GASTROINTESTINAL RESPONSE TO COPPER EXCESS: STUDIES ON COPPER (AND ZINC) LOADED RATS

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by

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Gastrointestinal Response to Copper Excess : Studies on Copper (and Zinc) Loaded Rats. By M. Hair-Bejo.

ABSTRACT

Copper associated diseases have been well documented in both man and animals, but little is known of the gastrointestinal (GIT) response to copper excess. Furthermore, rats can tolerate high dietary copper which may involve the GIT. Metallothionein (MT) a copper and zinc binding protein may play a role in the control of copper absorption. Antagonistic interaction between copper and zinc might be associated with MT.

The aims of this study were to explore the response of the GIT to chronic high dietary copper and to further clarify the role of MT in copper homeostasis at the absorptive site of copper and zinc loaded rats.

Ten-week-old male Wistar rats were fed a copper supplemented diet (1000 mg/kg) for 16 weeks and their body weights and food intakes recorded weekly. They were killed sequentially and the GIT was separated into the stomach, subdivisions of small intestine, caecum and colon. Tissue samples were fixed in 10% formalin or subjected to homogenisation with subsequent chromatographic separation; others were retained for MT and metal analysis. Fixed tissues were stained with HE and PAS for histological evaluation and with rhodanine and rubeanic acid for copper identification. Copper and zinc in whole tissues and the soluble and eluant fractions were analysed using Atomic Absorption Spectrophotometry (AAS). MT was detected in fixed tissues using a DNP-linked peroxidase method (University Wales College of Medicine) and in soluble fractions with an appropriate ELISA (Rowett Research Institute). Ultrastructural changes in the stomach, middle small intestine and caecum were examined using transmission EM and metal was detected using X-ray electron probe microanalysis (ICI, Alderley Edge) on tissues fixed with 2.5% glutaraldehyde with and without secondary fixation. In zinc supplemented (1000 mg/kg) rats with and without copper supplementation (1000 mg/kg) the small intestine was retained for MT and metal analysis. Copper and zinc contents in the liver were also analysed. Parenterally zinc induced MT was used as a reference marker. Copper (^{64} Cu) absorption in both copper and zinc loaded rats was determined using an automatic gamma counter after 4 hours intragastric administration of the isotope (Universities Research Reactor, Risley).

Studies showed that GIT mural copper retention in the copper loaded rats rose initially to a maximum level at week 5, but fell to normal by week 16, whilst zinc remained unchanged. Histological changes, particularly in the gastric mucosa, corresponded to copper content. Cytotoxic damage (single cell necrosis) occurred initially, but adaptation and recovery succeeded by week 16. Hyperplasia of mucous secreting cells and depletion of acid producing cells (parietal cells) were prominent throughout the trial, especially during the first 5 weeks. In the distal small intestine villus atrophy occurred during the first 5 weeks, succeeded by recovery thereafter. Rapid cellular turnover was indicated in the intestine with elevation of mitotic activity and depletion of goblet cell and intraepithelial leukocyte populations. Ultrastructurally, electron dense copper containing mucous granules were prominent in gastric surface mucous cells of the trial groups, whilst electron dense enterocyte lysosomes and copper containing Paneth cell granules were present in the intestine. The apical surfaces of the gastric mucous cells were covered with thickened electron dense membranes by week 16. Degenerative changes were confined to gastric parietal cells and enterocyte microvilli, but recovery of the microvilli had occurred by week 16. The GIT-MT concentration corresponded to copper content. MT was mainly localised in the cytoplasm of the enterocytes of the basal third of the villi and also the Paneth cells. Histochemical staining and chromatographic separation failed to detect copper and MT respectively. Oral zinc supplementation caused a lowering of hepatic copper in the control animals and a retardation in hepatic copper accumulation in copper supplemented groups. Intestinal MT elevation was of a very minor nature and could not be maintained and failed to be isolated chromatographically. ^{64}Cu absorption was reduced in both zinc and copper supplemented groups.

It was concluded that the GIT of rats can adapt to copper excess with repair of damage. The absorption of copper is reduced. Mechanisms responsible remain unclear, but are not directly associated with MT. Excess gastric mucous secretion and depletion of acid producing cells may alter the availability of copper for absorption. Copper binds to mucus, making it probably less toxic and less available for absorption. Adaptational changes within the enterocyte microvilli may prevent the uptake of the metal, whilst rapid cellular turnover may eliminate copper from the organ. Excess copper could also be excreted from Paneth cells. Furthermore, oral zinc inhibits copper absorption and may facilitate its removal. This interaction takes place at the luminal level and is not mediated by MT. It appears that MT may rather act as a temporary metal binding protein in transport across the absorptive cell than a barrier to copper absorption.

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Gastrointestinal Response to Copper Excess : Studies on Copper (and Zinc) Loaded Rats

GENERAL INTRODUCTION

I. BACKGROUND

Copper is an essential trace element in both man and animals, although toxic in excess and disturbances in the normal metabolism of the metal may lead to copper deficiency or toxicity. Copper associated disease is a complex problem and a great deal of research in this field, especially on the toxic damage and adaptive response of the liver and kidney to copper excess has grown dramatically in the last few years. However, knowledge of the disease including the significant variations in species, breed and even individual susceptibility and the mechanisms of toxicity are little understood. Furthermore, despite the vulnerablity of the gastrointestinal tract (GIT) to potential toxic insult very little is known about the response of the organ to copper excess. Metallothionein (MT), a low molecular weight copper and zinc binding protein may play a vital role in copper homeostasis at the absorptive site. The existence of the antagonistic interaction between copper and zinc might be associated with MT.

a. Copper

Copper has been identified in both plants and animals during the nineteenth century (Underwood, 1977), but the essential nature of the metal was not realised until the third decade of the twentieth century when it was demonstrated that copper was required for blood formation in rats (Hart et al., 1928). Shortly after this discovery, copper deficiency was reported in grazing sheep and cattle (Neal et al., 1931) and today naturally occuring copper deficiency has been reported widely (Underwood, 1977); it can even be genetic in origin as in Menkes' disease patients (Menkes et al., 1962) and brindled mice (Hunt, 1974). Furthermore, it has been

well documented that copper is required as part of the molecular structure of many enzymes including, superoxide dismutase, cytochrome oxidase, tyrosinase and lysyl oxidase and is vital to their functions, such as myelination of the spinal cord, keratinization and tissue pigmentation, bone formation and proper cardiac function (Evans, 1973 ; Underwood, 1977 ; Cousins, 1985).

Copper is absorbed from the gastrointestinal tract, bound to albumin in the portal circulation and is mainly accumulated in the liver with excess excreted in the bile (Evans, 1973 ; Cousins, 1985). A small amount of copper also passes directly from the plasma into the urine and intestinal epithelium, whilst a negligble amount is lost in the sweat (Underwood, 1971). Disturbances in copper homeostasis may lead to an excessive accumulation of this metal in body tissues, especially in the liver and can cause serious tissue injury and cell death. Copper toxicosis is a complex problem and it can be primarily due to excess copper intake or secondary to other factors and may be manifested in either the acute or chronic form.

Acute copper toxicity is usually a straight forward case due to a large dose of copper which may cause death within 24 to 72 hours post administration, mainly due to shock or hepatic and renal complications. The problem has been reported in man resulting from accidental or suicidal poisoning (Chuttani et al., 1965), whilst in farm animals such as sheep, it may be associated with the use of copper in the prevention of copper deficiency (swayback) (Ishmael et al., 1969) or treatment for internal parasites (Sholl, 1957).

Chronic copper toxicity is the most common form of copper poisoning and it may result in a significant economic loss in farm animals. The clinical signs of the disease occur after more prolonged periods of time following the continuous accumulation of sub-lethal amounts of copper in the the liver and other body tissues. The disease can be either genetic in origin as in Wilson's disease in man (Scheinberg and Sternlieb, 1976) and in Bedlington terriers (Twedt et al., 1979) or acquired as in sheep (Soli, 1980) and pigs (Higgins, 1981). Acquired copper toxicity has also been reported in other species of animals, such as in cattle (Marschang et al., 1980 ; Blackey et al.,

1982), goats (Soli and Nafstad, 1978), poultry (Henderson and Winterfield, 1975 ; Turska et al., 1978) and fishes (Lauren and Mc Donald, 1986).

b. Zinc

Zinc is essential for the structural and catalytic properties of many enzymes including in bone formation, cell mediated immunity, generalized host defense and a wide variety of factors related to tissue growth (Aggett, 1984 ; Cousins, 1985). Deficiency of the metal can be either genetic in origin as in acrodermatitis enteropathica in man and in Friesian cattle (Moynahan, 1984) or acquired due to low dietary zinc content or secondary to other factors (Underwood, 1977 ; Aggett, 1984 ; Cousins, 1985).

Zinc is absorbed from the intestinal tract, bound to albumin and accumulated initially in the liver before it is distributed further to the muscle and bone. Endogenous zinc is secreted from the intestinal tract, bile and pancreas (Underwood, 1977 ; Cousins, 1985). Apparently zinc is less toxic than copper (Underwood, 1977 ; Bises et al., 1989), although the incidence of acute and chronic zinc toxicity have been occasionally reported (Callender and Gentzkow, 1937 ; Mc Cord et al, 1926 ; Allen, 1968 ; Allen et al., 1983).

The existence of complex interactions between zinc and copper are well documented (Ritchie et al., 1963 ; Evans et al., 1970 ; Mills, 1974 ; Prasad et al., 1978 ; Fischer et al., 1984) and indeed, zinc has been used in the treatment of or to confer protection against copper toxicity in Wilson's disease patients (Brewer et al., 1983 ; Hoogenraad and Van den Hamer, 1983 ; Hill et al., 1987 ; Lipsky and Gollan, 1987), Bedlington terriers (Brewer, personal comunication) and in sheep (Bremner et al, 1976), although its usage is still far from universal and mode of action unknown.

II. COPPER ASSOCIATED DISEASES

a. Copper associated disease in animals

i. Copper toxicity in sheep

Chronic copper poisoning in sheep was first reported experimentally in 1925 (Mallory, 1925) and a few years later similar signs were described in grazing animals (Beijer, 1932). Since then, the occurrence of the disease has been reported world wide (Soli, 1980) and may arise as a consequence of different conditions. Chronic copper poisoning has been reported in association with the use of copper sulphate as molluscicides and herbicides (Rana and Kumar, 1983) and in sheep grazing on the grass land containing copper enriched pig manure slurry (Kneale and Howell, 1974). Several other elements such as molybdenum, sulphur (Dick, 1954 ; Ross, 1966, 1970 : Suttle, 1977), zinc (Bremner et al., 1976) and cadmium (Mills and Dalgarno, 1972) can influence the occurence of the disease. The disease has also also been reported in association with the liver damage due to the ingestion of plants containing pyrrolizidine alkaloids (Bull, 1949, 1964). Furthermore, it appears that some breeds of sheep are more susceptible to the disease than the others. Merino or pure breed sheep appears to be more resistant to copper than sheep of mixed breed (Marston and Lee, 1948 ; Wiener and Field, 1970), whilst the Ronaldsay breed, by contrast is extremely copper sensitive (Maclachlan and Johnston, 1982).

Chronic copper toxicity is considered to have three different phases: pre-haemolytic, haemolytic and post-haemolytic phases (Ishmael et al., 1971 ; Gooneratne et al., 1980). During the prehaemolytic phase copper is continously accumulated in the liver over a period of weeks or months with absence of clinical signs. However, the liver specific enzymes such as arginase, glutamic dehydrogenase, serum transaminase, lactic dehydrogenase and iditol dehydrogenase (formerly known as sorbitol dehydrogenase) are increased during this time (Ross, 1964, 1966 ; Ishmael et al., 1972). Histological examination of the early liver changes revealed necrosis of isolated parenchymal cells and occasional fatty changes distributed

mainly within the centrilobular zones. Copper containing granules were also observed within these regions using rubeanic acid stain (Ishmael et al., 1971).

More severe centrilobular hepatic necrosis, bile stasis and inflammatory cell infiltration have been demonstrated as the hepatic copper concentrations reached higher levels, up to 2000 to 4000 µg/g dry weight (Ishmael et al., 1971). Furthermore, the blood copper concentration increased up to ten fold followed by onset of a haemolytic crisis (Ishmael et al., 1972). The mortality is very high during this period and most animals die within 2 to 4 days after the onset of clinical signs (Soli, 1980). The gross lesions include, dull yellow carcass with yellow brown discolouration of the mucous membranes and body fat. The liver is yellow brown and the kidneys are enlarged and dark in appearance. Mild to marked abomasal mucosal and intestinal submucosal haemorrhages are also observed in some animals. Histologically, in addition to the hepatic lesions, many of the cortical and medullary tubules of the kidney are dilated and filled with numerous granular and hyaline casts (Ishmael et al., 1971 ; Gopinath et al., 1974). The epithelium of the proximal convoluted tubules contain large amount of brown granular pigment and in some places the cells are necrotic. Copper staining is demonstrated in both the tubular epithelium and casts. Kidney copper concentration is raised up to 300 to 500 µg/g dry weight (Ishmael et al., 1971).

Some animals may survive the haemolytic crisis. The histological changes in this animals include, periportal hepatic fibrosis and large amounts of bile pigment present in canaliculi and small bile ducts (Ishmael et al, 1971).

II. Copper associated disease in dogs

A genetic abnormality causing hepatic copper storage and progressive liver disease is recognised increasingly in some breeds of dogs. The disease was first reported in Bedlington terriers in USA (Hardy et al., 1975) and since then, a similar disease has been observed in the same breed in Australia (Robertson et al., 1983), Finland (Eriksson, 1983) and the United Kingdom (Kelly et al., 1984). A

familial copper storage disease has also been reported in West Highland white terriers (Thornburg et al., 1986a).

Copper associated liver disease which is more likely to be related to cholestasis or secondary to chronic liver disease of diverse aetiology rather than a primary defect of copper metabolism has been reported in Doberman pinschers (Johnson et al., 1982 ; Crawford et al., 1985), Pekingese poodles (Thornburg et al., 1986b), Labrador retrievers and Cocker spaniels (Thornburg et al., 1984) and recently, in Skye terriers (Haywood et al., 1988).

Bedlington terriers

Chronic copper storage disease in Bedlington terriers derives from an inherited disorder of copper metabolism leading to chronic hepatitis and ultimately cirrhosis (Twedt et al., 1979 ; Johnson et al., 1980). Early recognition of affected animals is important since the disease is inherited as an autosomal recessive trait (Johnson et al., 1980) and thus, the dam and sire of the affected dogs can be considered to be the carriers of the defective gene (Owen and Mc Call, 1983). It has been reported that about two thirds of the breed in USA are affected with the disease (Hardy and Stevens, 1978) and about one third in the United Kingdom (Herrtage et al., 1987 ; Haywood (personal communication)).

The clinical manifestation of the disease is generally associated with the progressive chronic accumulation of copper in the liver and is reflected in the age of the animals, although some considerable variations in the time of onset and manifestation of the disease may be observed (Hardy and Stevens, 1978). During the initial stage of hepatic copper accumulation, in the early life of the affected dogs, over a period of months or years, the disease is asymptomatic. The liver may appear histologically normal, although increased copper staining can be demonstrated using both rubeanic acid and rhodanine stains (Johnson et al., 1984 ; Thornburg et al., 1985).

