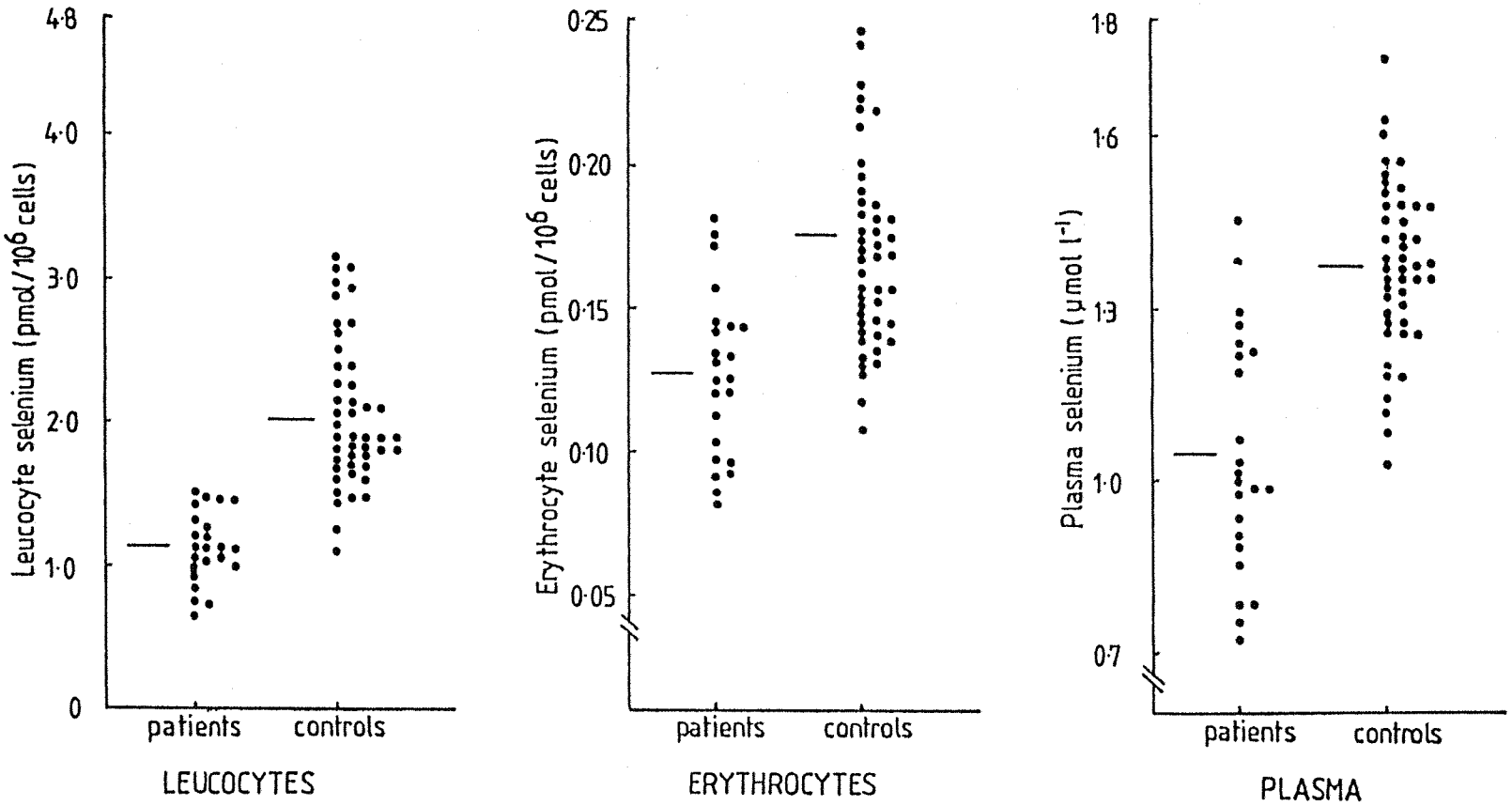


Figure 10.9

Scattergram of selenium concentrations in leucocytes, erythrocytes and plasma in patients with psoriasis and matched controls. Horizontal lines show the mean values of the data sets

Patients with eczema and psoriasis

In the present study, the use of topical skin preparations containing zinc were found to have no significant effect on plasma zinc concentrations in the patients with psoriasis. Plasma zinc concentrations were not significantly altered in a group of patients with psoriasis who were investigated before and during treatment with local zinc applications over a two-week period (Morgan et al, 1980). As all of the patients with eczema were receiving topical steroid treatment in the present study, it was not possible to evaluate the effect of this treatment on this group of patients although there was no significant effect on plasma zinc, copper or selenium concentrations from the use of topical corticosteroids by the group of patients with psoriasis.

Studies of plasma zinc concentrations in psoriatic patients have resulted in conflicting data, with decreased (Greaves and Boyde, 1967; Voorhees et al, 1969) and normal (Withers et al, 1968; Molokhia and Portnoy, 1970) levels reported. Decreased plasma zinc concentrations in patients with pustular psoriasis were particularly low during exacerbation of their disease, and were accompanied by a decrease in serum albumin concentrations (Thune, 1980). It has been suggested that the reason for the conflicting findings of plasma zinc levels in psoriatic patients may be due to failure to take into account the extent of skin involvement, and a relationship between surface area involvement and plasma zinc concentrations in psoriasis has been reported (Greaves, 1971). A more recent study also demonstrated significantly lower plasma zinc concentrations in psoriatic patients with greater than 10% surface area involvement compared with those who had less than 10% involvement, although no significant difference in plasma zinc concentrations was found between the total group of patients and age- and sex-matched controls (McMillan and Rowe, 1983). The present study failed to demonstrate any relationship between the extent of surface area involvement and the concentration of zinc, copper or selenium in leucocytes, erythrocytes and plasma for either of the patient groups.

Although there has been interest in the possibility of zinc deficiency in patients with eczema and psoriasis, no abnormality of Zn^{65} metabolism was identified in a small group of patients with dermatological diseases, including eczema and psoriasis (Hawkins et al, 1976). Furthermore, oral zinc therapy was found to be ineffective

in the treatment of patients with psoriasis (Voorhees et al, 1969; Greaves and Dawber, 1970), although an increase in the zinc concentration of psoriatic scale and uninvolved skin was demonstrated after zinc supplementation (Voorhees et al, 1969). It is of interest however that a case of severe eczema described in an elderly female responded to oral zinc supplementation (Owens et al, 1981). In the same study, a low plasma zinc concentration was reported before supplementation although the cause of this was not identified. It is possible that the decreased plasma zinc concentrations found in some studies of patients with psoriasis may be a reflection of the inflammatory nature of the skin disease rather than a result of zinc deficiency. In the present study, no significant difference was found in the concentration of zinc in leucocytes, erythrocytes or plasma in the patients compared with the control subjects, although it is interesting to note that there was a tendency towards decreased levels of leucocyte zinc in the patients with psoriasis, although not with eczema. The possibility remains that increased daily zinc loss via desquamation may be of importance in the development of a lowered zinc status in patients with psoriasis. A significantly higher concentration of zinc in involved (Molokhia and Portnoy, 1970; Molin and Wester, 1973) and uninvolved (Molokhia and Portnoy, 1970) psoriatic epidermis compared to normal epidermis has been reported. These patients were not receiving oral zinc supplementation, and the reason for the increased skin zinc concentration is unclear.

In the present study, the concentration of copper in leucocytes and erythrocytes in the patients with eczema and psoriasis did not differ significantly from the control subjects although plasma copper and caeruloplasmin concentrations were significantly increased in both patient groups. Increased plasma copper concentrations in patients with psoriasis have previously been reported (Lipkin et al, 1962; Kekki et al, 1966; Zackheim and Wolf, 1972). In contrast to the findings in the present study, Kekki et al (1966) attributed the increase in plasma copper levels in patients with uncomplicated psoriasis to the non-caeruloplasmin fraction although they determined the non-caeruloplasmin copper fraction indirectly. Although serum caeruloplasmin concentrations did not differ significantly in patients with uncomplicated psoriasis compared with control subjects, the plasma copper concentrations were not reported (Koskelo et al, 1966). Caeruloplasmin is an acute phase reactant protein and

the increased plasma copper and caeruloplasmin concentrations found in the patients in the present study can most probably be attributed to the inflammatory condition associated with the skin disease. Several studies have reported no significant difference in the copper concentration of normal and psoriatic epidermis (Molokhia and Portnoy, 1970; Molin and Wester, 1973).