Hepatic copper accumulation is progressively increased to maximum concentrations (7000 μg/g dry weight) between 2 to 4 years of age and is maintained at about the same levels thereafter, although there is some fall latterly (4000 μg/g dry weight) after above 10 years of age (Twedt et al., 1979). The hepatic damage can be identified by an elevation in serum alanine amino transferase (Robertson et al., 1983 ; Kelly et al., 1984). The dogs are usually presented with anorexia, vomiting, weakness, lethergy and dehydration (Twedt et al., 1979 ; Robertson et al., 1983 ; Kelly et al., 1984). Haemolytic episodes are rarely observed (Owen and Mc Call, 1983) and the serum ceruloplasmin remains unchanged or is slightly elevated (Su et al., 1982a). Increases in renal copper concentration have also been reported (Su et al., 1982a)

Animals may die after the onset of clinical signs and on post mortem examination the liver is usually uniformly shrunken and tan with a finely nodular capsular surface. Histologically, liver damage progresses from focal centrilobular necrosis to diffuse chronic hepatitis and cirrhosis. Copper staining granules are mainly distributed within the centrilobular regions (Twedt et al., 1979 ; Eriksson, 1983 ; Kelly et al., 1984 ; Rutgers and Haywood, 1988). In older dogs, despite some reduction in hepatic copper concentration, the hepatic damage is progressive and chronic active hepatitis and cirrhosis become prominant (Twedt et al., 1979).

West Highland white terriers

Chronic progressive hepatitis and cirrhosis in West Highland white terriers is associated with excess hepatic copper accumulation which may result from an inherited defect in copper metabolism similar to that observed in Bedlington terriers (Thornburg et al., 1986a). However, the hepatic copper accumulation in West Highland white terriers seems to be unrelated to the age of the animals and the hepatic copper concentration (maximum recorded: 3500 μg/g dry weight) is much lower than those in Bedlington terriers (Thornburg et al., 1986a).

Skye terriers

Copper associated hepatitis in Skye terriers appears to have some similarities to those of familial copper storage disease in the West Highland white terriers. High hepatic copper accumulation in Skye terriers does not correspond to the age of the animals and the hepatic copper does not decline with lesion maturity rather the converse (Haywood et al., 1988). Furthermore, the hepatic copper concentration is much lower (maximum recorded: 2300 µg/g dry weight) in this breed than in copper storage hepatitis in Bedlington terriers (Haywood et al., 1988). The distribution of the lesions and copper containing granules are pre-eminantly observed in the periacinar zones. In contrast to copper toxicosis in both Bedlington terriers and West Highland white terriers, intracanalicular cholestasis is one of the earliest and most characteristic hepatic lesions in Skye terrier hepatitis (Haywood et al., 1988). It appears that Skye terrier hepatitis is a distinct entity and may be derived from a disorder of intracellular bile metabolism culminating in disturbed bile secretion and accumulation of copper (Haywood et al., 1988).

iii. Copper toxicity in pigs

Copper has been commonly added to the ration (125 to 250 µg/g) of growing pigs since it was first discovered that the element can enhance growth rate and food conversion efficiency in the species (Barber et al., 1955). However, overt copper toxicosis was reported in association with unevenly mixed feed (Buntain, 1961 ; Higgins, 1981) or inadequate amounts of zinc and protein in the diets (Hatch et al., 1979). Affected animals are usually presented with anorexia, poor weight gain or weight loss, weakness, pale, yellow discolouration of the sclera and buccal mucous membranes, melena and jaundice (Buntain, 1961 ; Allen and Harding, 1962 ; Hatch et al., 1979 ; Higgins, 1981). Furthermore, microcytic hypochromic anaemia and increased erythrocyte glutathione concentration have also been reported (Hatch et al., 1979). The liver and kidney copper concentrations may increase up to 6000 and 5000 µg/g dry weight respectively (Allen and Harding, 1962).

Pigs may die after the onset of the clinical ilness and the gross lesions include jaundiced carcass, ulceration of the gastric mucosa and blood stained intestinal contents. The liver is friable, yellow tan to deep orange brown in appearance and the kidneys are enlarged (Buntain, 1961 ; Allen and Harding, 1962 ; Higgins, 1981). Histologically, hepatic centrilobular necrosis, hepatic fibrosis and bile duct proliferation are recorded. The renal damage ranging from tubular cell vacuolization to tubular necrosis (Buntain, 1961 ; Allen and Harding, 1962 ; Hatch et al., 1979). Copper staining granules are observed at the centrilobular region of the liver and at the epithelial cytoplasm of the renal convoluted tubules using rubeanic acid stain (Allen and Harding, 1962).

b. Copper associated disease in man

i. Wilson's disease

Wilson's disease (Wilson, 1912) is an inherited autosomal recessive copper storage disease characterised by cirrhosis of the liver, degeneration of the basal ganglia of the brain and the development of pigmented rings at the periphery of the cornea resulting from excess copper retention in these organs (Sternlieb, 1975 ; 1980 ; Shearman and Finlayson, 1989). The progress of the disease is age related as with in Bedlington terriers, however ceruloplasmin levels are generally reduced in Wilson's disease patients and central nervous system and corneal copper deposition are present in the human patient (Bern, 1972 ; Twedt et al., 1979 ; Shearman and Finlayson, 1989). Recognition of the disease at the early stage of hepatic copper accumulation can provide a better prognosis than after the onset of clinical manifestations.

Hepatic copper concentration in Wilson's disease patients is continously elevated up to 30 to 50 times normal (normal value: about 50 µg/g dry weight) upto about 4 years of age without the appearance of any clinical manifestations (Sternlieb, 1980 ; Shearman and Finlayson, 1989). However, mild hepatic damage can be demonstrated with the elevation of serum transaminase activities (Levi et al.,

1967 ; Sternlieb, 1975). Histologically, steatosis with fine or large droplets of triglycerides in hepatocytes is observed (Scheinberg and Sternlieb, 1959) and copper staining is diffuse in the cytoplasm of the hepatocytes (Sternlieb, 1975). Serum ceruloplasmin may decrease and urinary copper concentration rise at this time.

The clinical manifestations of the disease vary from patient to patient and usually occur between the age of 5 and 25 years. The hepatic copper concentration may now achieve 100 times normal values (Shearman and Finlayson, 1989), although the concentration is slightly reduced in the later stages (Sternlieb and Scheinberg, 1968). Copper concentrations in the blood, brain, cornea, kidney and urine are also increased (Bern, 1972 ; Sternlieb, 1980). Signs of hepatic insufficiency such as anorexia, weakness, upper abdominal pains, vomition, and jaundice are demonstrated at this period. Haemolysis, although uncommon, may be observed (Roche-Sicot et al., 1973 ; Iser et al., 1974). Serum transaminase and serum alkaline phosphatase are elevated (Sternlieb, 1975); conversely ceruloplasmin is reduced, although in some patients may remain unchanged (Scheinberg and Sternlieb, 1963 ; Frydman et al., 1985).

Histologically, severe hepatotoxic changes such as discrete or extensive necrosis of the hepatocytes with some hepatocytes containing hyaline inclusions, collapse of the parenchyma, infiltration of inflammatory cells, deposition of collagen, proliferation of bile ductules, and nodular regeneration and ultimately cirrhosis are observed (Sternlieb, 1975 ; Davies et al., 1989). Copper staining using both orcein and rhodanine are predominantly distributed in the periportal region, although positive staining may also be observed diffusely throughout some of the cirrhotic nodules (Davies et al., 1989). The intensity of the positive staining reaction is usually greater with orcein than with rhodanine stains (Jain et al., 1978 ; Goldfischer et al., 1980), however, the correlation between the histochemical demonstration of liver copper and hepatic copper determined by atomic absorption spectrophotometry is less reliable (Goldfischer et al., 1980). This lead to the

development of immunocytochemical techniques for the demonstration of MT as a better indicator of copper accumulation (Clarkson et al., 1985 ; Elmes et al., 1989). The pathological changes of the kidney include tubular necrosis and degenerative changes of the glomerular tuft (Wolff, 1964).

ii. Primary biliary cirrhosis

This is an uncommon disease usually affecting middle aged women associated with progressive damage of the bile ductules leading to severe hepatic damage and ultimately cirrhosis and liver failure (Smallwood et al., 1968 ; Sternlieb, 1980 ; Goldfisher et al., 1980). Hepatic copper accumulation may increase up to 700 μg/g dry weight (Goldfisher et al., 1980). Copper content in the eye can be also elevated (Frommer et al., 1977), but the concentration of the metal in the brain remains normal (Fleming et al., 1974). Marked elevation of serum alkaline phosphatase is identified with cholestasis (Sherlock, 1987), whilst raised serum alanine amino transferase reflects the hepatic damage (Mac Sween, 1979). Histologically, the changes include a chronic non suppurative destructive cholangitis which is followed by proliferation and destruction of ductules (Goldfischer et al., 1980). Copper granules are detected using both orcein and rhodanine stains primarily within the periportal zone of the liver lobules (Ludwig et al., 1979 ; Goldfischer et al., 1980), although it appears that rhodanine is more reliable than orcein stain (Ludwig et al., 1979).

iii. Indian childhood cirrhosis

This is a severe, progressive and fatal disease affecting children aged 1-3 years, originally confined to children of Indian sub-continent (Sternlieb, 1980 ; Tanner and Portmann, 1981), but now recognised in European children (Muller-Hocker et al., 1987, 1988). Hepatic copper concentrations can be elevated up to 4800 μg/g dry weight (Sternlieb, 1980). Histologically, the liver changes include massive necrosis, with some hepatocytes containing hyaline inclusions, and

ultimately cirrhosis similar to those observed in Wilson's disease (Nayak and Roy, 1976 ; Sternlieb, 1980 ; Shearman and Finlayson, 1989). Copper staining granules are distributed throughout the hepatic parenchyma with periportal predominance (Goldfischer et al., 1980). However, the concentration of copper in the brain and eye remained at the normal levels (Sternlieb, 1980) and ceruloplasmin moreover is elevated (Muller-Hocker et al., 1987). Cholestasis may occur in some patients (Sternlieb, 1980). The aetiology of the disease still remains unresolved, although, this appears to be an acquired copper disorder and is no longer thought to have a genetic basis (Sternlieb, 1980).

iv. Menkes' kinky hair syndrome

Menkes' disease, first described in 1962, has been shown to be associated with a familial defect in copper metabolism resulting in copper deficiency (the converse of Wilson's disease) and it can cause death in infant and early childhood (Menkes et al., 1962). The symptoms of the disease include neurological disorders such as severe mental retardation and seizures, pili torti or kinky hair, hypothermia and skeletal abnormalities. It appears that these changes reflect decreases in the activity of essential copper metalloenzymes such as cytochrome oxidase, dopamine beta hydroxylase, tyrosinase, amine oxidase and lysyl oxidase (Danks et al., 1972a ; Holtzman, 1976).

Hepatic copper, ceruloplasmin and plasma copper are depleted. However, intestinal, renal and urinary copper concentrations are elevated (Danks et al., 1972b ; Danks et al., 1973 ; Horn et al., 1975 ; Nooijen et al., 1981). Intestinal copper content is raised up to 70 µg/g dry weight (Danks et al., 1973) and absorptive studies using ^{64}Cu demonstrated a reduction in ^{64}Cu absorption after an oral administration of the isotopes, but normal plasma clearance and subsequent reappearence of labeled copper in ceruloplasmin has been demonstrated after parenteral introduction of the isotopes (Danks et al., 1972b ; Van den Hamer and Prins, 1978). Thus, it appears that copper deficiency in Menkes' disease is due to a

defect in transport of copper from the absorptive cell to the portal circulation (Danks et al., 1973). Studies using cultured fibroblasts from Menkes' disease patients has shown that excess copper retention within the cell is associated with an increase of copper bound to MT (Riordan and Jolicoeur-Paquet, 1982 ; Leone et al., 1985).

c. Copper toxicity and tolerance in rats

Laboratory rats have been extensively used as animal models in the study of the nature and role of copper in body systems (Underwood, 1971, 1977). Furthermore, the ability of rats to adapt and become tolerant to copper excess (Haywood, 1980, 1985) is an interesting phenomenon and can be useful in understanding complex mechanisms of copper homeostasis, possible treatment and prevention of copper toxicity in both man and animals.

The onset of the toxic effect and subsequent hepatotoxic changes in copper supplemented rats are dose related (Haywood, 1980, 1985). Furthermore, it appears that male rats accumulate copper more readily in their livers than the females and show more evidence of its toxic effect (Haywood, 1979). Copper is not randomly distributed within the rat liver and the median lobe of the organ contains the most copper (Haywood, 1981) and likewise the most severe hepatic changes (Haywood, 1980). Rats succumbed to severe toxicosis following dietary copper supplementation above 5000 $\mu g/g$ (Haywood, 1985). The animals lost weight or failed to gain weight at all, despite normal food intakes. Some of them died in the second week, meanwhile the survivors developed diarrhoea which may suggest additional gastrointestinal abnormalities.

The pattern of events in the liver in copper loaded rats can be divided into 3 phases: cumulative, critical and recovery phases (Haywood, 1980, 1985). In the cumulative phase, copper accumulates in the liver with little initial effect. Parenchymal cells become depleted of glycogen and become larger and more intensely stained. Single cell necrosis and foci of inflammatory cells were randomly

distributed throughout the lobules. Copper staining granules (rubeanic acid and rhodanine) were identified in the cytoplasm of hepatocytes within the periportal and mid zones and orcein stain (copper associated protein) showed a striking periportal distribution (Haywood, 1985). The plasma alanine aminotransferase activity was moderately elevated (Haywood and Comerford , 1980).

With further increases in hepatic copper (4800 µg/g dry weight) signs of hepatocellular disruption became more prominent. Grossly, affected lobes contained peripheral areas of necrotic tissue variable in size, pale, swollen and sharply demarcated from adjacent tissues (Haywood, 1980, 1985). The plasma alanine aminotransferase activity and ceruloplasmin were markedly elevated. However, the erythrocyte copper values and alkaline phosphatase activity remained unchanged and haemolysis did not occur (Haywood and Comerford, 1980).

Histologically, despite marked hepatic damage there were some variations in the severity and age of the lesions. Cellular necrosis was observed in the periportal region and frequently extended more deeply into the lobules. Hepatocyte and nuclear enlargement were often conspicuous. Inflammatory reaction consisting of both polymorphonuclear and mononuclear leukocytes were diffusely infiltrated throughout affected regions. However, histochemical staining using rubeanic acid, rhodanine and orcein stains now showed a less intense reaction despite high hepatic copper concentration in the organ (Haywood, 1980, 1985).

In the recovery phase, the hepatic copper concentration fell, but was still higher than normal. The inflammatory response subsided and regeneration advanced and was apparently complete by 15 weeks. The reconstituted lobules consisted of cells which displayed some nuclear heterogeneity, but were otherwise normal and had regained their normal floccular appearance. Bile duct hyperplasia, conspicuous hyalinized cells and distorted lobules were occasionally observed. Copper staining was limited to some pericanalicular granules in the periportal region (Haywood, 1980, 1985).

The sequence of histological events in the kidney followed a similar pattern to that occurring in the liver, although the elevated renal copper concentrations were maintained in the recovery phase. Initially copper retention in the organ has little cytotoxic effect. Eosinophilic droplets became increasingly detected in the cytoplasm of the proximal convoluted tubules especially at the apex of the cell or in the lumen and stained positively for copper (Haywood, 1985 ; Haywood et al., 1985a). As the renal copper concentration increased (up to 4200 µg/g dry weight) during the critical phase, extensive degeneration and necrosis of tubular cells occurred. Copper staining granules and droplets were concentrated within the proximal tubules and often appeared as aggregates occluding the tubular lumen. Mitotic activity and cellular pleomorphism of the tubular epithelium were more commonly observed at this time.

Regeneration of the proximal tubules was rapid and necrosis limited to individual cells during the recovery phase. Copper staining within the eosinophilic droplets were as numerous as previously observed and the cells continued to become detached and released into the tubule lumen (Haywood, 1980 ; Haywood et al., 1985a).

III. MECHANISMS OF RESPONSE TO COPPER

Significant variation in species susceptibility to copper intoxication, in which sheep as a species are more prone to the disease with some breeds more susceptible than others, is poorly understood. Neither is the tolerance as displayed in the copper loaded rat. However, such variations in copper sensitivity may be due to differences in copper homeostasis at the site of absorption, storage and excretion.

A defect in biliary copper excretion in both Wilson's disease (Frommer, 1974) and primary biliary cirrhosis patients (Smallwood et al., 1968) results in excess copper deposition in the liver. A similar mechanism has been suggested in Bedlington terrier toxicosis (Su et al., 1982b ; Owen and McCall, 1983) and sheep (Underwood, 1977), however, the hepatic centrilobular distribution of copper in

these species may suggest that the disease is principally due to a metabolic defect in copper homeostasis within the region rather than the biliary abnormalities (Fuentealba et al., 1989a). In contrast, a direct relationship between liver and biliary copper concentration occurs in pigs (Skalicky et al., 1978) and this species can adapt well to dietary copper supplementation. As with pigs, the hepatic copper content in copper tolerant rats has been shown to parallel biliary copper excretion (Evering, 1989). Furthermore, the removal of excess hepatic copper may be facilitated by redistribution of copper from the liver to the kidney and excretion in the urine and this mechanism appears to involve copper binding protein, identified as MT (Haywood et al., 1985a ; Evering, 1989).

Copper is bound intracellularly to proteins of both high and low molecular weight. The molecular association of copper has been suggested could determine the toxic effect of the metal (Mehra and Bremner, 1984). MT is an important low molecular weight (6000-7000 daltons) metal (copper) binding protein that has been isolated from both soluble (cytosolic) and particulate (non cytosolic) fractions of liver in copper supplemented pigs (Mehra and Bremner, 1984) and copper tolerant rats (Evering, 1989), but is not detected in the particulate fraction of copper loaded sheep (Mehra and Bremner, 1984). Furthermore, the synthesis of the protein in sheep in response to copper excess is limited (Saylor et al., 1980).

The detection of MT in the blood, urine and bile is consistent with the potential role of MT in copper transport (Bremner, 1987a ; Bremner et al., 1987 ; Evering, 1989). The binding of copper to MT in the liver may occur as a relatively short lived copper pool and may facilitate the excretion of the metal into the bile (Bremner, 1987b ; Evering, 1989). The direct correlation between MT and copper contents in the blood of copper tolerant rats (Evering, 1989) can possibly inhibit the occurrence of haemolysis.

Subcellular localisation of copper could also determine the toxic effect of the metal. Ultrastractural studies and X-ray microanalysis have demonstrated that excess hepatic copper is sequestrated by lysosomes in Wilson's disease patients

(Goldfischer and Moskal, 1966), Bedlington terriers (Twedt et al., 1979), sheep (Gooneratne et al., 1980) and rats (Fuentealba et al., 1989). It has been suggested that this may initiate lipid peroxidation damage to lysosomal membranes causing leakage of acid hydrolases into the cytoplasm with hepatic cell damage (Lindquist, 1968 ; Gooneratne et al., 1980). Furthermore, haemolysis in copper poisoned sheep may also be derived from lipid peroxidation induced red blood lysis (Soli, 1980). Conversely, copper deposited within lysosomes has been argued to constitute the defence mechanism against hepatotoxicity (Goldfischer and Sternlieb, 1968).

Subcellular fractionation of liver homogenates from copper tolerant rats found a distinct localisation of copper in the hepatic nuclear fraction in addition to lysosomal fractions. Furthermore, hepatic necrosis corresponded to excess copper accumulation in the nucleus rather than its lysosomal accumulation (Haywood et al., 1985b). X-ray microanalysis at the ultrastructural level confirmed that excess copper deposition occurred in the nucleus associated with chromatin condensation and karyolysis of these organelles, whilst the membrane integrity of the lysosomes remained intact. This appeared to support the hypothesis that the cytotoxic effect of copper is associated primarily with irreversible nuclear damage and not with disruption of lysosomes (Fuentealba and Haywood, 1988 ; Fuentealba et al., 1989b). The mechanism by which this occurs is little understood, although copper may destabilise DNA (Bryan and Frieden, 1967) and inhibit RNA polymerase activity of the nuclei (Novello and Stirpe, 1969).

Isolation of copper bound to MT from chicks (Starcher, 1969) and bovine duodenum (Evan et al., 1970) suggest that this protein may play a vital role in copper homeostasis at the absorptive site. The binding of much copper in the intestinal tract has been speculated can facilitate the absorption of the metals (Starcher, 1969 ; Evans, 1979). Conversely, copper bound to intestinal MT may inhibit the transfer of the metal from the absorptive cell to the portal circulation (Evans and Johnson, 1978). Elevation of copper retention within the intestinal mucosa of brindled mice (Evans and Reis, 1978), Menkes' disease patients (Leone et

al., 1985), and in rats treated with estrogen (Cohen et al., 1979) resulted in a decrease in copper absorption and appeared to be associated with excess copper bound to MT. The failure to detect intestinal MT from copper loaded sheep may be reflected in the susceptibility of the species to copper poisoining (Saylor et al., 1980). However, it has also been reported that the mucosal content of radioactive copper bound to the protein was unassociated with the reduction of copper absorption (Bremner et al, 1979).

IV. GASTROINTESTINAL RESPONSE TO COPPER

a. Gastrointestinal maturity and copper retention

Cellular morphology of the neonatal gastrointestinal tract when compared to the adults can be considered as two entirely different organs. The gastric glands (Kammeraad, 1942 ; Hill, 1956), small intestine (Williams and Beck, 1969 ; Clarke, 1977) and large intestine (Helander, 1973) of rats are progressively developed during the first three weeks of life and a typical adult appearance of the organs occurs at about two months of age (Clarke, 1977) similar to the liver (Mc Kellar, 1949).

Copper retention within the intestinal tract of the neonatal rats was elevated initially, reaching a maximum level of 300 µg/g dry weight, but regressed to about the adult level (10 µg/g dry weight) by the third week (Schwartz and Kirchgessner, 1975 ; Johnson and Evans, 1980). These changes are paralleled by the gradual process of cellular maturation within this period and the ability of neonatal animals to regulate the uptake of copper by pinocytosis which is diminished as the neonatal cells are replaced by the adult cell type (Mistilis and Maerrick, 1969 ; Williams and Beck, 1969 ; Dinsdale et al., 1986). Furthermore, copper was found to be associated primarily with a low molecular weight protein (MT) initially, but this association diminished by the third week (Hurley et al., 1980 ; Johnson and Evans, 1980 ; Mason et al., 1981).

b. Gastrointestinal response to copper toxicosis

The gastrointestinal response to copper toxicosis has been little investigated, although it has been reported that the pathological changes mainly occurred at the proximal part of the organ , within the stomach and proximal small intestine. In acute copper toxicosis in man, superfical to deep erosions and ulceration of the gastric mucosa have been reported (Chuttani et al., 1965) and intense inflammation with marked necrosis of the mucosa and submucosa of the abomasum in lambs have also been observed (Sholl, 1957). Gastric ulceration in pigs (Allen and Hardling, 1962 ; Hatch et al., 1979 ; Higgins, 1981) and haemorrhage of the abomasal mucosa in goats (Adam et al., 1977) have been reported in chronic poisoning.

c. Copper absorption

i. Sites and mechanisms of copper absorption

The absorption of copper from the gastrointestinal tract seems to be at sites determined by species. Copper appears to be absorbed from the stomach and upper small intestine in rats (Van Campen and Mitcheall, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984), whilst in sheep, it appears that considerable net absorption of copper takes place from the large intestine (Grace, 1975). Studies with stable and radioactive copper indicate that ingested copper is poorly absorbed (Underwood, 1977). Furthermore, regulatory control of copper absorption is indicated by reduced response to high ^{64}Cu dosage (Crampton et al., 1965 ; Mistilis and Farrer, 1968 ; Marceau et al., 1970).

The mechanisms which regulate the absorption of copper are little understood. It has been suggested that copper may be transported through the intestinal mucosa in the form of both ionic copper and in the form of complexes (Mills, 1956 ; Kirchgessner and Grassman, 1970). Two copper binding ligands have been identified in the gastrointestinal tract: a high molecular weight and low molecular weight (10000 daltons) ligand (Evans and Johnson, 1978). The identity and function of

high molecular weight ligand remain unknown, whilst the low molecular weight ligand was identified as MT (Evans and Johnson, 1978).

Studies using isolated small intestine suggest that the absorption of the metal seems to involve two separate mechanisms: the uptake of copper from the lumen into the absorptive cell across the brush border and the transfer of the metal from the cell to the portal circulation (Crampton et al, 1965). A special energy dependent mechanism of copper transport seems to be involved in the second step of copper absorption (Crampton et al., 1965). Furthermore, copper might be transported as a copper amino acids complex, as it was reported that amino acids can facilitate the transport of copper across cellular membranes (Harris and Sass-Kortsak, 1967).

II. Factors affecting copper absorption

Some evidence suggests that copper absorption is regulated in accordance with bodily copper needs. It has been shown in rats and mice that the absorption of copper was higher in copper depleted than in copper adequate animals (Underwood, 1971). Pregnant rats were also noted to absorb about twice more of a single dose of ^{64}Cu than the non pregnant animals (Davies and Williams, 1976). Furthermore, young lambs prior to weaning appear to utilize four to seven times the amount of copper than the mature sheep (Suttle, 1975).

Relatively little is known about the chemical forms and combinations in which copper exists in food and the changes that might occur in the gastrointestinal tract. It has been reported that the absorption of copper of cupric sulphide occurs much less efficiently than that of copper sulphate in pigs (Bowland et al., 1961). Studies on the absorption of copper compounds in cattle showed that $CuCO_3$ was absorbed most rapidly, followed by the absorption of $Cu(NO_3)_2$, $CuSO_4$ and $CuCl_2$ (Underwood, 1977). The absorption of fresh herbage, in which copper is mainly in a natural or ionic complex form in copper deficient rats, is more rapid and greater liver copper storage occurs than with an equivalent amount of copper as copper sulphate (Mills,

1956). Transitional elements such as zinc, cadmium, molybdenum could inhibit copper absorption (Evans, 1973 ; Cousins, 1985).

iii. Antagonistic interaction between copper and zinc

Like copper the absorption of zinc appears to involve two separate mechanisms: the uptake of zinc from the lumen into the enterocyte and the transfer of the metal from the enterocyte to the portal circulation (Evans et al., 1975 ; Smith et al., 1978 ; Jackson et al., 1981). These mechanisms also seem to be regulated by one or more metal binding ligands, whose chemical nature and functions are still poorly understood (Evans and Johnson, 1978 ; Cousins, 1985). However, zinc is primarily absorbed from the small intestine (Van Campen and Mitchell, 1965).

The antagonistic interaction between copper and zinc seems to take place during the process of absorption, at the small intestine (Van Campen and Scaife, 1967 ; Oestreicher and Cousins, 1985). This could possibly occur within the lumen or brush border and in the absorptive cell since copper and zinc do not appear to share the same binding sites on the albumin molecule (Cousins, 1985). Reduction of copper absorption in zinc supplemented rats has been reported to be associated with excess copper bound to MT within the intestinal mucosa suggesting that MT might play a vital role in this antagonistic interaction within the absorptive cell (Richards and Cousins, 1977 ; Ogiso et al., 1979 ; Hall et al., 1979 ; Fischer et al., 1981, 1983).

Zinc is a better inducer of MT than copper (Oestreicher and Cousins, 1985), however, MT has a higher affinity to bound copper than zinc (Bremner, 1980). Zinc induced MT has been speculated could sequester copper within the absorptive cell and make it unavailable for absorption (Richards and Cousins, 1977 ; Ogiso et al., 1979 ; Hall et al., 1979 ; Fischer et al., 1981, 1983). Conversely, it has been suggested that zinc could inhibit the absorption of copper by binding to and displacing copper from MT (Starcher, 1969 ; Evans et al., 1970). Alternatively, absorptive studies using copper isotopes appear to suggest that the antagonistic effect between copper

and zinc is primarily associated with the interaction of the metals at the luminal level (Van Campen and Scaife, 1967 ; Oestreicher and Cousins, 1985) and may not be asociated with MT.

V. METALLOTHIONEIN

Metallothionein is a low molecular weight (6000-7000 daltons), cysteine rich, metal binding protein (4-5 atoms/mole), first isolated from the equine kidney (Margoshes and Vallee, 1957) and today it has been detected in many other tissues, with the highest concentration in the liver, kidney and intestine (Kagi and Nordberg, 1979 ; Hamer, 1986). This protein has 61 amino acid peptides containing 20 cysteines, 6-8 lysines, 7-10 serines and no aromatic or histidine residues (Hamer, 1986 ; Richards, 1989). However, chicken thionein consists of 63 amino acids including one histidine residue and amino acid not present in mamalian MT (Richards, 1989). MT is poorly absorbed at 280nm and usually isolated at the same elution volume as globular proteins of about 10000 daltons using chromatographic separation (Bremner and Davies, 1975 ; Richards and Cousins, 1977).

The protein can be induced in response to both dietary and parenteral administration of essential trace elements such as copper and zinc as well as the potentially toxic elements such as cadmium (Hamer, 1986 ; Bremner, 1987a ; Richards, 1989). MT has a binding capacity of 11-12 g atoms/mole of copper and 7 g atoms/mole of zinc and cadmium (Bremner, 1987b). The binding of these metals to MT occur exclusively through the cysteinyl residues by formation of metal thiolate lingkage. Copper is tightly bound to the protein when compared to those of cadmium and zinc (Dunn et al., 1987). The induction of MT synthesis is blocked by actinomycin D (Richards and Cousins, 1975) and is accompanied by increases in translatable MT mRNA suggesting that heavy metals act at the level of mRNA synthesis, processing or degradation (Hamer, 1986). MT induction can also be initiated by other agents such as glucocorticoids interferon, interlukin I and activator protein kinase (Hager and Palmiter, 1981 ; Cousins, 1985).

Two principal isoforms of MT have been identified in mamalian tissue designated as MT I and MT II based on their eluant position during ion exchange chromatography (Bremner and Davies, 1975 : Jackson et al, 1986 ; Lehman-McKeeman et al., 1988). MT I is more easily released from ion exchange chromatography, and is less stable and degraded faster than MT II (Lehman-McKeen et al., 1988). The degradation of copper induced MT is much faster than zinc or cadmium MT. The mechanisms involved in the degradation of the protein is little understood, although it seems to take place in the lysosome (Bremner, 1987b).

The function of MT is still a matter of considerable conjecture, although it appears that this protein may play an important role in the detoxification of certain heavy metals and in the metabolism of copper and zinc (Webb and Cain, 1982 ; Hamer, 1986 ; Bremner, 1987a ; Richards, 1989).

VI. TREATMENTS FOR COPPER TOXICITY

The main aim of the treatment for copper toxicity is to achieve a negative copper balance and this can be done by increasing the excretion of endogenous copper and/or inhibiting the absorption of copper from the gastrointestinal tract. Based on these concepts various types of drugs have been used in the treatment for the disease.

a. Penicillamine

Penicillamine or dimethyl cysteine was first introduced in 1956 (Walshe, 1956) for the treatment of Wilson's disease patients. This overwhelming drug of choice for the disease is a copper chelating agent and is readily absorbed from the intestine and rapidly excreted in the urine. Initial dose of 500 mg, orally three times daily is indicated and once the disease in remission the dose may be reduced (Walshe, 1975). However, penicillamine is relatively toxic (Brewer et al., 1983). Acute sensitivity reactions including skin eruptions, fever, eosinophilia, leukopenia and thrombocytopenia have been reported. Furthermore, chronic administration of the drug can also produce many side effects, including nephrotic syndrome, retinal

haemorrhage, episodes of acute polyarthritis and excessive wrinkling of the skin (Brewer et al., 1983).

Penicillamine has also been used in copper storage disease in Bedlington terriers. Daily administration of 250 mg of the drug is recommended to achieve a negative copper balance and reduce hepatic copper concentration (Ludwing et al., 1980), although the hepatic copper concentration in some dogs may remain unchanged (Robertson et al., 1983). Furthermore, the therapy probably is not effective in dogs with acute hepatic failure (Hardy, 1983). The side effects of the drug such as anorexia, nausea and vomiting have also been reported (Twedt et al., 1979 ; Hardy, 1983).