Reduced concentrations of selenium were observed in leucocytes, erythrocytes and plasma in both groups of patients in the present study. A Swedish study reported depressed erythrocyte glutathione peroxidase activity in patients with various skin disorders which included eczema and psoriasis, and it was suggested that this indicated a possible selenium deficiency although selenium levels were not directly measured (Juhlin et al, 1982). In some of the cases of eczema and psoriasis however, the low activity of glutathione peroxidase was attributed to malnutrition, alcohol abuse and vegetarian diet rather than to the disease itself.

The reason for the lowered selenium status in the patients in the present study is not clear. None of the patients were following exclusion diet regimes and a reduced intake of selenium is therefore unlikely. Furthermore, there was no correlation between body mass index and selenium concentrations in leucocytes, erythrocytes or plasma. An increased epidermal cell turnover as a possible source of loss of selenium is unlikely. The distribution of selenium in abdominal skin is similar to that for zinc, with the content higher in the epidermis than the dermis (Molokhia et al, 1979). In normal individuals, the estimated daily loss via desquamation is 306-612 nmol for zinc and 6-13 nmol for selenium (Molin and Wester, 1976). Depletion of selenium via this route would therefore probably be associated with a zinc depletion, and the patients in the present study did not have a significantly reduced body zinc status. Another consideration is the association between malabsorption and skin disease. The concept of dermatogenic enteropathy was introduced by Shuster and Marks (1965) following their observation that steatorrhoea occurring in patients with widespread skin disease, including psoriasis and eczema, improved following treatment of the skin alone. Subsequently, they showed a relationship between the area of skin involved and fat malabsorption in patients with psoriasis, although the degree of mucosal abnormality found on jejunal biopsy was not related to the extent of the psoriasis (Shuster et al, 1967). In a

further report by this group however, there appeared to be no difference between the jejunal mucosa from cases of eczema and psoriasis compared with the mucosa from a local control population (Marks and Shuster, 1970). In contrast to these findings, Barry et al (1971) reported changes in small bowel mucosal architecture in patients with severe psoriasis compared with normal controls. These authors however also reported similar changes in patients who were ill and losing weight for reasons other than psoriasis, and emphasised the importance of the careful selection of control subjects for clinical studies. Malabsorption of selenium in patients with eczema and psoriasis may therefore be a possible explanation for the lowered selenium status in the patients in the present study.

The clinical importance of a lowered selenium status in these patients has yet to be decided. Both eczema and psoriasis are inflammatory skin diseases. Increased free radical generation is likely in inflammatory conditions (Halliwell, 1987), and there may be an increased bodily need for selenium. Low selenium levels may exacerbate the skin condition by reducing the activity of glutathione peroxidase, and therefore antioxidant protection. Beneficial effects of combined oral selenium and vitamin E therapy in skin patients, including a small number with psoriasis and eczema, have been demonstrated although the effect on the skin lesions was difficult to evaluate since other treatments were also given (Juhlin et al, 1982). A similar study using the same therapy regime reported good results in patients with acne vulgaris, another inflammatory skin condition, who also had a decreased activity of erythrocyte glutathione peroxidase (Michaelsson and Edqvist, 1984).

GENERAL DISCUSSION

CHAPTER 11

GENERAL DISCUSSION AND RECOMMENDATIONS FOR FURTHER WORK

11.1 GENERAL DISCUSSION

The essentiality of zinc, copper and selenium for the normal performance of many biochemical processes in man is well established. Whilst frank deficiencies have been reported, there is an increasing awareness that marginal deficiency states of these trace elements may complicate a wide variety of clinical conditions. Nevertheless, it is clear that inadequate methodology still exists for the diagnosis of nutritional status with regard to trace elements. Whilst plasma or serum measurements are undoubtedly the most straightforward, in the context of disease or even in normal altered physiology such as pregnancy or aging, their interpretation can be difficult. In the present study, a decrease in plasma zinc and increase in plasma copper levels were observed in the apparently healthy elderly people. At present, the effect of advancing age on trace element metabolism is poorly understood. Interestingly, ingestion of food in fasting healthy subjects under the age of 50 years resulted in a decrease in plasma zinc concentrations. This observation is obviously of importance when establishing a reference range for plasma zinc, and for correct interpretation of plasma zinc measurements. Oral contraceptive agents were shown to increase plasma copper levels, and steroid intake had an apparent effect on the circulating concentrations of zinc and copper in the patients with Crohn's disease. Increased plasma copper concentrations were demonstrated in patients with skin disease and alcoholic liver disease, presumably as a result of the inflammatory nature of the condition. In addition, decreased plasma zinc concentrations in patients with alcoholic liver disease were associated with a decrease in plasma albumin levels. Clearly, findings from the present study emphasise the limitations of the use of plasma zinc and copper for the diagnosis of deficiency states of these trace elements. Plasma selenium appears to be less influenced by non-nutritional factors, although Lloyd et al (1983) demonstrated

an increase in plasma selenium levels in women taking oral contraceptive agents.

As a result of the difficulties associated with the use of plasma or serum concentrations of the trace element for the diagnosis of deficiency states, some workers have proposed determination of the trace elements in erythrocytes or leucocytes. Erythrocyte zinc and copper however have not generally been used as an index of body status, and the relatively long life of the red cells limits their potential usefulness. It is of interest in the present study that there was a significant relationship between erythrocyte and plasma copper concentrations in the young healthy control subjects and patients with coeliac disease. No such relationship was noted in the patients with Crohn's disease, eczema or psoriasis, who demonstrated increased plasma, but not erythrocyte, copper levels either as a result of steroid intake or the inflammatory condition. Whilst no significant correlation was found between erythrocyte and plasma zinc concentrations in any of the subject groups, there was a consistent significant relationship between erythrocyte and plasma selenium levels. Erythrocyte selenium is generally considered to be a useful measurement for the diagnosis of selenium deficiency, although unsatisfactory for the investigation of short-term changes in selenium status.

Leucocytes have some advantages and some disadvantages compared to erythrocytes. Although they are arguably more representative of body tissue, they are not so easily available and are more difficult to handle than red cells. However, the method developed for the separation of white cells in the present study was shown to be both practicable and reliable, with no evidence of cell clumping. Other workers have repeatedly encountered the problem of cell clumping with leucocyte separation techniques, thus making it impracticable to express the results in terms of cell number.

Of particular interest in the present study was the highly significant relationship between leucocyte zinc concentration and zinc retention in the housebound elderly people who had an overall negative zinc balance. Prasad et al (1978) demonstrated that simple dietary deprivation of zinc in healthy subjects induced a reduction in leucocyte zinc concentration. In the present study, the housebound elderly people were shown to have a markedly reduced intake of zinc and decreased leucocyte zinc levels compared with the healthy elderly

people. Significantly decreased leucocyte zinc levels were also demonstrated in the patients with alcoholic liver disease, indicating a lowered zinc status. Although these patients are at risk of a decreased zinc intake, other factors such as malabsorption and increased urinary excretion of the element may be in part responsible for the apparent zinc deficiency.

The use of leucocyte copper and selenium as an index of body status of these trace elements in this project is somewhat conjectural. No significant relationship was found between leucocyte copper concentration and copper retention in the housebound elderly people. It is noteworthy however that these subjects were in negative copper balance and also demonstrated reduced leucocyte copper levels. Of further interest is the significant relationship between selenium concentrations in leucocytes and erythrocytes, and leucocytes and plasma in all the subject groups. No similar relationships were noted for copper or zinc. Although the value of leucocyte copper and selenium measurements has yet to be established, steroid intake, use of oral contraceptive agents and inflammation had no significant effect on these parameters, or on leucocyte zinc levels. Perhaps leucocyte measurements may prove to be most useful for group comparison studies, or studies involving supplementation, rather than as an isolated measurement for the diagnosis of trace element deficiency.