Penicillamine has also been successfully used in copper toxicity in sheep, but it is probably too expensive for routine use under field conditions (Soli et al., 1978).

b. Ammonium tetrathiomolybdate

The oral administration of 50 to 500 mg of ammonium molybdate and 300 to 1000 mg sodium sulphate can prevent or reduce the incidence of copper toxicity in sheep (Hatch, 1977). It appears that molybdate reacts with sulphide in the rumen to form thiomolybdate and this subsequently combines with dietary and tissue copper to form complexes in which copper is unavailable (Mills, 1980 ; Gooneratne et al., 1981). Intravenous injections of ammonium tetrathiomolybdate, an organic compound containing molybdenum and sulphur, at 100 mg of the compound, twice weekly can prevent the accurrence of haemolytic crisis in sheep and minimize tissue damage (Gooneratne et al., 1981). Recently, subcutaneous injection of ammonium tetrathiomolybdate, at 3.4 mg/kg body weight each, for 3 doses, given on alternate days has been reported to be effective for the treatment of chronic copper poisoning in sheep (Humphries et al., 1988).

c. Zinc

Oral zinc therapy at 50 mg of zinc, 3 times daily is effective in controlling copper balance in Wilson's disease patients (Brewer et al., 1983 ; Hill et al., 1987), although some patients may not respond well to the therapy (Walshe, 1984). It has been speculated that copper is bound to zinc induced metallothionein in the absorptive cell and is unavailable for further absorption (Richards and Cousins 1977 ; Hall et al., 1979 ; Mills, 1980). The oral administration of 150 mg of zinc, daily in copper storage disease in Bedlington terriers has also been observed can supress ^{64}Cu absorption (Brewer, personal communication). Furthermore, zinc supplementation at 420 ppm has been reported to prevent the incidence of copper toxicosis in sheep (Bremner et al., 1976).

d. Tetramine compound

Triethylene tetramine dihydrochloride (Trientine) is a copper chelating agent which is capable of facilitating urinary copper excretion has been effectively used at the dose of 400 to 800 mg, three times daily, orally in the treatment of Wilson's disease patients, although it may induce iron deficiency and the long term side effect of the drug is still unknown (Walshe, 1982, 1986). In Bedlington terrier hepatotoxicosis, oral therapy of 300 to 600 mg tetramine compound, daily has been successfully used in reducing hepatic copper concentration in the dogs (Twedt et al., 1988).

e. Other drugs

This includes parenteral administration of 200 to 300 mg of BAL (2,3-dimercaptopropanal) twice daily to induce negative copper balance in Wilson's disease patients, although in some patients severe toxic reactions to BAL such as skin rashes, fever and even coma have been reported (Bearn, 1956). However, it appears that BAL is not effective in the treatment of copper toxicosis in sheep (Soli, 1980). Other chelating agents such as carbacrylamide and potassium sulphide (Strickland et

al, 1971) and disodium calcium ethylenediamine (CaEDTA) (Soli, 1980) have proved equally unsuccessful.

AIMS AND OBJECTIVES

The aims of the present study are to explore the response of the gastrointestinal tract to copper excess and to further clarify the possible role of metallothionein (MT) in copper homeostasis at the absorptive site of copper and zinc loaded rats.

The specific objectives will be:

i. The identification of copper in the gastrointestinal tract and its effect at both the light and ultrastructural levels employing EM transmission and X-ray probe microanalysis.

ii. A clarification of the relationship between copper concentration and MT status within the gastrointestinal tract employing immunoreactive techniques for the detection of MT.

iii. A clarification of the antagonistic interractions between copper and zinc and its correlation to MT status within the intestinal tract employing immunoreactive techniques for the detection of MT.

iv. The effect on copper absorption in both copper tolerant and zinc supplemented rats using a radioactive ^{64}Cu isotope.

CHAPTER : 1

Gastrointestinal Response to Copper Excess: Metal Accumulation and Pathological Changes

1.1. INTRODUCTION

Copper is an essential trace element, although toxic in excess and any disturbance in bioavailability may predispose to either copper deficiency or toxicity. Copper is absorbed from the gastrointestinal tract and normally stored in the liver without damage, however unrestricted accumulation may cause hepatic injury. Copper toxicosis occurs as a familial copper storage disorder with liver and kidney injury in Wilson's disease in man (Scheinberg and Sternlieb, 1976) and in Bedlington terriers (Twedt et al., 1979). Alternatively, copper poisoning may be acquired as in sheep (Soli, 1980) and less commonly in cattle (Marschang et al., 1980) and pigs (Higgins, 1981). In contrast, the Dominican toad and Mute swan (Sternlieb, 1980) are apparently resistant to excess liver copper and rats have been shown to adapt to copper overload and became tolerant (Haywood, 1980, 1985).

Reasons for differences in copper susceptibility and the development of tolerance are poorly understood. Haywood (1980) suggested that copper tolerance could be associated with the ability of the liver and kidney to unload excess copper and indeed, Evering (1989) has shown greatly increased biliary and urinary copper excretion in copper supplemented rats. However, this adaptation may also involve other homeostatic mechanisms, especially those concerned with the uptake and absorption of the metal from the gastrointestinal tract.

The regulatory mechanisms concerned with the absorption of copper are still incompletely understood. In newborn animals facilitated uptake of copper by pinocytosis occurs (Mistilis and Mearrick, 1969), whereas in adults absorption is effected by two separate mechanisms which may involve both passive diffusion

and energy dependent transport (Scheinberg and Morell, 1957 ; Crampton et al., 1965). Studies with both stable and radioactive copper have shown that this metal is poorly absorbed (Underwood, 1977) and further regulatory control is indicated by the reduced response to high ^{64}Cu dosage (Mistilis and Farrer, 1968 ; Marceau et al., 1970).

Furthermore, despite the vulnerablity of the gastrointestinal tract to potential toxic insult very little is known about the effect of high copper concentrations on this organ. Single very large doses of copper are known to be associated with a haemorragic gastritis in man (Chuttani et al.,1965) and in lambs (Sholl, 1957) and in the longer term ulcerative gastritis has been described in pigs (Allen and Harding, 1962 ; Hatch et al.,1979 ; Higgins, 1981). Such an omission compared with studies of liver and kidney is additionally surprising in view of the important role of the gastrointestinal tract in copper metabolism.

It is the aim of this study to assess the effect of chronic exposure to high dietary copper on the gastrointestinal tract in relation to copper retention by this organ and associated changes.

1.2. MATERIALS AND METHODS

1.2.1. Animals

Ten-week-old male Wistar rats of uniform weight (323 ± 4 g (mean ± SE)) were randomly selected into thirteen groups of four animals and each group was allocated into plastic cages with woodchips bedding. A pelleted diet with copper content of 1000mg/kg (Labsur Animal Diet, Lavender Mill, Manea Cambridgeshire) was fed to ten groups of animals, whilst the remaining three groups which acted as controls were fed with the unsupplemented diet containing 14 mg copper/kg (Appendix, 1.0). The zinc content for both diets was constant (61 mg/kg). Food and tap water were freely available. The animals were regularly observed and weighed at weekly intervals (Appendix, 1.1). The food was also weighed and dietary intake per group was recorded weekly (Appendix, 1.2). Groups of copper supplemented and control rats were killed at intervals of 1, 2, 3, 4, 5, 6, 8, 10, 12, and 16 weeks, and 1, 8, 15 weeks respectively. Prior to killing the animals were isolated and deprived overnight of food and their feces retained. Euthanasia was performed by carbon dioxide inhalation and subsequent cervical dislocation. A midline incision was made into the abdominal cavity, the gastrointestinal tract was immediately removed and placed on ice and was separated into the stomach, caecum, colon and small intestine (Fig, 1.0). The latter was further divided into proximal, middle and distal portions. Samples were taken from each specimen for histology, metal analysis and chromatographic separation (Chapter, 2). All sampling procedures were in the same sequence, starting from the proximal, middle and distal parts of small intestine, colon, stomach and the caecum.

1.2.2. Histology

1.2.2.I. Sampling procedure

a. Stomach

Fatty tissue was removed from the stomach serosa and a midline incision was made longitudinally at the greater curvature of the organ. Approximately 2.0 cm

length and 0.4 cm width of glandular stomach including a small non glandular portion was isolated. This tissue was slowly flushed with freshly prepared 10% formalin and when free from stomach content was placed on a piece of filter paper (Whatman Ltd., Maidstone, England). The filter paper was folded over to cover the mucosa and serosal surface of the stomach and was loosely ligated externally to maintain the tissue in place. The sample was then immersed in 10% formalin for further fixation.

b. Small intestine

Approximately 5 cm length was isolated from the middle of each intestinal subdivision (Fig, 1.0). The selected sites can be identified by their distance from the pylorus and this distance can be expressed as percent of the length of the small intestine that was respectively 15-20% (proximal), 50% (middle) and 80-85% (distal) of this length (Altmann and Leblond, 1970). The isolated portion was ligated at the proximal end, into the lumen of which 10% formalin was slowly injected and the intestinal contents gently flushed away. When the outflow was clear a second ligature was placed at the distal end. Formalin (10%) was then further injected until the intestinal lumen was filled and a diameter achieved of about 0.4 cm. The sample was then immersed in 10% formalin for further fixation.

c. Caecum

The sampling procedure was similar to that described for the stomach. The fatty tissue was removed and a midline longitudinal incision was made at the convex surface of the caecum. A portion of about 2.5 cm length and 0.6 cm width was removed and immediately flushed with 10% formalin, cleared from caecal contents and placed on filter paper and further retained in 10% formalin.

d. Colon

About 3 cm from the middle section of the colon was isolated and treated similarly to that described for the small intestine except that the lumen was

distended with 10% formalin to a diameter of about 0.8 cm.

1.2.2.ii. Tissue processing

All tissue samples were further fixed by immersion for at least 48 hours in freshly prepared 10% formalin. The stomach and caecum samples were bisected and blocks prepared. Both longitudinal and transversely sectioned blocks were prepared from fixed small intestine and colon. The blocks were subsequently dehydrated in alcohol, cleared with xylene and embedded in paraffin wax using an automatic tissue processor (Elliot, Liverpool, UK). They were vertically sectioned at right angles at about 5 μm on a Wetzler Microtome (Leizt, West Germany) and mounted on glass slides.

1.2.2.iii. Staining methods

a. Haematoxylin and Eosin (HE)

Sections were stained with Mayer's haemalum for 5 minutes, washed in running water until they were blue (about 5 minutes) and counterstained in 0.25% eosin (to which 1 drop of acetic acid had been added) for approximately 5 minutes. They were then rinsed in running water to differentiate the eosin, dehydrated in graded series of alcohols and two changes of xylene, before being mounted in Canada balsam or DPX (Lillie, 1965a).

b. Rubeanic acid

A staining method for the demonstration of copper as described by Lillie (1965b) was utilised. The paraffin sections were deparaffinised and dehydrated before they were placed in a 100 ml of 70% ethyl alcohol solution containing 0.1 g rubeanic acid (dithioxemide). Sodium acetate (0.2 g per 100ml solution) was added 20 minutes later and the sections were then incubated for 72 hours at room temperature. They were rinsed in two changes of 70% ethyl alcohol for a total of 3 hours. These were followed by a final rinse with 100% ethyl alcohol for 24 hours,

counterstained with neutral red or metanil yellow for 30 seconds, dehydrated, cleared and permanently mounted in Canada balsam or DPX.

c. Rhodanine

The modified method of Okamoto and Utamara was employed (Lindquist, 1969). The paraffin sections were deparaffinised and dehydrated before being incubated in rhodanine working solution at 37 ^{o}C for 18 hours. They were then rinsed in several changes of distilled water, stained with diluted hematoxylin for 10 minutes and quickly rinsed in borax solution. A final rinse with distilled water was followed and they were then dehydrated, cleared, and mounted.

d. Alcian blue

The method described by Pearse (1960) was used. Sections were stained in freshly filtered 0.1% solution of Alcian blue in 3% acetic acid for 20 minutes. They were then rinsed in distilled water, stained in Ehrlich's haemalum (5 to 10 minutes), differentiated in 1% alcohol and washed in running water for 10 to 20 minutes. Sections were then dehydrated in alcohol, cleared in xylene and mounted in Canada balsam or DPX.

e. Periodic Acid Shiff (PAS)

A method described by Mc Manus and Mowry (1960) was employed. Sections were immersed in 0.5% aqueous periodic acid for 10 minutes, rinsed in distilled water and placed in Schiff's reagent for 15 minutes. They were then directly transfered into three changes of sulphurous acid (1 minute each), rinsed in running water (5 to 10 minutes) and counterstained with Mayer's haemalum for 2 minutes. Sections were washed in running water (5 to 10 minutes), dehydrated, cleared, and mounted.

1.2.2.iv. Histological evaluation

Sections from all subdivisions of the GIT were stained with HE and at least one from each group was further stained with PAS. Stomach tissues were also stained with Alcian blue. Tissues were carefully examined using X10 and X40 objectives and the histological changes were classified and subjectively graded accordingly: nil (0), mild (+), mild to moderate (++), moderate (+++), and marked (++++).

a. Stomach

The descriptive terminology established by Stevens and Leblond (1953) was used in the identification of cells of the typical gastric mucosa. Generally, surface mucous cells were distributed at the pit and isthmus, parietal cells were abundant at the isthmus and neck, mucous neck cells were confined to the neck region and peptic cells occupied the base of the gland (Fig, 1.1). Regional differences concerning the distribution of cell types were observed within the gastric mucosa near the junction of glandular and non glandular stomach. This area was almost exclusively occupied by mucous secreting cells (surface mucous and mucous neck cells). Parietal cells were mainly observed at the base of the glands, whereas peptic cells were rarely found.

Histological changes of the gastric mucosa were classified as: single cell necrosis (apoptosis), surface mucous and mucous neck cell "hyperplasia" and parietal cell "atrophy". The presence of mitotic figures and leukocytes were also recorded. Apoptosis was characterized by the appearence of shrunken eosinophilic cells with pyknotic or fragmented nuclei. Hyperplasia was gauged from an increase in depth of gastric pits, increased numbers of PAS positive surface mucous cells and mucous neck cells. Parietal cell atrophy and depletion or deletion are terms used synonymously to denote a decrease in numbers of parietal cells (Barker and Van Dreumel, 1985). Mitotic figures were identified under a X40 objective and graded as to field number: 0 = nil, + = 0 to 1, ++ = 1 to 3, +++ = 3 to 5 and ++++ = more than 5.

b. Small intestine

Both transverse and longitudinal sections of the subdivided parts of small intestine were initially examined. Villus morphology of the transverse section was irregular and varied from a finger like projection to a flat dome shape (Fig, 1.2a). As a result histological evaluation was only carried out on the longitudinal section (Fig, 1.2b).

The villus of the small intestine was lined by simple columnar epithelium (enterocytes or absorptive cells), and goblet cells were scattered between the enterocytes. Intraepithelial lymphocytes or intraepithelial leukocytes (IEL) (mainly lymphocytes but occasionally eosinophils and macrophages) were also found in this region. Goblet cell numbers were estimated from the mucous granule content of well orientated villi on both HE and PAS stained sections. The appearance of the IEL was also recorded from the same villi. The crypt lining was composed of mainly undifferentiated cells, goblet cells (mucous cells), and Paneth cells. The presence of leukocytes were also recorded and mitotic figures were identified under a X40 objective and graded as the number of mitoses per crypt: 0 = nil, + = 1 to 2, ++ = 2 to 3, +++ = 3 to 5 and ++++ = more than 5.

c. Caecum and colon

The epithelial cells of the caecum and colon mainly consisted of the enterocytes, goblet cells and undifferentiated cells. Apoptosis, mucous cell populations (goblet cells and undifferentiated cells), leukocytes and mitotic figures were estimated using similar criteria to those of the small intestine. In the crypts of the caecum, at the luminal surface basophilic stained material was observed and identified as bacterial colonies. Their frequency of appearance was recorded.