A consistent finding in the patients investigated in the present study was the reduced levels of selenium in leucocytes, erythrocytes and plasma. In general, a decreased intake of selenium appeared to be a contributing factor to the lowered selenium status in these patients. Selenium is an essential component of the enzyme glutathione peroxidase, an important antioxidant which helps to protect against free radical-mediated damage. According to Halliwell (1987), increased free radical reactions are likely in any disease, and in inflammatory conditions in particular there may be increased activity of phagocytic cells which would result in an increased formation of the superoxide radical. Superoxide dismutase removes the superoxide radical by converting it to hydrogen peroxide, and this in turn is removed by glutathione peroxidase which uses hydrogen peroxide to oxidise reduced glutathione to oxidised glutathione (Willson, 1987). Deficiency of selenium may result in conditions in which levels of hydrogen peroxide rise. An iron-catalysed reaction may then

take place that results in the formation of the hydroxyl radical and a subsequent increase in lipid peroxidation (Diplock, 1984). As discussed in Chapter 2, vitamin E is an important chain-breaking antioxidant, and in the circumstances just described an adequate supply of this vitamin would be of particular importance in order to react with the peroxy radicals formed during lipid peroxidation, thus preventing damage to cell membranes which may otherwise result.

Of further relevance with regard to the lowered selenium status of the patients studied is the possible relationship between dietary selenium intake and susceptibility to carcinogensis. As already discussed, alcholic subjects and patients with coeliac disease have an increased risk of cancers of the gastrointestinal tract. In a recent review of the available information from animal studies on the effectiveness of selenium in cancer prevention, Ip (1986) concluded that the risk of developing cancer appears higher with a low selenium intake and decreases proportionally with dietary selenium levels above the recommended requirement. On the basis of this evidence it is tempting to speculate that selenium supplementation might be of benefit to these patients by reducing the risk of developing cancer, particularly in view of their lowered selenium status. However, animal studies to date on the anticarcinogenic effect of selenium are far from conclusive. The correlation between the amount of supplementation and the degree of inhibition of carcinogensis obviously depends on the strain of animals, the composition of the diet and the form of selenium used, as well as the specificity and dose of the carcinogen. Caution must therefore be applied in administering selenium to humans with the aim of preventing cancer.

11.2 RECOMMENDATIONS FOR FURTHER WORK

Several studies in humans have been carried out to assess the usefulness of leucocyte zinc for the diagnosis of zinc deficiency. The use of leucocyte analyses to assess body copper and selenium status requires similar investigations. Studies in humans of the relationship between the concentrations of copper and selenium in leucocytes and various body tissues, such as muscle or liver, would be necessary. In the case of selenium, it would also be helpful to perform balance studies on subjects both in good health and with a

lowered selenium status, in order to determine any relationship between leucocyte selenium levels and selenium retention.

A number of results presented in this thesis constitute good evidence of a lowered selenium status in patients with Crohn's and coeliac disease, eczema and psoriasis, and in alcoholic subjects. It is now necessary to evaluate the relevance of these findings. Of particular importance would be the determination of the antioxidant status of these patients. Decreased vitamin E levels were observed in the alcoholic subjects, and the vitamin E status of the other patient groups should be determined together with erythrocyte glutathione peroxidase activity. As the patients studied demonstrated reduced leucocyte selenium concentrations, measurement of glutathione peroxidase activity in leucocytes would also be of interest.

The reason for the lowered selenium status of these patients also requires investigation. Patients with coeliac disease are perhaps particularly at risk of a reduced intake of selenium due to their exclusion diet regime, and it is therefore necessary to determine the selenium content of their diet and compare this with a normal diet. It would also be of interest to investigate the absorption of dietary selenium, particularly in the case of patients with eczema and psoriasis, and alcoholic subjects, and it may be that these studies would be most suitably carried out by using stable isotopes.

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APPENDICES

APPENDIX 1

DIURNAL VARIATION OF PLASMA COPPER AND SELENIUM CONCENTRATIONS IN HEALTHY SUBJECTS EATING NORMALLY

No.	Sex	Age	OCA*	Plasma copper concentration μmol l^{-1}					Plasma selenium concentration μmol l^{-1}				
				0900h+	1100h	1300h+	1500h	1700h	0900h+	1100h	1300h+	1500h	1700h
1	F	30	No	16.1	16.3	16.0	17.0	17.1	1.09	1.14	1.11	1.09	1.11
2	F	23	No	11.4	11.4	11.0	11.9	11.5	1.05	1.03	1.01	1.06	1.08
3	F	31	No	17.5	17.5	16.7	17.7	16.7	1.10	1.06	1.11	1.09	1.08
4	F	19	No	14.8	15.6	15.6	15.2	15.6	0.76	0.80	0.79	0.81	0.77
5	F	26	B	19.0	19.2	19.0	19.5	19.0	1.00	0.95	0.98	0.96	1.00
6	M	35	-	12.9	12.9	13.5	13.3	12.9	1.03	1.06	1.08	1.05	1.04
7	F	47	No	19.4	18.6	19.2	18.9	19.0	0.95	0.95	0.91	0.96	0.94
8	F	37	No	15.1	16.0	15.6	16.0	15.6	0.96	0.99	1.01	1.01	0.98
9	F	34	No	16.3	16.0	16.7	16.4	16.5	1.17	1.13	1.18	1.19	1.18
10	M	30	-	11.6	11.6	11.6	12.0	11.0	1.08	1.04	1.03	1.06	1.09
11	M	25	-	15.6	16.0	16.7	16.0	15.8	1.17	1.22	1.15	1.15	1.19
12	F	43	No	22.2	23.2	22.2	22.0	22.8	1.14	1.15	1.18	1.19	1.18
13	M	36	-	13.7	14.1	14.1	14.1	14.1	1.23	1.25	1.20	1.19	1.20
14	F	28	A	19.4	19.0	19.4	19.8	19.8	0.94	0.90	0.95	0.91	0.91
15	M	39	-	11.8	12.5	11.7	12.4	12.6	0.94	0.95	0.92	0.94	0.92
16	M	20	-	17.5	17.1	17.1	17.1	16.7	1.30	1.28	1.32	1.27	1.32
17	F	34	No	17.6	17.2	17.3	17.0	17.7	0.98	1.01	0.99	1.01	0.96
18	F	24	No	16.1	17.0	16.1	16.7	16.0	0.96	0.96	0.92	0.95	0.99
19	F	39	No	14.4	13.8	14.7	13.7	14.5	1.01	1.00	1.03	1.00	1.01

* OCA, oral contraceptive agent: A, Ovranette (Wyeth); B, Microgynon (Schering): Levonorgestrel + Ethinyloestradiol

\+ 0900h, fasting blood sample. Subjects ingested food immediately after 0900h and 1300h

APPENDIX 2

DIURNAL VARIATION OF PLASMA ZINC CONCENTRATIONS IN HEALTHY SUBJECTS EATING NORMALLY (A) AND FASTING SUBJECTS (B)

No.	Sex	Age	OCA*	A: Plasma zinc concentration μmol l^{-1}					B: Plasma zinc concentration μmol l^{-1}				
				0900h+	1100h	1300h+	1500h	1700h	0900h	1000h	1100h	1200h	1300h
1	F	30	No	12.2	10.5	10.2	10.8	10.8	12.6	12.8	12.8	12.7	12.9
2	F	23	No	13.1	11.8	11.3	11.3	11.7	13.3	13.2	13.0	13.3	13.3
3	F	31	No	12.8	11.8	11.0	11.8	11.0	12.5	12.5	12.2	12.3	12.4
4	F	19	No	13.3	12.5	12.5	11.7	12.6	13.0	12.8	13.1	13.1	12.9
5	F	26	B	14.1	13.2	13.0	12.7	13.3	14.3	14.4	14.6	14.3	14.6
6	M	35	-	15.0	14.0	14.0	13.7	14.3	14.7	14.9	14.9	14.7	14.5
7	F	47	No	12.3	10.9	11.2	11.2	10.9	12.5	12.3	12.2	12.5	12.3
8	F	37	No	14.1	13.2	13.2	13.5	13.0	13.7	13.7	13.8	13.6	13.6
9	F	34	No	12.6	11.2	11.5	12.0	11.8	12.6	12.4	12.2	12.4	12.7
10	M	30	-	15.1	14.3	14.0	14.5	14.0	15.0	15.3	15.4	15.4	15.2
11	M	25	-	13.8	12.4	12.7	12.5	12.0	14.4	14.5	14.2	14.1	14.3
12	F	43	No	13.0	12.1	12.0	12.3	12.1	13.3	13.3	13.3	13.2	13.4
13	M	36	-	16.1	15.5	14.9	15.7	15.2	15.6	15.7	15.8	15.9	15.6
14	F	28	A	13.6	12.7	12.9	12.6	12.9	13.2	13.5	13.3	13.5	13.4
15	M	39	-	15.0	13.9	14.0	14.4	14.0	15.0	14.8	14.9	14.9	15.0
16	M	20	-	13.4	12.2	12.5	12.0	12.2	13.6	13.5	13.3	13.5	13.5
17	F	34	No	14.6	13.3	13.0	13.6	13.1	14.3	14.5	14.2	14.0	14.2
18	F	24	No	13.4	12.7	12.7	12.7	12.4	13.5	13.5	13.3	13.4	13.1
19	F	39	No	12.6	11.1	11.2	11.5	10.9	12.2	12.2	12.5	12.5	12.2

* OCA, oral contraceptive agent: A, Ovranette (Wyeth); B, Microgynon (Schering): Levonorgestrel + Ethinyloestradiol

\+ 0900h, fasting blood sample. Subjects ingested food immediately after 0900h and 1300h.

APPENDIX 3

HEALTHY CONTROL SUBJECTS : RESULTS

					Zinc				Copper				Selenium			
No.	Sex	Age	OCA*	Smoker	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
1	F	23	No	Yes	147	18.5	13.5	94	11.2	1.22	17.0	16.0	1.94	0.143	1.46	1.53
2	F	22	No	Yes	70	15.2	12.6	78	15.1	1.13	14.5	13.6	2.09	0.219	1.40	1.75
3	F	31	A	Yes	100	17.3	14.4	77	10.3	0.93	30.5	21.6	2.84	0.269	1.86	2.18
4	M	45	-	No	88	20.1	18.8	101	7.0	1.15	19.0	16.1	1.93	0.175	1.39	1.61
5	F	23	No	No	139	14.8	10.0	76	8.4	1.24	14.5	13.7	2.27	0.171	1.23	1.52
6	F	22	A	No	110	18.9	14.9	95	15.4	1.25	25.5	20.2	1.53	0.164	1.39	1.55
7	M	27	-	No	114	17.9	12.4	92	8.6	1.12	15.5	13.8	1.30	0.188	1.22	1.58
8	M	39	-	Yes	113	17.3	11.5	92	11.5	1.08	16.5	13.8	1.80	0.156	1.29	1.49
9	M	22	-	Yes	151	17.0	14.5	89	9.7	1.28	16.0	14.9	3.25	0.219	1.52	1.89
10	M	22	-	No	156	14.8	17.7	85	9.6	1.12	18.7	15.3	3.01	0.214	1.46	1.89
11	F	36	No	No	136	17.9	13.8	86	9.7	1.16	17.0	15.1	1.51	0.161	1.23	1.49
12	M	22	-	No	135	20.1	15.3	98	8.8	1.23	16.6	15.7	1.85	0.191	1.38	1.68
13	F	22	B	Yes	106	15.5	13.4	84	8.0	1.04	22.0	17.9	2.17	0.163	1.38	1.56
14	M	30	-	Yes	82	13.1	13.2	84	8.8	1.05	17.0	14.1	2.10	0.147	1.29	1.51
15	F	21	No	No	113	16.8	14.3	87	9.2	0.81	17.0	13.4	1.48	0.181	1.47	1.70
16	M	24	-	Yes	146	13.2	13.7	79	12.2	1.33	15.7	15.3	1.80	0.174	1.46	1.71
17	M	21	-	No	104	13.6	14.1	89	8.3	1.36	12.7	14.6	2.68	0.174	1.61	1.80
18	M	23	-	Yes	122	16.3	14.3	88	13.2	1.04	16.1	13.4	2.25	0.167	1.55	1.82
19	F	22	No	Yes	86	18.0	12.8	90	4.9	1.22	23.5	19.4	2.30	0.172	1.27	1.55

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent; A, Ovranette (Wyeth); B, Microgynon (Schering): Levonorgestrel + Ethinyloestradiol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
20	F	24	B	No	79	17.1	11.0	96	4.1	1.37	23.5	18.9	2.72	0.184	1.63	1.81
21	M	22	-	No	105	12.1	12.8	72	17.3	1.22	12.7	13.3	1.96	0.151	1.09	1.44
22	F	24	A	No	121	16.0	11.9	90	9.2	1.10	26.5	21.3	1.27	0.140	1.34	1.56
23	M	21	-	No	92	16.8	13.2	98	11.5	1.22	19.6	16.8	2.65	0.193	1.56	1.84
24	F	21	A	No	172	15.9	12.1	84	12.6	0.98	23.1	18.2	2.40	0.230	1.86	2.17
25	M	23	-	No	105	14.6	11.7	93	13.6	0.80	19.9	14.7	1.67	0.161	1.11	1.51
26	F	22	No	Yes	108	17.8	11.5	94	16.0	0.96	21.4	15.9	1.58	0.164	1.38	1.56
27	M	18	-	No	149	13.4	12.6	76	7.6	1.01	17.0	14.6	2.53	0.172	1.40	1.65
28	F	22	No	No	183	18.5	12.3	84	15.4	1.45	15.7	15.2	1.97	0.194	1.39	1.55
29	M	21	-	No	191	16.4	14.8	94	10.0	0.99	17.5	11.3	2.58	0.207	1.51	1.90
30	M	23	-	No	98	16.2	14.3	90	11.2	1.01	18.5	14.9	2.33	0.189	1.44	1.75
31	M	23	-	Yes	89	10.4	12.3	70	12.4	1.31	17.0	15.4	1.25	0.162	1.21	1.58
32	M	23	-	Yes	107	17.2	12.2	100	13.1	1.04	16.5	14.3	1.95	0.169	1.36	1.62
33	F	21	No	No	98	17.2	12.3	82	11.8	1.00	22.0	17.2	2.10	0.288	1.38	2.10
34	F	37	No	Yes	109	18.4	13.2	82	10.8	1.00	20.5	16.5	1.58	0.224	1.46	1.75
35	F	34	No	No	101	16.5	13.6	93	6.6	1.08	15.9	14.5	1.51	0.157	1.34	1.52
36	F	35	No	No	95	16.6	13.4	78	4.2	1.36	15.1	15.5	2.98	0.224	1.29	1.74
37	F	46	No	Yes	120	16.1	12.8	88	5.4	1.28	18.5	16.6	1.93	0.148	1.27	1.43
38	F	32	No	No	97	15.9	14.2	75	8.3	0.81	15.1	11.6	2.42	0.220	1.63	1.99
39	F	51	No	Yes	121	18.6	12.2	91	3.7	0.85	20.4	17.0	1.80	0.169	1.61	1.74
40	M	47	-	No	103	18.4	15.6	100	10.2	1.06	15.5	14.0	1.76	0.135	1.38	1.44
41	M	64	-	No	117	16.9	13.2	94	14.8	1.32	14.7	14.2	2.13	0.183	1.52	1.74

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: A, Ovranette (Wyeth); B, Microgynon (Schering): Levonorgestrel + Ethinyloestradiol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
42	M	42	-	Yes	106	17.6	14.4	106	6.4	1.20	14.7	13.8	1.84	0.194	1.81	2.03
43	M	59	-	Yes	96	18.0	13.4	97	14.8	1.26	15.5	13.5	1.98	0.146	1.19	1.38
44	M	22	-	Yes	93	16.6	12.6	96	11.1	1.06	15.5	13.8	2.57	0.203	1.74	2.01
45	F	50	No	No	81	15.5	14.0	83	4.4	1.04	19.3	17.0	3.12	0.246	1.74	2.23
46	M	54	-	No	100	19.2	15.2	100	15.1	1.33	14.9	14.6	2.19	0.197	1.56	1.82
47	M	19	-	No	112	19.8	13.2	108	7.0	0.93	18.9	14.5	2.23	0.184	1.53	1.72
48	M	50	-	No	146	15.5	14.2	91	5.3	1.46	18.0	16.7	1.63	0.170	1.36	1.60
49	F	52	No	Yes	89	16.9	12.4	90	10.1	0.85	19.6	14.8	1.93	0.181	1.42	1.68
50	M	64	-	Yes	109	19.5	13.6	107	6.7	1.42	15.5	15.6	1.67	0.128	1.20	1.32
51	F	38	No	No	76	15.0	13.4	77	11.9	1.13	19.1	16.0	1.91	0.213	1.61	1.91
52	M	38	-	No	81	12.3	16.7	84	9.3	1.27	15.1	15.4	3.17	0.169	1.46	1.77
53	F	25	B	No	113	11.8	14.2	72	9.3	1.20	27.6	22.4	1.27	0.135	1.42	1.52
54	F	22	A	Yes	163	13.2	12.5	70	19.4	0.99	21.1	17.8	2.81	0.234	1.72	2.13
55	F	25	No	No	90	14.3	11.4	76	9.8	0.92	13.3	12.1	1.70	0.188	1.09	1.49
56	F	24	A	No	105	16.9	14.1	88	12.5	1.07	18.4	15.1	2.18	0.251	1.72	2.10
57	F	27	A	No	127	11.3	12.3	61	17.2	1.08	22.5	17.0	2.36	0.204	1.32	1.71
58	F	24	C	Yes	112	19.6	12.1	112	9.2	0.93	21.3	16.6	1.74	0.132	1.15	1.32
59	F	22	B	No	108	12.8	12.1	68	11.3	0.94	23.6	18.4	1.58	0.176	1.27	1.55
60	F	23	No	No	133	15.6	13.4	80	13.7	1.41	15.0	15.4	2.36	0.184	1.20	1.53
61	F	24	No	Yes	128	14.9	13.2	80	14.6	1.26	12.9	13.6	1.30	0.136	1.08	1.46
62	M	49	-	Yes	140	19.1	12.9	92	13.3	1.11	18.4	15.8	1.77	0.177	1.39	1.57