1.2.2.v. Histochemistry

Sections were stained with rubeanic acid and rhodanine and were examined under X10 and X40 objectives for the presence of copper staining material as

described by Irons et al. (1977).

1.2.2.vi. Villus and crypt measurements

Villus and crypt measurements were made on well orientated longitudinal sections of the three subdivisions of small intestine. Only those villi and crypts with the plane section perpendicular to the surface with one layer of cellular epithelium cut along their length were measured (Fig, 1.3). Measurements were made on 10 villi and 10 crypts of each animal in the group with an eyepiece micrometer at X10 and X40 fields respectively (Clarke, 1970). Villus height and crypt depth are expressed in µm as mean ± standard error of mean of 4 rats in a group (or as otherwise stated).

1.2.3. Copper and zinc analysis

All glass ware was cleaned and soaked in 0.1 N Nitric acid (Analar grade, BDH Chemical) for at least 48 hours. This was followed by three washes (24 hours each) in de-ionised water, rinsed and dried in an oven at 70 ^{o}C.

1.2.3.i. Sampling procedure

The intestinal lumen of the remaining portions was gently flushed with cold physiological saline and the content was collected on filter paper (Whatman Ltd., Maidstone). The intestine was opened and further flushed with physiological saline before being subdivided into eight equal sections. Alternate samples were removed and pooled for chromatographic studies (Chapter, 2) while the remainder were also pooled and used for copper and zinc analysis. This procedure was repeated throughout the small intestinal divisions and colon. A similar procedure was also used in the caecum and stomach, except flushing was only done on an exposed lumen and samples were taken from one half of the organ only. Fecal samples were collected from the total overnight fecal output, soaked in physiological saline pooled and uniformly mixed. Triplicate pooled samples from 4 rats in the group were taken for acid

digestion. Replicate samples were taken from the middle part of small intestinal tissues and caecal contents only and used for estimation of recovery.

1.2.3.ii. Acid digestion

All samples were oven dried in plastic containers at 70 ^{o}C until they reached a constant weight. The dry weights of the samples were recorded and they were then digested in a pyrex glass tube (150 mm x 18 mm) with 70% Aristar grade nitric acid (BDH Chemical Ltd.) and 60% Spectrosol grade perchloric acid (BDH Chemical Ltd.) in 2 to 1 (v/v) respectively. Fifty µl (1mg/ml) of Spectrosol grade cupric nitrate and zinc nitrate solutions (BDH Chemical Ltd.) were added into each recovery tube. All tubes were covered with glass marbles and left overnight. They were then heated in a heating block (Tecam, Di-Block, DB-4) at 100 ^{o}C for 30 minutes, 200 ^{o}C for 60 minutes and 250-300 ^{o}C until all the samples were completely digested (about 120 minutes) and changed from dark brown to colourless.

1.2.3. iii. Atomic Absorption Spectrophotometery (AAS)

Digested samples were diluted in distilled water to 10 ml in volumetric flasks and further diluted if required. Copper and zinc contents were analysed in an IL Atomic Absorption Spectrophotometer (Instrumentation Labroratory Inc., USA) at wavelengths of 324.7 nm and 213.9 nm respectively. The spectrophotometer was standardized with a solution containing 2.00 and 4.00 µg/ml of copper and 0.50 and 1.00 µg/ml of zinc prepared from Spectrosol grade cupric nitrate and zinc nitrate (1mg/ml) (BDH Chemical Ltd.) in 0.1M HCl/litre (Analar, BDH Chemical Ltd.). Cation-Cal Calibration References (American Dale) was used as the reference value and the performance of the spectrophotometer was frequently monitored and restandardised if required. Copper and zinc concentrations of both the gastrointestinal tissues and their contents and feces (Appendix, 1.3, 1.4) are expressed as the average data from 3 sample readings (Appendix, 1.5, 1.6, 1.7, 1.8,

1.9) and the mean ± standard error of mean of selected groups in µg/g dry weight.

1.2.4. Statistical analysis.

Statistical analysis was performed using Student's t-test in cricket software (version 1.1) for Macintosh (Rafferty et al., 1985).

1.3. RESULTS

1.3.1. Gross patholological changes

There were no remarkable changes in the gastrointestinal tract of either control or copper supplemented rats throughout the trial, apart from the darker contents of the latter groups.

1.3.2. Histological changes

1.3.2.i. Stomach

Histological changes within the stomach of control and copper supplemented rats are as shown in table 1.0.

a. Control

There were no remarkable variations in the gastric morphology between the control groups throughout the trial. Mitotic figures were occasionally observed at the isthmus and rarely at the neck of the glands. A low incidence of apoptosis was observed in all animals. Enlarged parietal cells, with poorly stained cytoplasm were occasionally observed at the dorsal region of the isthmus. A mild diffuse scattering of cells which included both polymorphonuclear and mononuclear leukocytes occurred within the lamina propria at the base of the glands. However, in two rats (week 1 and week 8 each) moderate accumulations of predominantly polymorphonuclear neutrophil leukocytes were found concentrated particularly at the junction of the glandular and non glandular region. Increased mitotic activity was also found in one of these rats (week 1).

b. Copper supplementation

Weeks 1 to 3

During the first 2 weeks of copper supplementation the numbers of apoptotic cells were increased within the gastric pits and occasionally observed at the isthmus and neck of the glands. Gastric pits extended more deeply into the mucosa and the surface mucous cells which lined the pits were more prominent and numerous. At the

neck of the glands also there were increased numbers of mucous cells. Hyperplasia of mucous secreting cells was at the expense of the parietal cell population of the region which was correspondingly depleted. However, parietal cells were present as normal at the base of the glands and no changes could be identified in the peptic cells. Leukocyte numbers were also slightly increased. Mitotic activity had increased. Histological changes became more pronounced by week 3, at which time, apoptosis (Fig, 1.4 a) was maximal with corresponding hyperplasia of mucous cells and parietal cell atrophy (Figs, 1.5 a, b). Enhanced mitotic activity and leukocyte numbers were sustained.

Weeks 4 to 6

Apoptosis was still prominent. Mitotic activity increased up to week 5 (Fig, 1.4b) and generally the mucous hyperplasia was maintained with corresponding parietal cell atrophy. Leukocyte numbers declined at week 6.

Weeks 8 to 16

The incidence of apoptosis declined to week 16 at which time it approximated the control values. Some mucous glandular hyperplasia persisted in nearly all animals with considerable individual fluctuations and corresponding variations in parietal cell depletion (Fig, 1.6b). The leukocyte populations fell to within the normal range although a few small lymphoid aggregates were frequently observed. Mitotic activity was maintained at variable though generally slightly elevated levels above the normal.

1.3.2.ii. Small Intestine

Histological changes of the proximal, middle and distal small intestine of control and copper supplemented groups are shown in tables 1.1, 1.2 and 1.3 respectively.

a. Control

Generally the histological features of the subdivided parts of the small intestine remained unchanged throughout the trial. Apoptosis was rarely observed and

occasionally Paneth cells were found to be poorly stained with HE. The population of goblet and Paneth cells were greater in the distal part of the organ than the upper portions.

b. Copper supplementation

Proximal

Mild depletion of villus goblet cells was observed overall during the first 12 weeks, but the normal populations were regained at week 16. However, crypt goblet cells remained unchanged. Mitotic activity increased early and continued throughout the experiment (Fig, 1.8a), although no increase in apoptosis was recorded. Numbers of intraepithelial leukocytes were decreased from week 2 and thereafter.

Middle

Mild to moderate reduction of villus goblet cells and intraepithelial leukocytes were generally observed from week 2 and thereafter. Mitotic activity mildly increased during the first 6 weeks, after which it fell to normal. The crypt epithelial cell populations remained unchanged.

Distal

A modest depletion in villus goblet cells was observed from week 2 to week 5 (Fig, 1.7b). However in the latter weeks, overall populations were generally regained. Mitotic activity was somewhat reduced during the first 3 weeks, but was regained in the latter weeks. Variations in leukocyte numbers were also observed in some groups of animals. The crypt epithelial cell populations remained unchanged.

1.3.2.iii. Caecum

Histological changes of control and copper supplemented rats are as shown in table 1.4. The experimental groups displayed no variations from the control groups throughout the trial with the exception that in the former group bacterial colonies

were more often recorded within the lumen of the crypts. However, this was not associated with an increased leukocyte activity.

1.3.2.iv. Colon

Histological changes of a consistent nature were not observed either within the control or experiment groups (Table, 1.5). Apoptosis was recorded in week 12 only, whereas some elevation of leukocyte activity was observed during the first 10 weeks of copper supplementation.

1.3.3. Histochemical staining

Copper was not identified with either rubeanic acid or rhodanine in the stomach, small intestine, caecum and colon of either the control or copper supplemented groups (Figs, 1.8b, c)

1.3.4. Villus and crypt measurements

Villus height and crypt depth of the proximal, middle and distal small intestine are as shown in tables 1.6 and 1.7 respectively (Figs, 1.9, 1.10, 1.11). Villus height in the proximal and distal parts of the organ were reduced ($p<0.05$) during the first week and first 5 weeks of copper supplementation respectively, whilst the crypt depth remained unchanged, although some reductions ($p<0.05$) were inconsistently observed in the middle small intestine of the trial groups. A reduction ($p<0.05$) in villus height was also observed at week 15 in the distal small intestine of the control group compared with week 1. Overall, villus height along the tract decreased ($p<0.05$) distally, whilst the crypt depth remained unchanged ($p>0.05$).

1.3.5. Copper and zinc concentrations

a. Stomach

Copper concentration was markedly elevated ($p<0.05$) to nearly three times normal value (26.4 ± 1.2 µg/g) during the first 5 weeks of copper supplementation,

falling subsequently from week 6 - 12 (15.4 ± 0.5 μg/g) and was further reduced to about the normal level at week 16 (10.0 μg/g). Overall copper remained unchanged in the control groups throughout the trial period (9.7 ± 0.3 μg/g). Zinc concentration remained unchanged ($p>0.05$) in both controls (106.8 ± 3.7 μg/g) and copper supplemented groups (107.5 ± 3.4 μg/g) (Tables, 1.8, 1.9 ; Figs, 1.12a, b).

b. Small intestine

Proximal

Copper concentrations increased ($p<0.05$) during the first 5 weeks of copper loading to about twice the normal (16.3 ± 1.2 μg/g) and despite some subsequent fluctuations, fell to approximately the control levels at week 16 (9.3 μg/g). Copper remained constant (8.5 ± 0.4 μg/g) in the controls throughout the trial. Zinc content remained unchanged ($p>0.05$) in both control and copper supplemented groups (Tables, 1.8, 1.9 ; Figs, 1.13a. b).

Middle.

Copper increased ($p<0.05$) during the first 5 weeks (18.6 ± 1.7 μg/g) in copper supplemented groups, falling subsequently from week 6 onward to the control value at week 16. Copper content within the control groups remained constant through the trial (10.3 ± 0.7 μg/g). Zinc concentration despite minor fluctuations in the copper loaded groups did not deviate overall from the control levels (Tables 1.8, 1.9 ; Figs, 1.14a, b).

Distal

The copper content rose markedly ($p<0.05$) during the first 5 weeks of copper loading (22.3 ± 4.5 μg/g), falling from week 6-12 (16.7 ± 0.7 μg/g) and thereafter when it achieved approximately normal levels at week 16 (10.3 μg/g). Control copper contents varied little (9.3 ± 0.9 μg/g) and likewise zinc content in both control and experimental groups remained unchanged (Tables, 8, 9 ; Figs, 1.15a. b).

c. Caecum.

Copper concentrations rose markedly ($p<0.05$) during the first 5 weeks of copper supplementation (38.0 ± 4.1 µg/g) and despite some group fluctuations, falling thereafter but still remaining elevated at week 16 (17.5 µg/g) when compared with the control (9.9 ug/g). Control copper contents remained constant (9.5 ± 0.9 µg/g) throughout the trial. Zinc concentrations also remained unchanged ($p>0.05$) in both control (117.9 ± 6.8 µg/g) and experimental groups (121.1 ± 2.3 µg/g) (Tables, 8, 9 ; Figs, 1.16a, b).

d. Colon

Copper concentrations increased ($p<0.05$) during the first 5 week of copper supplementation (19.7 ± 0.9 µg/g), afterwards progressively falling from week 6-12 (15.8 ± 1.0 µg/g) and further to equal the control values at week 16 (9.3 µg/g). Copper content of controls remained constant (9.6 ± 0.3 µg/g). Zinc content of both control (129.2 ± 3.0 µg/g) and experimental groups (131.4 ± 2.2 µg/g) remained unchanged ($p>0.05$) (Tables, 8, 9 ; Figs, 1.17a, b).

The estimated recovery for the copper and zinc concentrations during acid digestion were 102 ± 8 % and 105 ± 4 % (mean ± SD) respectively.

Table 1.0

Histological changes of the stomach of control and copper supplemented rats

Histological changes	Control			Copper supplementation				
	W-1	W-8	W-15	W-1	W-2	W-3	W-4	W-5
1. Necrosis (apoptosis)								
i. Gastric pits	+ (4)	+ (4)	+ (4)	+ (1) ++ (3)	++ (4)	++ (3) +++ (1)	++ (4)	++ (4)
ii. Gastric glands	0 (4)	0 (4)	0 (4)	0 (1) + (3)	+ (3) ++ (1)	+ (1) ++ (2) +++ (1)	+ (3) ++ (1)	+ (3) ++ (1)
2. Hyperplasia								
i. Surface mucous cells	0 (4)	0 (4)	0 (4)	+ (1) ++ (2) +++ (1)	++ (3) +++ (1)	++ (1) +++ (3)	++ (1) +++ (3)	++ (4)
ii. Mucous neck cells	0 (4)	0 (4)	0 (4)	+ (1) ++ (2) +++ (1)	++ (3) +++ (1)	++ (2) +++ (2)	++ (2) +++ (2)	++ (4)
3. Atrophy Parietal cells	0 (4)	0 (4)	0 (4)	+ (3) ++ (1)	+ (3) ++ (1)	++ (4)	++ (4)	+ (1) ++ (3)
4. Mitotic figures	+ (3) ++ (1)	+ (3) ++ (1)	+ (4)	+ (2) ++ (2)	+ (2) ++ (2)	+ (1) ++ (3)	++ (2) +++ (2)	++ (1) +++ (3)
5. Leukocytes	+ (3) ++ (1)	+ (3) ++ (1)	+ (4)	+ (2) ++ (2)	+ (3) ++ (1)	+ (2) ++ (2)	+ (3) ++ (1)	+ (2) ++ (2)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats. W = weeks.

...continued from table 1.0

Histological changes	Copper supplementation				
	W-6	W-8	W-10	W-12	W-16
1. Necrosis (apoptosis)					
i. Gastric pits	++ (4)	++ (4)	+ (3) ++ (1)	+ (3) ++ (1)	+ (3) ++ (1)
ii. Gastric glands	++ (3) +++ (1)	+ (1) ++ (3)	+ (3) +++ (1)	+ (4)	0 (1) + (3)
2. Hyperplasia					
i. Surface mucous cells	++ (1) +++ (3)	+ (1) ++ (2) +++ (1)	++ (1) +++ (3)	0 (2) ++ (1) +++ (1)	0 (1) + (1) ++ (1) +++ (1)
ii. Mucous neck cells	++ (1) +++ (3)	+ (2) ++ (1) +++ (1)	+++ (4)	0 (2) +++ (2)	0 (1) + (1) ++ (1) +++ (1)
3. Atrophy Parietal cells	0 (1) ++ (3)	0 (1) + (2) ++ (1)	++ (4)	0 (2) ++ (2)	0 (1) + (2) ++ (1)
4. Mitotic figures	+ (1) ++ (3)	+ (4)	+ (2) ++ (2)	++ (3) +++ (1)	+ (2) ++ (2)
5. Leukocytes	+ (4)	+ (4)	+ (3) ++ (1)	+ (3) ++ (1)	+ (4)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats . W = weeks.