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: A, Ovranette(Wyeth); B, Microgynon (Schering); C, Eugynon (Schering): Levonorgestrel + Ethinyloestradol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
63	M	32	-	Yes	129	17.1	14.7	97	9.0	1.06	13.1	12.3	1.85	0.109	1.36	1.32
64	F	33	No	Yes	82	18.4	12.9	86	7.0	1.07	19.2	15.6	1.93	0.246	1.65	1.96
65	F	26	A	No	131	15.5	14.7	88	9.0	1.04	28.6	21.5	1.55	0.232	1.46	1.96
66	F	27	A	No	114	14.7	11.8	77	11.0	1.04	25.9	20.0	2.72	0.219	1.79	2.09
67	F	37	No	No	144	14.8	12.0	82	5.0	1.17	12.5	11.4	2.20	0.288	1.48	2.20
68	M	37	-	Yes	94	17.7	14.3	102	5.7	0.90	13.8	12.3	1.85	0.204	1.51	1.85
69	M	31	-	No	126	16.2	13.6	95	6.4	1.39	13.3	14.3	2.08	0.151	1.36	1.49
70	F	29	No	No	116	15.1	12.3	81	13.3	1.18	17.1	14.7	2.29	0.197	1.38	1.65
71	F	31	No	Yes	91	17.0	9.2	83	12.2	1.04	18.6	14.9	2.75	0.224	1.49	1.81
72	F	46	No	Yes	159	16.9	11.0	89	7.2	1.05	15.3	14.3	2.98	0.178	1.38	1.68
73	M	41	-	No	153	15.1	12.6	95	10.6	1.14	14.8	14.8	1.48	0.136	1.43	1.55
74	F	24	A	No	142	20.4	11.8	92	16.0	1.53	25.2	22.0	2.67	0.190	1.61	1.77
75	F	22	B	Yes	88	16.1	14.4	86	6.3	0.88	23.1	16.2	1.58	0.132	1.29	1.80
76	F	27	A	No	78	19.4	16.4	99	10.0	1.14	15.7	14.0	2.32	0.215	1.37	1.76
77	M	32	-	Yes	146	16.5	14.8	97	18.8	1.17	17.0	15.5	1.96	0.119	1.13	1.33
78	M	26	-	No	95	17.5	13.4	95	14.0	1.18	13.8	14.1	2.72	0.196	1.71	1.93
79	M	31	-	No	134	16.4	11.4	97	6.6	1.36	17.6	17.1	2.41	0.155	1.33	1.53
80	M	29	-	No	88	16.0	13.1	84	10.8	1.14	17.4	15.6	2.86	0.218	1.53	1.91
81	F	24	A	No	90	14.2	12.2	70	10.9	1.27	29.3	23.9	2.58	0.161	1.38	1.56
82	M	52	-	No	92	11.6	12.9	67	7.2	0.91	15.9	13.3	1.89	0.142	1.19	1.41
83	F	39	No	Yes	161	11.9	12.9	64	17.8	1.00	16.1	14.1	2.19	0.230	1.29	1.80

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: A, Ovranette (Wyeth); B, Microgynon (Schering): Levonorgestrel + Ethinyloestradol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
84	F	42	No	Yes	177	17.7	12.6	92	10.6	1.19	12.7	12.8	2.90	0.229	1.56	2.03
85	M	51	-	Yes	127	18.1	14.8	123	14.1	1.28	18.2	16.7	2.14	0.131	1.30	1.44
86	F	21	No	No	124	16.9	11.0	81	11.1	1.41	13.6	13.0	2.05	0.208	1.27	1.55
87	F	44	No	No	115	17.4	12.6	84	13.1	0.92	19.0	15.6	2.33	0.178	1.29	1.56
88	F	48	No	Yes	96	18.0	11.6	96	4.9	1.49	14.0	15.4	1.62	0.172	1.49	1.75
89	F	26	C	No	88	18.9	12.6	86	16.8	0.90	22.7	18.4	2.18	0.165	1.13	1.39
90	F	33	No	No	143	19.2	11.0	91	8.2	1.04	15.1	13.6	1.25	0.163	1.25	1.48
91	M	27	-	Yes	94	18.5	11.4	105	15.2	1.16	18.4	13.0	1.32	0.203	1.46	1.85
92	F	32	A	No	91	16.1	11.4	81	7.0	1.27	29.3	23.3	2.55	0.200	1.48	1.79
93	F	25	B	No	120	18.0	8.9	94	11.9	1.28	21.7	19.3	2.44	0.202	1.53	1.89
94	F	34	No	Yes	117	18.5	10.0	91	12.7	1.24	15.1	14.1	2.10	0.196	1.47	1.74
95	F	33	No	Yes	153	15.5	11.2	85	12.7	1.25	15.0	14.2	2.63	0.148	1.52	1.63
96	M	29	-	Yes	88	16.9	10.9	98	10.6	1.24	19.4	13.6	2.82	0.201	1.71	2.00
97	F	30	A	Yes	97	16.0	9.9	81	11.6	1.29	27.4	21.9	1.38	0.166	1.39	1.56
98	F	54	No	No	91	18.6	12.7	101	16.5	1.47	15.1	15.3	3.24	0.288	1.62	2.34
99	M	22	-	Yes	200	15.1	11.1	83	10.6	0.91	15.6	14.8	1.11	0.153	1.22	1.51
100	M	27	-	No	172	16.4	11.0	89	18.0	1.31	13.3	14.2	2.06	0.215	1.51	1.86
101	M	39	-	No	128	15.9	10.6	90	7.0	1.07	14.8	14.9	1.48	0.172	1.36	1.67
102	M	39	-	Yes	79	16.3	9.1	89	10.0	1.35	14.8	15.3	1.61	0.153	1.36	1.55
103	M	50	-	Yes	103	20.5	13.1	104	10.3	1.10	12.6	12.0	1.89	0.171	1.55	1.71
104	F	24	No	No	109	11.7	7.7	68	10.9	1.10	13.9	13.0	1.29	0.154	1.37	1.58

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: A, Ovranette(Wyeth); B, Microgynon(Schering);C, Eugynon(Schering): Levonorgestrel + Ethinyloestradol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
105	F	49	No	Yes	148	14.3	10.2	71	8.1	1.12	17.8	16.3	1.11	0.202	1.30	1.65
106	F	30	No	No	101	14.5	13.4	82	14.6	1.12	13.5	12.5	1.36	0.171	1.20	1.52
107	M	29	-	No	91	17.8	11.0	101	16.5	1.17	16.0	14.2	3.32	0.184	1.51	1.77
108	M	23	-	No	167	15.4	11.2	98	6.0	1.03	17.3	14.7	2.27	0.172	1.56	1.80
109	M	43	-	Yes	110	18.2	10.0	110	10.1	1.01	12.8	13.4	1.93	0.138	1.49	1.55
110	F	33	No	No	103	16.6	13.1	83	15.1	1.18	14.8	13.2	3.17	0.200	1.47	1.75
111	M	30	-	Yes	124	20.9	10.4	107	11.4	1.03	14.2	12.3	2.27	0.158	1.27	1.47
112	M	22	-	No	115	13.7	8.5	78	11.0	0.84	15.5	12.4	2.56	0.181	1.36	1.72
113	F	57	No	Yes	118	15.2	12.0	78	7.4	1.41	13.9	15.2	2.15	0.174	1.27	1.57
114	F	34	No	No	131	16.0	10.4	82	11.4	1.11	17.8	15.2	2.94	0.210	1.39	1.76
115	M	21	-	No	109	15.7	11.3	85	14.3	0.84	14.6	12.0	2.95	0.183	1.56	1.79
116	M	24	-	No	103	12.9	12.0	75	17.0	1.20	15.5	12.3	1.37	0.156	1.11	1.46
117	M	19	-	No	114	14.8	11.4	97	19.3	0.89	16.7	13.3	2.44	0.168	1.34	1.67
118	F	55	No	Yes	191	16.5	11.2	83	16.1	1.00	14.3	13.4	2.52	0.195	1.37	1.74
119	M	23	-	No	96	16.0	11.6	86	13.0	0.96	17.1	14.5	2.75	0.197	1.57	1.89
120	M	18	-	No	104	17.1	13.7	85	12.2	1.19	15.7	13.5	1.46	0.157	1.44	1.55
121	M	22	-	Yes	158	15.2	17.5	90	9.5	1.29	18.9	17.1	2.53	0.174	1.61	1.80
122	F	59	No	No	107	18.6	11.3	93	12.4	0.99	15.5	13.8	3.17	0.171	1.34	1.57
123	M	49	-	No	164	15.1	12.8	97	13.4	0.98	18.2	15.7	1.74	0.164	1.55	1.71
124	F	45	No	No	87	14.3	16.6	80	6.9	1.33	19.1	17.3	2.42	0.163	1.39	1.57
125	F	51	No	No	128	16.0	15.0	86	14.0	0.97	18.1	15.7	2.75	0.129	1.20	1.32
126	F	32	No	Yes	96	16.3	14.0	81	7.9	1.22	14.8	14.7	1.89	0.175	1.39	1.60

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
127	M	59	-	Yes	117	12.5	13.6	79	10.2	1.32	15.8	14.8	2.94	0.171	1.36	1.60
128	F	22	D	No	89	16.9	10.8	86	5.1	1.19	23.7	19.5	1.74	0.164	1.38	1.56
129	M	38	-	No	138	13.5	16.9	83	9.1	1.02	14.9	13.8	1.46	0.182	1.41	1.75
130	M	22	-	Yes	155	15.1	9.3	90	16.5	1.11	15.6	14.5	2.08	0.163	1.24	1.61
131	F	49	No	Yes	106	16.7	15.0	88	15.3	1.21	17.9	16.8	1.38	0.178	1.23	1.55
132	F	42	No	No	125	16.3	14.2	79	14.1	1.08	15.6	14.7	2.19	0.215	1.29	1.74
133	M	42	-	No	111	20.5	11.8	93	17.2	1.09	12.9	12.3	1.77	0.173	1.49	1.61
134	M	42	-	No	139	14.9	11.5	88	3.3	1.13	16.7	14.6	1.93	0.172	1.49	1.70
135	M	50	-	Yes	135	12.8	9.7	73	8.8	0.89	13.7	12.5	2.90	0.177	1.49	1.71
136	M	59	-	Yes	87	14.3	12.4	89	3.2	0.97	12.5	12.9	2.19	0.160	1.41	1.62
137	M	59	-	No	182	19.4	11.2	107	13.9	0.93	17.5	14.2	1.61	0.151	1.37	1.52
138	M	60	-	No	172	17.5	10.3	102	10.3	1.31	16.5	15.3	1.85	0.184	1.49	1.75
139	F	26	No	No	115	15.1	13.5	80	13.3	1.25	16.3	15.4	2.30	0.174	1.27	1.55
140	F	50	No	No	103	16.3	13.6	85	12.1	1.26	14.8	15.2	2.81	0.279	1.41	2.19
141	F	35	No	Yes	163	13.5	14.5	66	3.5	1.09	10.6	11.8	2.42	0.168	1.63	1.75
142	F	38	No	Yes	118	15.8	12.4	83	11.0	1.21	14.8	13.9	1.58	0.163	1.44	1.57
143	M	61	-	No	117	15.3	9.7	102	18.0	0.93	22.2	12.4	2.75	0.179	1.48	1.82
144	F	22	No	No	136	17.7	10.9	83	19.1	1.10	23.4	19.1	1.80	0.215	1.71	1.96
145	F	22	No	No	107	13.9	13.2	78	8.4	0.98	22.2	18.3	2.30	0.137	1.15	1.32
146	M	37	-	Yes	86	14.3	12.1	76	6.9	1.13	12.6	12.8	1.84	0.147	1.49	1.57
147	F	27	No	No	155	14.8	16.2	73	12.8	1.06	15.5	13.4	1.90	0.181	1.32	1.62
148	M	49	-	Yes	100	15.8	10.0	92	9.2	0.97	18.0	15.2	2.75	0.247	1.49	2.10

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: D, Trinordiol (Wyeth): Levonorgestrel + Ethinyloestradiol

APPENDIX 3 continued

No.	Sex	Age	OCA*	Smoker	Zinc				Copper				Selenium			
					Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++	Leu+	Eryth+	Plasma++	WB++
149	M	55	-	Yes	106	13.7	11.1	77	5.0	1.13	20.5	17.6	1.85	0.147	1.52	1.58
150	M	50	-	No	129	13.9	13.0	75	10.8	1.03	15.9	13.9	1.09	0.137	1.04	1.28
151	F	28	A	Yes	133	19.8	13.9	88	14.4	1.23	17.1	15.2	2.09	0.209	1.38	1.72
152	F	31	A	No	150	16.3	13.6	79	10.6	1.14	18.6	16.8	2.42	0.239	1.72	2.10
153	F	25	A	No	119	17.6	14.4	85	3.2	0.92	19.4	16.2	2.75	0.234	1.81	2.13

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

+ pmol/10^6 cells; ++ µmol l^{-1}

* OCA, oral contraceptive agent: A, Ovranette (Wyeth): Levonorgestrel + Ethinyloestradiol

APPENDIX 4

ELDERLY SUBJECTS : RESULTS

Healthy elderly subjects

	Zinc		Copper	
No.	Leu*	Plasma+	Leu*	Plasma+
1	125	12.0	11.7	21.4
2	120	11.0	11.3	20.4
3	89	11.6	15.9	20.2
4	101	10.9	7.9	17.2
5	100	10.0	12.4	22.3
6	121	12.1	11.5	15.9
7	171	9.8	9.4	20.2
8	100	12.9	9.8	21.8
9	97	11.6	8.7	23.1
10	136	10.7	7.7	14.7
11	125	13.4	7.4	18.1
12	129	8.0	13.8	26.9
13	168	11.1	18.7	22.7
14	100	10.9	9.9	20.2
15	113	10.7	10.5	21.8
16	110	12.0	11.2	12.4
17	126	11.1	7.9	19.7
18	136	12.7	8.6	15.5
19	78	10.7	11.6	17.5
20	87	11.8	10.8	18.1
21	129	10.5	13.2	16.4
22	95	8.7	11.9	18.7
23	136	10.7	11.6	16.8
24	144	10.9	21.8	23.5

Housebound elderly subjects

	Zinc		Copper	
No.	Leu*	Plasma+	Leu*	Plasma+
1	107	10.1	5.9	32.6
2	88	10.8	8.7	24.4
3	101	10.8	7.2	21.2
4	142	11.1	14.4	24.0
5	78	11.1	7.2	22.9
6	72	11.8	8.3	20.6
7	92	12.6	9.4	26.8
8	83	14.4	10.1	17.1
9	131	9.2	13.2	20.8
10	81	8.6	8.8	22.3
11	92	9.3	9.3	19.7
12	78	12.0	9.5	19.6
13	107	13.1	6.7	22.3
14	135	12.4	11.3	8.6
15	124	11.1	8.5	18.4
16	84	13.3	6.1	20.6
17	88	10.2	7.4	24.9
18	80	9.9	5.3	26.2