Table 1.1

Histological changes of the proximal small intestine of control and copper supplemented rats

Histological changes	Control W-1	Control W-8	Control W-15	Copper supplementation W-1	Copper supplementation W-2	Copper supplementation w-3	Copper supplementation W-4	Copper supplementation W-5
Villus epithelial cells								
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+ (1) ++ (3)	+ (1) ++ (3)	+ (1) ++ (3)	+ (2) ++ (2)	+ (3) ++ (1)	+ (4)	+ (4)	+ (4)
3. Intraepithelial leukocytes (nos.)	++ (2) +++ (2)	+++ (4)	+++ (4)	++ (1) +++ (3)	+ (2) ++ (1) +++ (1)	+ (4)	+ (3) ++ (1)	+ (2) ++ (2)
Crypts epithelial cells								
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
Leukocytes	++ (1) +++ (3)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	++ (2) +++ (2)	+++ (4)	++ (1) +++ (3)
Mitotic figures	++ (3) +++ (1)	++ (4)	++ (4)	+++ (4)	++ (1) +++ (3)	++ (1) +++ (3)	++ (2) +++ (2)	++ (2) +++ (2)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats . W = weeks.

...continued from table 1.1

Histological changes	Copper supplemetation				
	W-6	W-8	W-10	W-12	W-16
Villus epithelial cells					
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells	+ (2)	+ (3)	+ (4)	+ (4)	+ (1)
	++ (2)	++ (1)			++ (3)
3. Intra epithelial leukocytes (nos.)	++ (4)	+ (3)	+ (3)	+ (4)	+ (1)
		+++ (1)	++ (1)		++ (2)
					+++ (1)
Crypts epithelial cells					
1. Enterocytes (degeneration/apoptosis.)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
Leukocytes	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
Mitotic figures	++ (1)	++ (2)	++ (3)	++ (1)	++ (3)
	+++ (3)	+++ (2)	+++ (1)	+++ (3)	+++ (1)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate) and ++++ (marked). Figure in the brackets is the number of rats . W = weeks.

Table 1.2

Histological changes of the middle small intestine of control and copper supplemented rats

Pathological changes	Control			Copper supplementation				
	W-1	W-8	W-15	W-1	W-2	W-3	W-4	W-5
Villus epithelial cells								
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	.++ (1) +++ (3)	++ (1) +++ (3)	+++ (4)	++ (1) +++ (3)	++ (3) +++ (1)	++ (4)	++ (4)	++ (3) +++ (1)
3. Intraepithelial leukocytes (nos.)	.++ (1) +++ (3)	+++ (4)	+++ (4)	++ (1) +++ (3)	+ (1) ++ (3)	+ (3) ++ (1)	+ (1) ++ (3)	+ (1) ++ (3)
Crypts epithelial cells								
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)
Leukocytes	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
Mitotic figures	++ (2) +++ (2)	++ (4)	++ (4)	++ (2) +++ (2)	++ (3) +++ (1)	++ (2) +++ (2)	++ (2) +++ (2)	++ (3) +++ (1)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats . W = weeks.

...continued from table 1.2

Histological changes	Copper supplementation				
	W-6	W-8	W-10	W-12	W-16
Villus epithelial cells					
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells	+++ (4)	++ (3) +++ (1)	++ (4)	++ (3) +++ (1)	++ (4)
3. Intraepithelial leukocytes (nos.)	++ (4)	+ (2) + (2)	+ (1) ++ (3)	+ (3) ++ (1)	+ (3) ++ (1)
Crypts epithelial cells					
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)
Lymphocytes	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
Mitotic figures	++ (3) +++ (1)	++ (4)	++ (4)	++ (4)	++ (4)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats .
W = weeks .

Table 1.3

Histological changes of the distal small intestine of control and copper supplemented rats

Histological changes	Control			Copper supplementation				
	W-1	W-8	W-15	W-1	W-2	W-3	W-4	W-5
Villus epithelial cells								
1.Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	++ (3) +++ (1)	++ (3) +++ (1)	++ (3) +++ (1)	++ (3) +++ (1)
3. Intraepithelial leukocytes (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	++ (1) +++ (3)	++ (3) +++ (1)	++ (3) +++ (1)	++ (3) +++ (1)
Crypts epithelial cells								
1.Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
Leukocytes	+++ (4)	+++ (4)	++ (1) +++ (3)	+++ (4)	++ (1) +++ (3)	++ (3) +++ (1)	++ (3) +++ (1)	++ (1) +++ (3)
Mitotic figures	++ (2) +++ (2)	++ (3) +++ (1)	++ (3) +++ (1)	++ (4)	++ (3) +++ (1)	++ (4)	++ (3) +++ (1)	++ (3) +++ (1)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in the brackets is the number of rats. W = weeks.

...continued from table 1.3

Histological changes	Copper supplementation				
	W-6	W-8	W-10	W-12	W-16
Villus epithelial cells					
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	++ (1) +++ (3)	++ (2) +++ (2)	++ (2) +++ (2)	+++ (4)
3. Intraepithelial leukocytes (nos.)	++ (2) +++ (2)	++ (3) +++ (1)	++ (2) +++ (2)	++ (2) +++ (2)	++ (2) +++ (2)
Crypts epithelial cells					
1. Enterocytes (degeneration/apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Goblet cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Paneth cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
Leukocytes	++ (2) +++ (2)	++ (1) +++ (3)	++ (2) +++ (2)	++ (3) +++ (1)	++ (2) +++ (2)
Mitotic figures	++ (3) +++ (1)	++ (3) +++ (1)	++ (2) +++ (2)	++ (2) +++ (2)	++ (2) +++ (2)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats.
W = weeks.

Table 1.4

Histological changes of the caecum of control and copper supplemented rats

Histological changes	Control			Copper supplementation				
	W-1	W-8	W-15	W-1	W-2	W-3	W-4	W-5
1. Enterocytes(apoptosis)	0 (3) + (1)	0 (4)	0 (4)	0 (4)	0 (4)	0 (3) + (1)	0 (4)	0 (4)
2. Mucous cells (nos.)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)
3. Mitotic figures	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
4. Bacterial colonies	0 (3) + (1)	0 (2) + (1) ++ (1)	0 (2) + (2)	0 (1) + (1) ++ (2)	++ (4)	+ (2) ++ (2)	+ (2) ++ (2)	+ (3) ++ (1)
5. Leukocytes	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats. W = weeks

...continued from table 1.4

Histological changes	Copper supplementation				
	W-6	W-8	W-10	W-12	W-16
1. Enterocytes (apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Mucous cells (nos.)	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)
3. Mitotic figures	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
4. Bacterial colonies	+ (3) ++ (1)	0 (1) + (1) ++ (2)	+ (3) ++ (1)	0 (2) + (2)	+ (1) ++ (3)
5.Leukocytes	++ (4)	++ (4)	++ (4)	++ (4)	++ (4)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats. W = weeks.

Table 1.5

Histological changes of the colon of control and copper supplemented rats

Histological changes	Control			Copper supplementation				
	W-1	W-8	W-15	W-1	W-2	W-3	W-4	W-5
1. Enterocytes (apoptosis)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)
2. Mucous cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Mitotic figures	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
4. Leukocytes	+ (3) ++ (1)	+ (3) ++ (1)	+ (2) ++ (2)	++ (4)	++ (4)	+ (2) ++ (2)	+ (1) ++ (3)	+ (1) ++ (3)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild), ++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats. W = weeks.

...continued from table 1.5

Histological changes	Copper supplementation				
	W-6	W-8	W-10	W-12	W-16
1. Enterocytes (apoptosis)	0 (4)	0 (4)	0 (4)	0 (1) + (3)	0 (4)
2. Mucous cells (nos.)	+++ (4)	+++ (4)	+++ (4)	+++ (4)	+++ (4)
3. Mitotic figures	+ (4)	+ (4)	+ (4)	+ (4)	+ (4)
4. Leukocytes	++ (4)	++ (4)	++ (4)	+ (2) ++ (2)	+ (2) ++ (2)

Relative intensities of histological changes are estimated from: 0 (nil), + (mild),++ (mild to moderate), +++ (moderate), and ++++ (marked). Figure in brackets is the number of rats . W = weeks.

Table 1.6

Villus height of the small intestine of copper supplemented and control rats

Time (weeks)	Villus height, μm (mean ± SEM) Proximal	Middle	Distal
	Copper supplementation		
1	436 ± 10* (4)	431 ± 7 (4)	292 ± 21* (2)
2	493 ± 10 (4)	421 ± 16 (4)	283 ± 13* (4)
3	479 ± 1 (2)	390 ± 3 (2)	284 ± 26* (4)
4	458 ± 16 (2)	388 ± 43 (3)	269 ± 12* (4)
5	477 ± 29 (4)	454 ± 10 (4)	277 ± 15* (4)
6	501 ± 5 (3)	418 ± 16 (3)	334 ± 15 (2)
8	485 ± 5 (4)	482 ± 51 (4)	322 ± 11 (4)
10	484 ± 10 (4)	460 ± 8 (4)	268 ± 10 (3)
12	504 ± 9 (4)	489 ± 23 (4)	280 ± 23 (4)
16	469 ± 12 (4)	422 ± 28 (4)	277 ± 11 (4)
	Control		
1	484 ± 14 (4)	430 ± 11 (4)	372 ± 9 (4)
8	459 ± 11 (4)	411 ± 34 (3)	340 ± 23 (4)
15	469 ± 14 (4)	436 ± 15 (3)	314 ± 12 (4)

Figure in brackets is the number of rats. (*) $p<0.05$.

Table 1.7

Crypt depth of the small intestine of copper supplemented and control rats

Time (weeks)	Crypt depth, µm (mean ± SEM) Proximal	Middle	Distal
	Copper supplementation		
1	103 ± 5 (4)	118 ± 3 (4)	107 ± 8 (2)
2	110 ± 3 (4)	113 ± 7 (4)	110 ± 2* (4)
3	107 ± 4 (4)	113 ± 7 (4)	114 ± 7 (4)
4	112 ± 5 (4)	101 ± 4* (4)	107 ± 5 (4)
5	110 ± 5 (4)	102 ± 4* (4)	117 ± 5 (4)
6	116 ± 6 (3)	121 ± 8 (3)	153 ± 8 (2)
8	117 ± 6 (4)	128 ± 9 (4)	130 ± 5 (4)
10	109 ± 4 (4)	111 ± 2* (4)	123 ± 9 (3)
12	107 ± 4 (4)	113 ± 5 (4)	107 ± 1 (4)
16	100 ± 3 (4)	101 ± 4* (4)	118 ± 4 (4)
	Control		
1	116 ± 4 (4)	126 ± 11 (4)	131 ± 5 (4)
8	100 ± 6 (4)	121 ± 6 (3)	119 ± 8 (4)
15	101 ± 6 (4)	124 ± 6 (4)	123 ± 5 (4)

Figure in brackets is the number of rats. (*) $p < 0.05$.

Table 1.8

Copper concentration of the gastrointestinal divisions of copper supplemented and control rats

Time (weeks)	Stomach	Prox.	Mid.	Distal	Caecum	Colon
	Copper supplementation					
1	21.9	12.4	12.3	13.9	32.7	18.1
2	26.6	17.1	17.8	16.3	25.7	17.8
3	28.2	15.9	21.6	28.0	46.5	19.7
4	27.1	6.6	19.9	21.3	47.6	20.1
5	28.1	19.6	21.4	32.0	37.7	22.6
6	15.8	15.8	16.9	17.8	27.0	17.2
8	14.1	13.3	14.7	15.8	23.4	17.9
10	16.5	17.4	17.1	17.1	26.6	13.7
12	14.8	13.2	12.0	16.1	19.3	14.3
16	10.0	9.3	8.8	10.3	17.5	9.3
Mean	20.3*	15.1*	16.3*	18.9*	30.4*	17.1*
± SEM	2.2	1.0	1.4	2.1	3.3	1.2
	Control					
1	10.2	9.3	11.5	11.0	10.7	10.1
8	9.5	8.5	10.3	9.1	7.8	9.0
15	9.4	7.8	9.0	7.9	9.9	9.7
Mean	9.7	8.5	10.3	9.3	9.5	9.6
± SEM	0.3	0.4	0.7	0.9	0.9	0.3

Copper concentration (μg/g dry weight)

Each value is the mean of pooled samples of 4 rats in the group. Prox. = proximal, Mid. = middle and distal small intestine. (*) $p<0.05$.

Table 1.9

Zinc concentration of the gastrointestinal divisions of copper supplemented and control rats

Time (weeks)	Zinc concentration (μg/g dry weight)					
	Stomach	Prox.	Mid.	Distal	Caecum	Colon
	Copper supplementation					
1	111.3	114.8	135.5	204.1	120.0	128.2
2	108.1	112.1	140.8	183.5	124.0	133.7
3	111.6	113.4	131.6	170.5	113.1	131.7
4	126.0	131.2	169.1	212.7	124.5	138.0
5	116.8	126.9	149.6	209.1	135.3	142.7
6	103.3	144.6	143.6	190.1	115.1	126.6
8	102.9	123.6	142.6	193.1	125.7	120.5
10	108.3	124.4	143.2	192.8	126.4	135.7
12	102.1	114.7	129.2	159.3	114.2	122.0
16	85.0	106.6	131.5	164.5	112.5	134.4
Mean	107.5	118.2	141.7	188.0	121.1	131.4
± SEM	3.4	2.5	3.7	5.8	2.3	2.2
	Control					
1	113.9	118.8	140.9	218.4	130.5	134.2
8	104.9	112.2	140.0	154.4	116.1	123.8
15	101.5	105.0	127.7	158.1	107.1	129.6
Mean	106.8	112.0	136.2	177.0	117.9	129.2
± SEM	3.7	4.0	4.3	20.7	6.8	3.0

Each value is the mean of pooled samples of 4 rats in the group. Prox. = proximal, Mid. = middle, and distal small intestine.

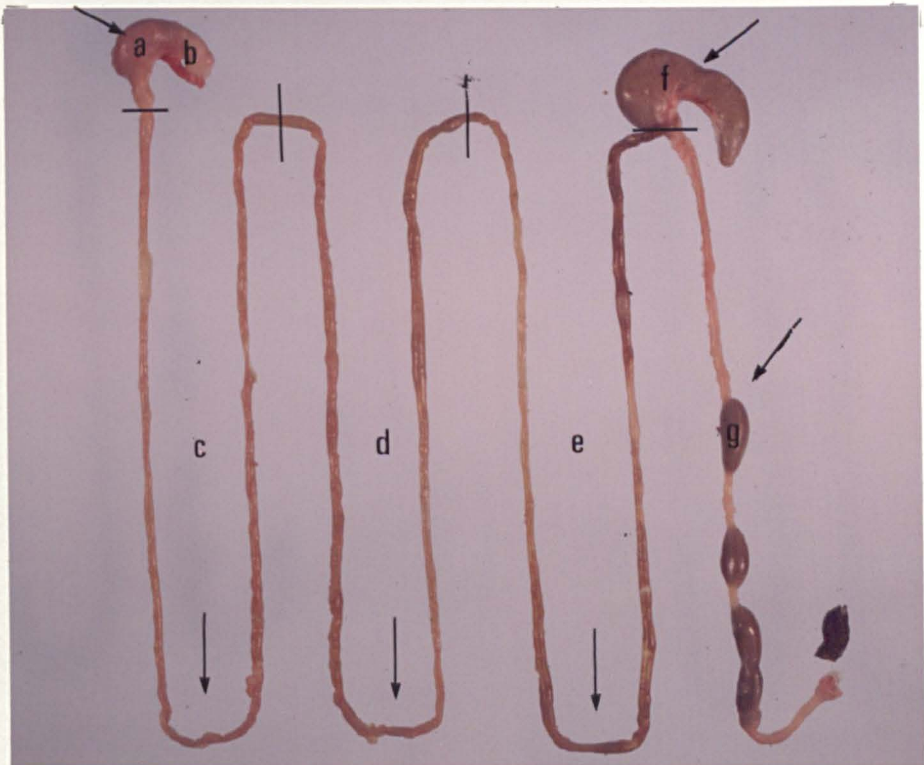


Fig. 1.0. **Gastrointestinal tract.** (a) Glandular and (b) non glandular stomach, (c) proximal, (d) middle and (e) distal small intestine, (f) caecum and (g) colon. Sites of sampling for histology (arrow).