Abbreviations: Leu, leucocytes * pmol/10^6 cells, + µmol l^{-1}

APPENDIX 5

CROHN'S PATIENTS : RESULTS

	Zinc				Copper				Selenium			
No.	Leu^{+}	Eryth^{+}	Plasma^{++}	WB^{++}	Leu^{+}	Eryth^{+}	Plasma^{++}	WB^{++}	Leu^{+}	Eryth^{+}	Plasma^{++}	WB^{++}
1	97	20.2	10.9	96	3.3	1.13	20.6	19.4	0.85	0.093	0.95	0.98
2	82	17.3	11.2	88	18.6	1.27	18.0	16.4	0.85	0.081	0.86	0.88
3	79	17.9	12.6	85	5.1	1.33	16.0	15.1	1.05	0.143	1.30	1.37
4	97	19.9	8.3	86	8.3	0.89	22.1	16.9	1.57	0.174	1.25	1.46
5	147	15.2	10.3	82	16.4	1.10	22.9	18.3	1.56	0.118	1.17	1.25
6	90	16.0	10.1	71	13.4	0.95	28.1	21.8	1.99	0.137	1.32	1.39
7	88	16.4	9.9	79	8.2	1.41	17.5	16.8	1.75	0.092	0.96	0.99
8	104	15.5	16.0	84	14.6	0.87	16.6	13.5	2.47	0.219	1.67	2.00
9	126	13.9	13.9	72	13.2	1.14	17.2	15.5	1.58	0.177	1.36	1.63
10	89	12.1	10.4	76	11.0	0.99	12.5	12.3	1.32	0.135	1.25	1.44
11	91	16.1	13.7	87	6.4	1.10	18.5	16.1	1.65	0.129	1.37	1.42
12	90	17.8	12.8	85	7.8	1.11	16.0	14.7	1.09	0.141	1.14	1.32
13	112	19.7	9.4	95	7.5	1.32	25.5	20.8	1.36	0.185	1.44	1.68
14	127	19.1	12.6	99	13.9	1.22	14.1	13.8	1.10	0.100	0.89	0.94
15	93	13.5	11.2	74	9.2	0.75	27.8	18.8	1.95	0.190	1.49	1.75
16	148	12.8	10.1	79	8.8	1.06	22.4	17.5	1.62	0.099	1.05	1.11
17	100	17.4	11.9	101	10.7	0.87	21.6	16.4	1.67	0.116	1.27	1.32
18	147	16.8	14.4	80	7.4	1.30	14.0	14.0	2.66	0.251	1.53	1.99
19	151	14.8	8.1	70	6.5	0.88	35.5	25.2	2.06	0.179	1.60	1.75
20	97	12.6	12.0	80	4.2	0.97	17.0	14.5	1.17	0.163	1.24	1.60

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

$^{+}$ pmol/10^{6} cells; $^{++}$ µmol l^{-1}

APPENDIX 6

COELIAC PATIENTS : RESULTS

	Zinc				Copper				Selenium			
No.	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]
1	103	18.6	12.2	92	3.8	0.84	15.4	12.9	1.96	0.150	1.28	1.44
2	181	20.1	9.3	98	14.3	0.86	8.7	9.0	0.87	0.125	1.03	1.17
3	156	16.9	9.3	79	9.9	1.16	9.5	13.9	1.53	0.123	0.92	1.10
4	120	15.2	13.9	83	9.0	0.80	11.7	10.4	0.98	0.084	0.75	0.90
5	103	14.7	9.3	81	4.2	1.20	22.5	18.4	1.66	0.137	1.19	1.34
6	110	15.9	13.0	99	12.0	1.29	21.4	18.1	1.96	0.239	1.44	2.13
7	80	15.7	12.5	92	7.1	1.08	22.7	17.9	0.92	0.092	0.77	0.91
8	93	16.8	14.4	93	11.8	1.22	15.4	15.0	1.60	0.143	1.14	1.37
9	87	15.5	11.0	78	15.2	1.19	18.8	16.5	1.47	0.207	1.46	1.80
10	105	16.5	10.9	99	8.8	0.84	13.9	11.9	0.73	0.099	1.04	1.09
11	99	14.8	12.2	78	6.7	0.85	17.0	14.0	1.30	0.204	1.14	1.63
12	98	20.5	12.2	102	13.0	0.94	13.9	12.5	1.32	0.202	1.24	1.65
13	94	18.0	11.2	93	5.9	1.20	24.7	19.6	2.18	0.220	1.53	1.92
14	93	14.4	10.8	78	6.3	1.28	18.5	16.3	1.15	0.091	0.77	0.87
15	118	13.7	11.5	80	12.9	1.03	16.0	14.2	1.13	0.103	0.85	1.01
16	90	12.8	10.5	68	15.0	1.13	22.5	18.8	1.53	0.119	1.18	1.27

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

[+] pmol/10^6 cells ; [++] µmol l^{-1}

APPENDIX 7

ALCOHOLICS WITH ESTABLISHED LIVER DISEASE : RESULTS

	Zinc		Copper		Selenium	
No.	Leu*	Plasma+	Leu*	Plasma+	Leu*	Plasma+
1	120	10.2	16.2	18.6	1.08	0.79
2	63	11.1	13.7	25.9	0.89	0.65
3	102	10.2	4.3	21.1	1.04	0.86
4	113	8.7	13.1	23.5	1.15	0.92
5	94	8.0	7.1	20.7	0.89	0.68
6	107	7.9	19.3	22.3	1.15	0.92
7	70	8.5	13.2	18.2	0.95	0.66
8	100	11.1	17.3	17.8	0.99	0.76
9	69	13.1	5.6	18.2	1.29	1.18
10	124	10.7	9.1	14.2	1.13	0.76
11	75	9.4	10.6	14.2	1.23	0.82
12	82	10.7	8.7	17.8	1.58	1.06
13	72	10.4	12.0	17.6	1.49	1.09
14	66	11.1	9.7	18.2	1.63	1.19
15	79	15.4	8.3	18.8	1.03	0.71
16	64	12.4	6.8	18.8	1.79	1.60
17	69	10.4	11.0	15.0	1.75	1.05
18	105	12.0	9.9	19.8	1.11	0.87

Abbreviation: Leu, leucocytes * pmol/10^6 cells + µmol l^{-1}

APPENDIX 8

ALCHOLICS IN THE COMMUNITY : RESULTS

	Zinc		Copper		Selenium	
No.	Leu*	Plasma+	Leu*	Plasma+	Leu*	Plasma+
1	97	13.3	6.0	18.1	1.60	1.04
2	129	9.5	18.3	16.8	1.43	0.99
3	78	15.0	12.5	18.9	1.77	1.20
4	94	14.3	7.7	15.5	1.17	1.09
5	80	11.3	11.7	17.0	1.34	1.14
6	79	13.5	9.5	16.6	1.74	1.47
7	76	10.8	10.9	12.6	1.04	0.91
8	85	14.1	5.8	14.6	1.25	1.11
9	103	12.8	8.3	16.2	1.24	1.09
10	119	11.4	13.9	16.0	1.18	0.99
11	134	10.2	12.3	15.3	1.79	1.20
12	91	12.2	15.8	15.1	1.23	0.91
13	118	12.4	6.6	18.4	1.65	1.06

Abbreviation: Leu, leucocytes * $pmol/10^6$ cells + $\mu mol\ l^{-1}$

APPENDIX 9

PATIENTS WITH ECZEMA : RESULTS

	Zinc				Copper				Selenium			
No.	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]
1	118	15.5	15.8	79	12.7	0.78	19.5	15.4	1.56	0.174	1.19	1.51
2	111	18.0	14.1	89	7.4	1.34	17.1	15.9	1.18	0.093	0.72	0.84
3	110	17.7	15.8	79	7.2	1.37	33.8	25.7	0.90	0.109	0.99	1.02
4	143	18.5	14.5	101	7.1	1.11	23.2	18.1	0.99	0.124	1.10	1.22
5	97	14.9	15.7	80	8.8	1.06	19.1	16.1	1.01	0.122	0.99	1.15
6	129	18.7	13.6	103	10.0	0.80	21.2	15.8	1.17	0.114	1.00	1.13
7	81	15.3	13.2	82	14.7	1.19	26.0	20.4	1.33	0.206	1.41	1.79
8	112	12.0	12.6	76	6.1	1.35	23.5	20.5	1.11	0.118	1.13	1.29
9	119	17.0	12.5	73	9.4	1.15	24.5	19.3	1.28	0.194	1.22	1.48
10	156	16.5	14.6	98	8.6	1.22	20.0	17.5	1.00	0.074	0.82	0.95
11	93	13.8	12.2	80	5.9	1.04	22.0	17.6	1.27	0.126	1.38	1.43
12	113	16.6	13.6	82	11.0	0.83	26.6	19.4	1.15	0.092	0.89	0.94
13	160	14.4	14.5	97	17.9	0.96	20.8	16.7	0.99	0.058	0.60	0.65
14	97	15.7	13.8	78	16.3	1.22	30.3	23.4	0.79	0.081	0.71	0.79
15	96	18.4	14.1	106	5.5	1.14	17.6	15.8	1.11	0.124	0.95	1.18
16	128	16.7	11.8	89	7.6	1.18	30.5	22.9	1.56	0.152	1.01	1.27
17	84	17.0	13.4	82	15.8	0.83	24.8	19.2	1.15	0.127	1.22	1.43
18	83	18.0	14.3	93	7.1	1.22	28.5	22.8	1.32	0.190	1.39	1.69
19	138	14.9	13.9	84	6.7	0.96	18.6	15.1	1.30	0.148	1.20	1.42
20	132	15.8	12.9	80	14.5	1.29	14.5	16.7	0.96	0.066	0.56	0.63
21	86	17.5	13.6	97	15.7	1.41	16.5	16.3	1.32	0.217	1.60	1.99
22	98	18.4	14.0	100	6.1	1.24	13.0	13.5	1.23	0.178	1.24	1.58
23	106	13.6	12.5	70	4.7	1.14	15.5	14.5	1.25	0.192	1.48	1.77
24	136	13.9	6.8	80	16.0	1.20	16.0	15.1	1.91	0.191	1.82	2.01

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

[+] $pmol/10^6$ cells; [++] $\mu mol\ l^{-1}$

APPENDIX 10

PATIENTS WITH PSORIASIS : RESULTS

	Zinc				Copper				Selenium			
No.	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]	Leu[+]	Eryth[+]	Plasma[++]	WB[++]
1	86	20.4	15.1	116	9.0	0.95	20.3	15.8	0.68	0.087	0.73	0.85
2	93	15.1	16.4	93	17.1	1.12	20.8	17.8	1.08	0.099	0.94	1.08
3	81	15.4	16.4	95	6.8	0.81	16.5	13.6	0.75	0.093	0.79	0.95
4	99	13.9	15.5	74	9.5	0.96	14.7	13.0	1.46	0.147	1.08	1.32
5	157	15.5	13.9	76	6.4	1.34	15.7	13.2	1.53	0.132	1.25	1.36
6	96	17.6	16.2	89	5.8	1.22	17.0	15.5	0.99	0.144	0.98	1.23
7	144	12.6	14.3	80	5.4	1.14	22.8	18.2	0.77	0.083	0.86	0.92
8	88	17.6	17.2	89	14.2	1.43	20.7	18.8	1.37	0.127	1.22	1.30
9	91	14.9	12.2	82	9.6	0.82	18.6	15.1	1.22	0.104	1.00	1.11
10	88	14.4	14.3	72	15.4	1.18	30.0	22.9	1.51	0.172	1.39	1.58
11	131	17.5	17.2	101	5.4	0.88	21.2	16.3	1.06	0.134	0.99	1.24
12	129	12.0	17.0	77	13.4	1.13	13.0	13.2	1.10	0.093	0.89	0.99
13	84	17.1	11.5	89	6.2	1.27	23.0	19.2	1.28	0.145	1.46	1.53
14	89	17.0	11.5	91	7.7	1.25	18.0	16.3	1.14	0.159	1.23	1.48
15	108	19.1	8.8	91	7.1	1.31	25.0	19.9	0.85	0.122	1.04	1.13
16	85	18.5	13.6	93	6.1	1.08	12.0	12.3	1.48	0.176	1.28	1.55
17	148	15.7	14.2	93	9.2	1.03	18.0	15.0	1.05	0.143	1.20	1.41
18	103	17.9	13.5	106	8.1	1.17	23.0	17.5	1.28	0.128	0.99	1.19
19	123	16.2	13.3	93	4.8	0.93	14.5	13.3	1.11	0.097	0.90	1.03
20	116	15.5	14.4	84	16.0	1.29	22.8	19.8	1.13	0.123	0.76	1.05
21	99	17.6	11.8	97	12.7	1.43	16.6	16.2	1.00	0.134	1.03	1.24
22	107	16.6	11.9	95	5.1	1.31	20.0	18.0	0.90	0.114	0.79	1.04
23	113	14.2	13.2	80	17.0	1.09	18.5	15.8	1.38	0.182	1.30	1.65

Abbreviations: Leu, leucocytes; Eryth, erythrocytes; WB, whole blood

[+] pmol/10^6 cells; [++] µmol l^{-1}

APPENDIX 11

MANUFACTURERS AND SUPPLIERS

BDH Chemicals Ltd.
Parham Drive
Boyatt Wood Industrial Estate
Eastleigh
Hampshire

Coulter Electronics Ltd.
Northwell Drive
Luton
Bedfordshire

Dow Corning
Barry
Glamorgan

Fisons Scientific Apparatus
Bishop Meadow Rd.
Loughborough
Leicestershire

Hawksley and Sons Ltd.
12 Peter Rd.
Lancing
Sussex

Hoechst UK Ltd.
Hoechst House
Salisbury Rd.
Hounslow
Middlesex

MSE Scientific Instruments
Manor Royal
Crawley
West Sussex

Perkin-Elmer Ltd.
Post Office Lane
Beaconsfield
Buckinghamshire

Tecator Ltd.
Thornbury
Bristol

Techne (Cambridge) Ltd.
Duxford
Cambridge

Teklab Ltd.
9 Dorothy Terrace
Sacriston
Co. Durham

Weddel Pharmaceuticals Ltd.
Wrexham
Clwyd

APPENDIX 12

PUBLICATIONS IN SUPPORT OF CANDIDATURE

Hinks L J, Colmsee M, Delves H T (1982)*
Determination of zinc and copper in isolated leucocytes.
Analyst 107: 815-823

Hinks L J, Colmsee M, Delves H T (1983)*
Measurement of zinc and copper in leucocytes.
In: Bratter P, Schramel P, eds. Trace element and analytical chemistry in medicine and biology. Proceedings of the 2nd International Workshop, Neuherberg, Federal Republic of Germany. Berlin, Walter de Gruyter; 885-892

Hinks L J, Clayton B E, Lloyd R S (1983)*
Zinc and copper concentrations in leucocytes and erythrocytes in healthy adults and the effect of oral contraceptives.
Journal of Clinical Pathology 36: 1016-1021

Bunker V W, Hinks L J, Lawson M S, Clayton B E (1984)*
Assessment of zinc and copper status of healthy elderly people using metabolic balance studies and measurement of leucocyte concentrations.
American Journal of Clinical Nutrition 40:1096-1102

Hinks L J, Inwards K D, Lloyd B, Clayton B E (1984)*
Body content of selenium in coeliac disease.
British Medical Journal 288:1862-1863

Stead R J, Hinks L J, Hodson M E, Redington A N, Clayton B E, Batten J C (1985)
Selenium deficiency and possible increased risk of carcinoma in adults with cystic fibrosis.
Lancet 2: 862-863

Tanner A R, Bantock I, Hinks L , Lloyd B, Turner N R, Wright R (1986)
Depressed selenium and vitamin E levels in an alcoholic population.
Digestive Diseases and Sciences 31: 1307-1312

Foote J W, Hinks L J (1987)
Reduced leucocyte zinc and albumin-bound zinc in blood of haemodialysis patients.
Annals of Clinical Biochemistry 24:198-202

*Publications which are based on work presented in this thesis

Foote J W, Hinks L J, Lloyd B (1987)
Reduced plasma and white blood cell selenium levels in haemodialysis patients.
Clinica Chimica Acta 164:323-328

Hinks L J, Young S, Clayton B (1987)*
Trace element status in eczema and psoriasis.
Clinical and Experimental Dermatology 12:93-97

Bunker V W, Hinks L J, Stansfield M F, Lawson M S,Clayton B E*
Metabolic balance studies for zinc and copper in housebound elderly people, and the relationship between zinc retention and leucocyte zinc concentrations.
American Journal of Clinical Nutrition. In Press

Cornell M S, Hinks L J, Singha H S K, Walker V, Willmott F E
Zinc and genital infections.
Genitourinary medicine. In press

Hinks L J, Inwards K D, Lloyd B L, Clayton B*
Reduced concentrations of selenium in mild Crohn's disease.
Journal of Clinical Pathology. In Press

*Publications which are based on work presented in this thesis

The following published papers were included in the bound thesis. These have not been digitised due to copyright restrictions, but the links are provided.

https://doi.org/10.1039/an9820700815

https://doi.org/10.1136/jcp.36.9.1016

https://doi.org/10.1093/ajcn/40.5.1096

https://doi.org/10.1136/bmj.288.6434.1862