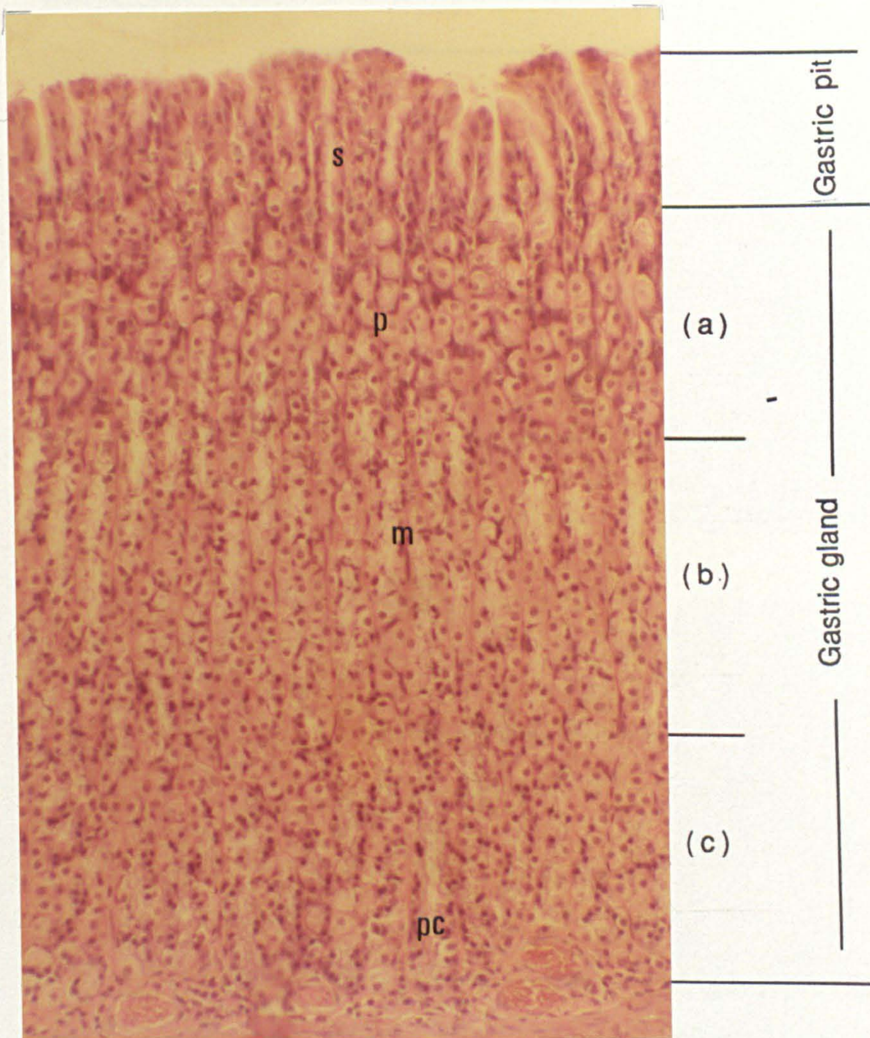
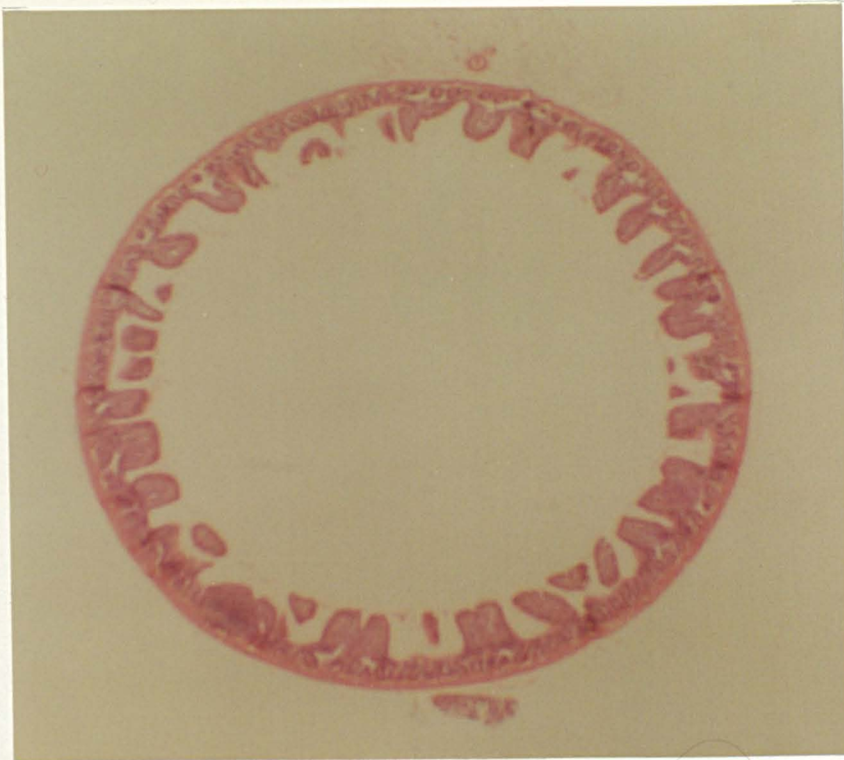
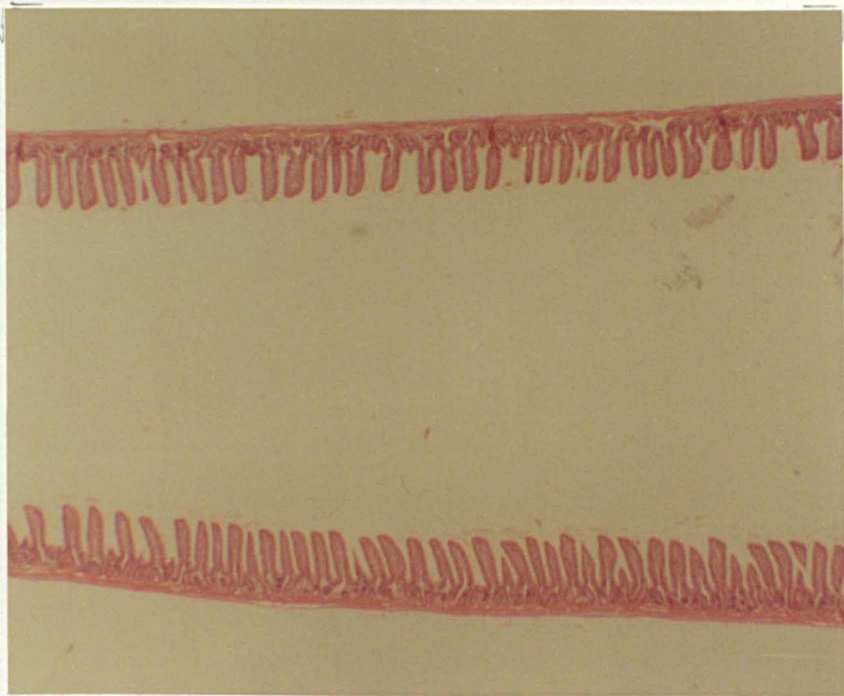


Fig. 1.1. **Gastric mucosa.** Control. The mucosa is divided into (i) gastric pit and (ii) gastric gland. The latter is further classified into the (a) isthmus (b) neck and (c) base of the gland. Surface mucous cell (s), mucous neck cell (m), parietal cell (p) and peptic cell (pc). HE (X 125).



(a)



(b)

Fig. 1.2. **Distal small intestine.** Copper supplementation. Week 4. (a) **Transverse section**. Villus morphology was irregular and varied from a finger like projection to a flat dome shaped. (b) **Longitudinal section**. Finger like villi were more obvious. HE (X 16).

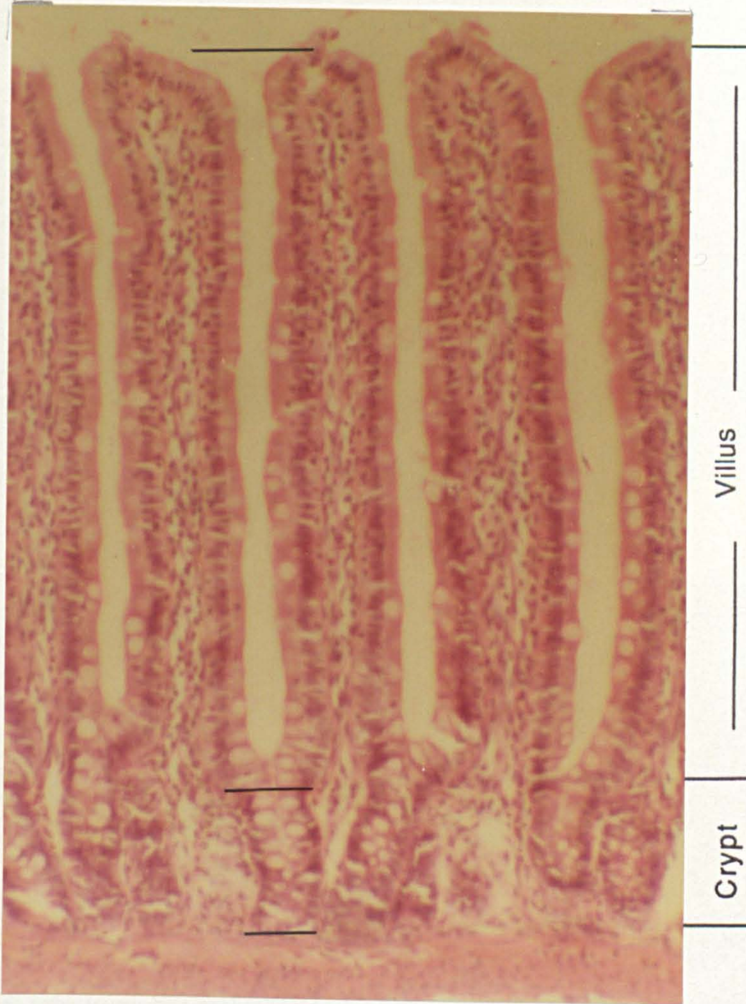
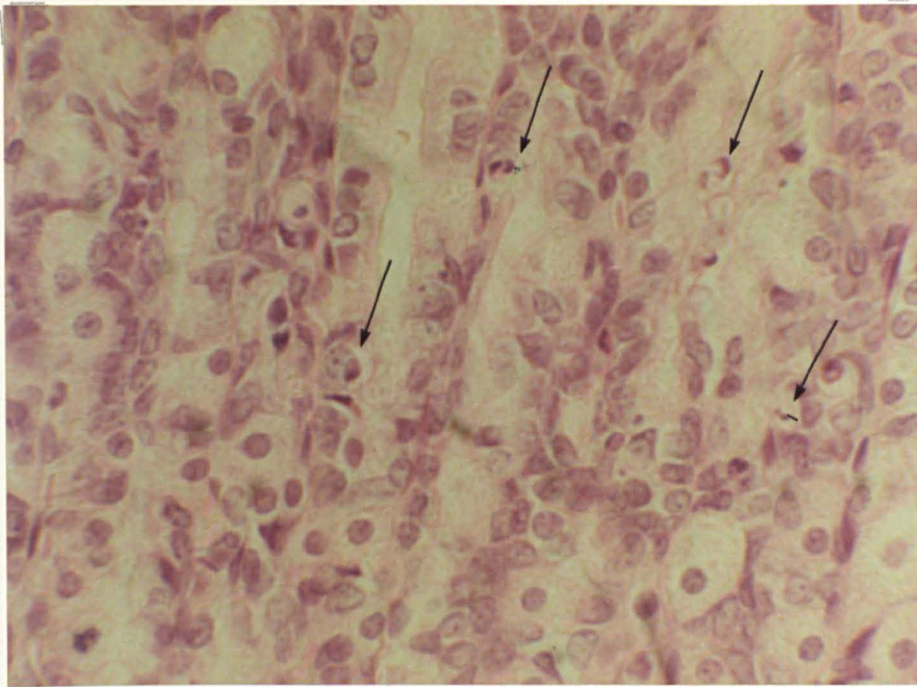
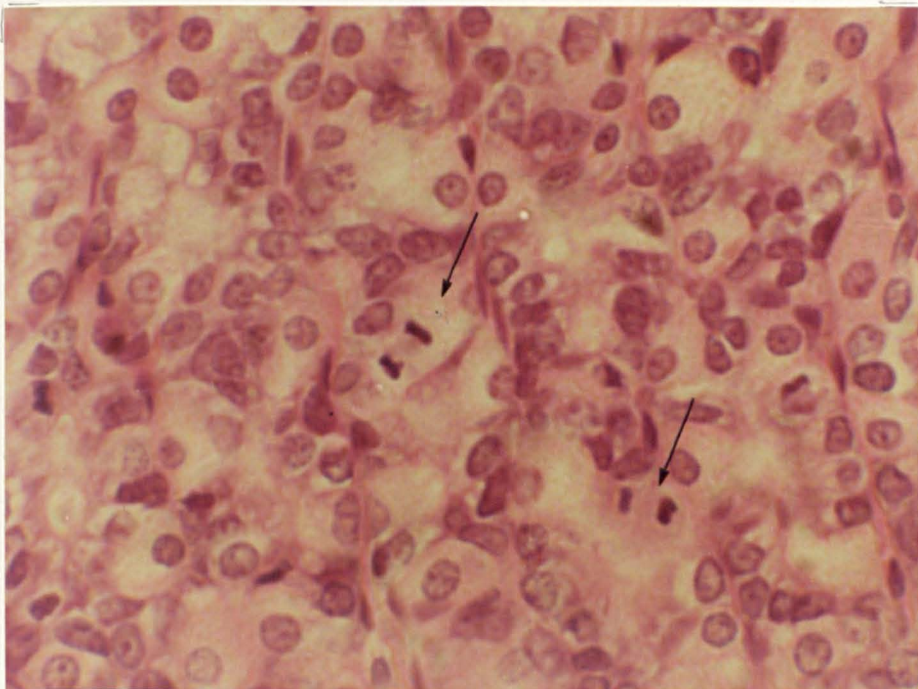


Fig. 1.3. **Middle small intestine.** Copper supplementation. Week 5. Villus and crypt measurements. (a) Villus height and **(b) Crypt depth.** HE (X 125).

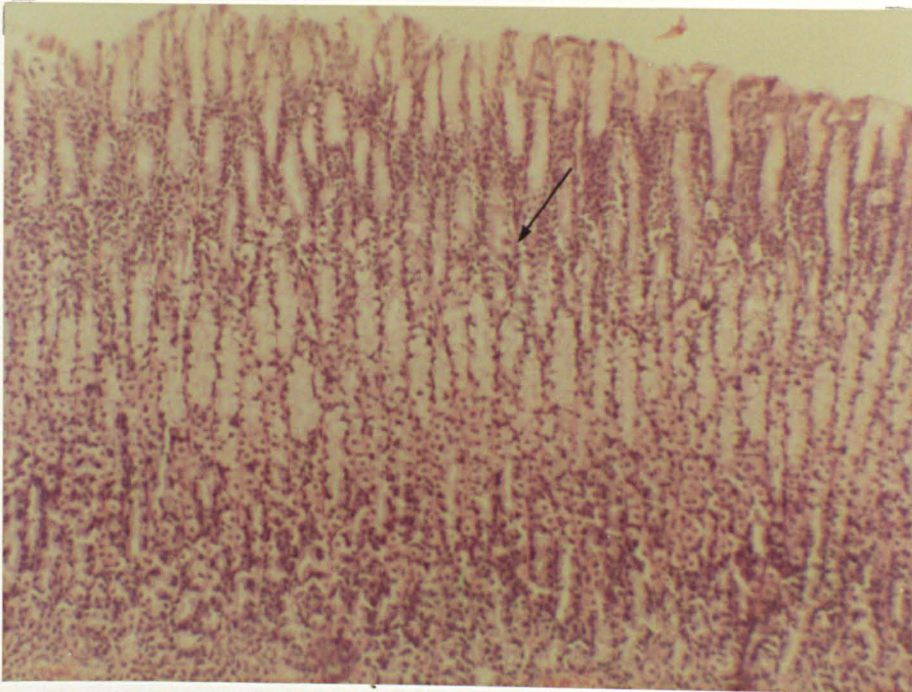


(a)

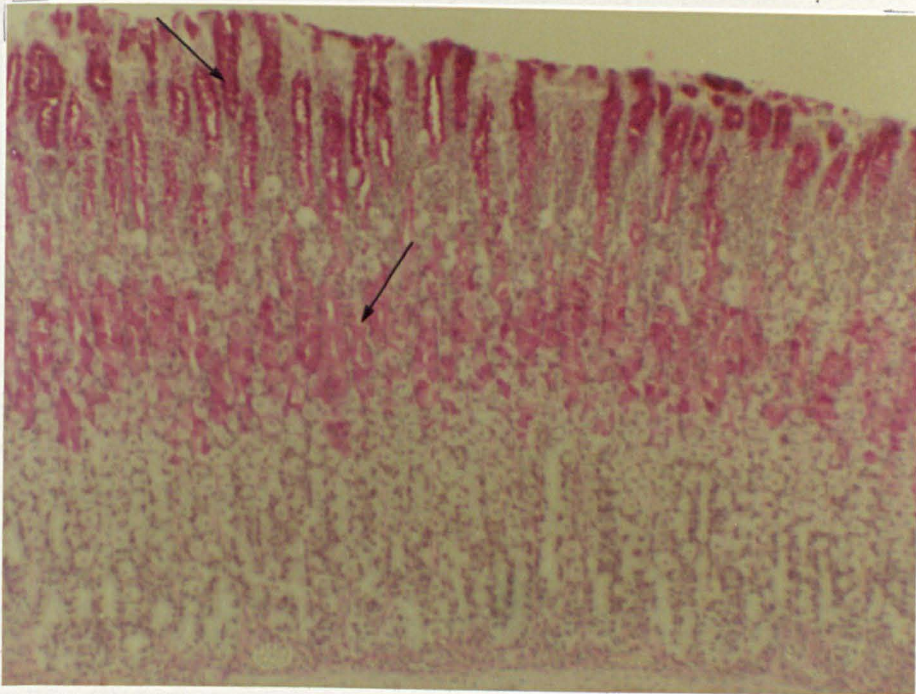


(b)

Fig. 1.4. **Copper supplementation.** Gastric gland (a) **Single cell necrosis (apoptosis)** (Week 3) (+++) (arrow). (b) **Mitotic cell** (Week 5) (+++) (arrow). HE (X 500).

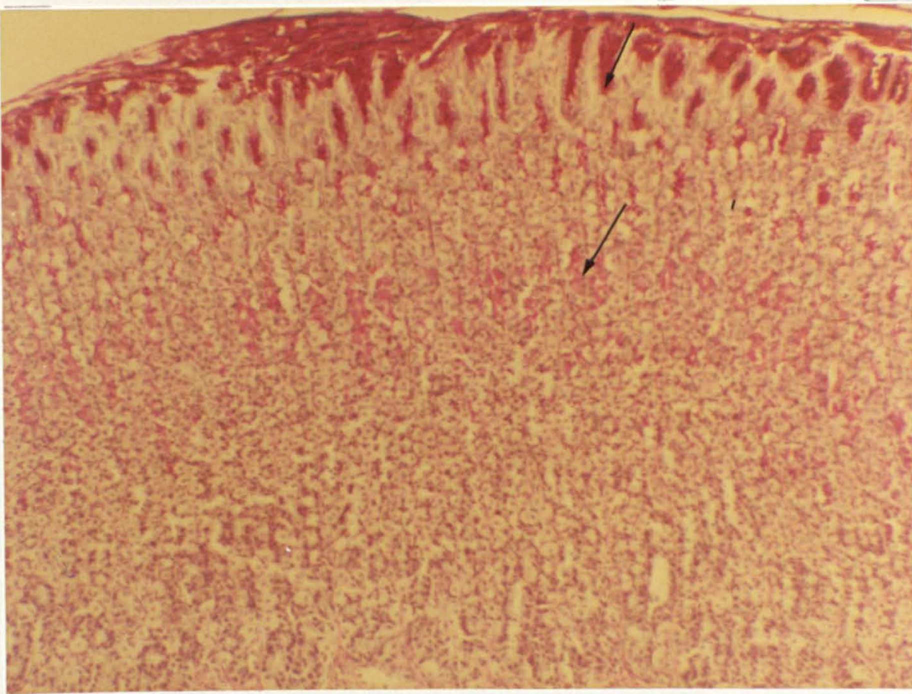


(a)

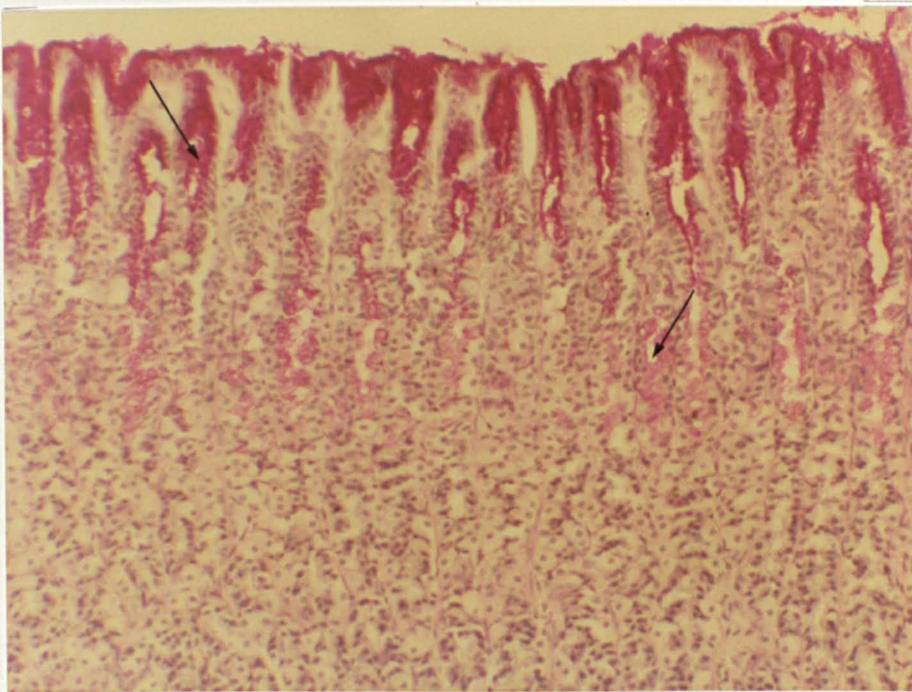


(b)

Fig. 1.5. **Copper supplementation. Week 3.** Gastric mucosa of rat. **Mucous secreting cell hyperplasia** (+++) and **parietal cell atrophy** (+++) (arrow). (a) HE and (b) PAS (X 80).



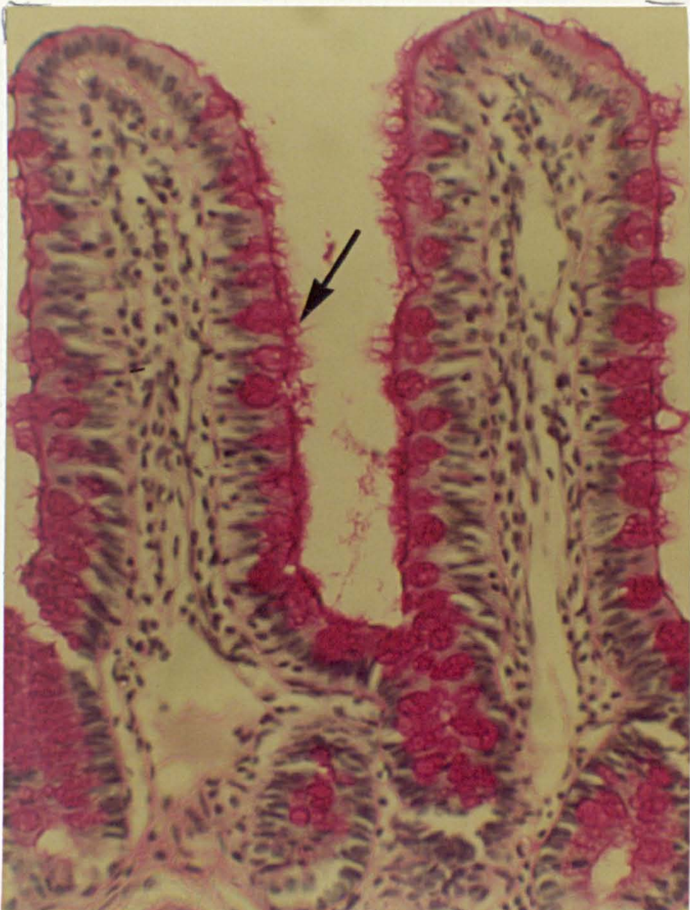
(a)



(b)

Fig. 1.6. **Gastric mucosa.** (a) **Control** (X 100). (b) **Copper supplementation.** Mucous secreting cell hyperplasia partially regressed to about control by **Week 16** (X 125) (arrow) . PAS .

(a)



(b)

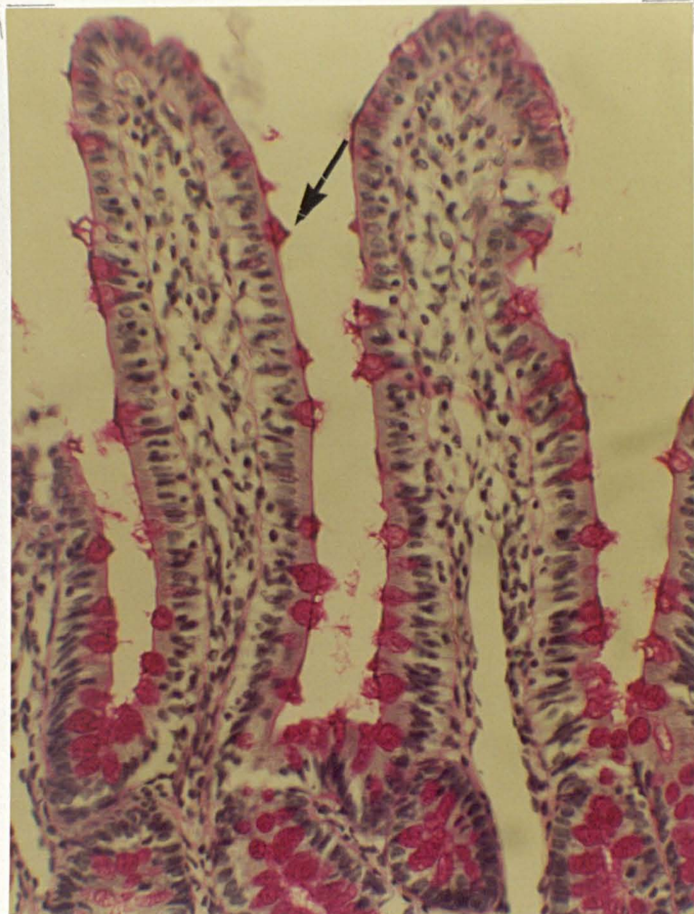


Fig. 1.7. **Distal small intestine.** (a) **Control.** Goblet cell mucous granules (arrow) decreased in (b) **Copper supplemented groups**. Week 5. PAS (X 200).

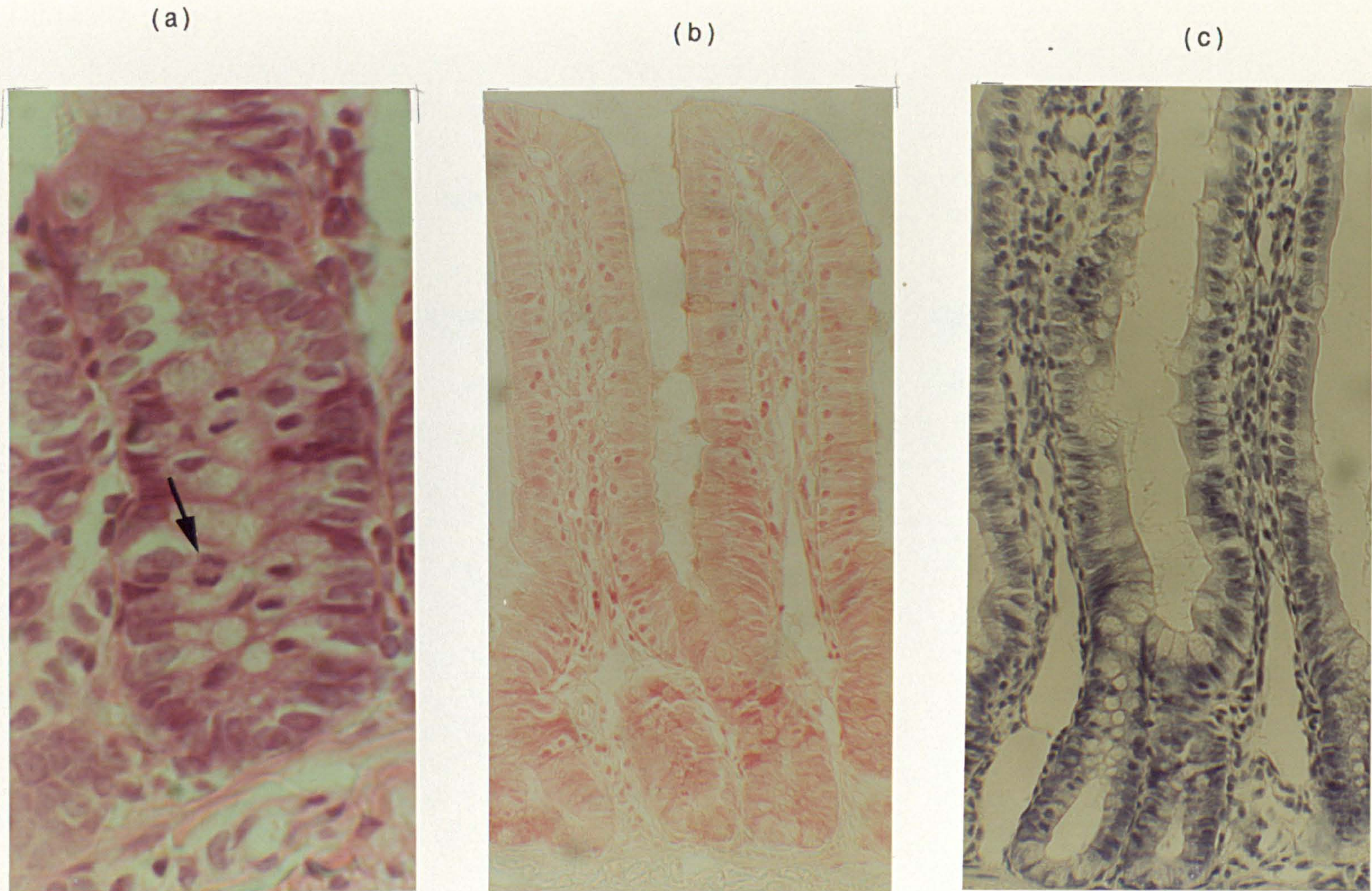


Fig. 1.8. **Small intestine. Copper supplementation. Week 5.** (a) **Mitotic cells** (HE. X 500) (arrow). Histochemical staining (b) **Rhodanine** and (c) **Rubeanic acid** (absence of copper staining granules) (X 200).

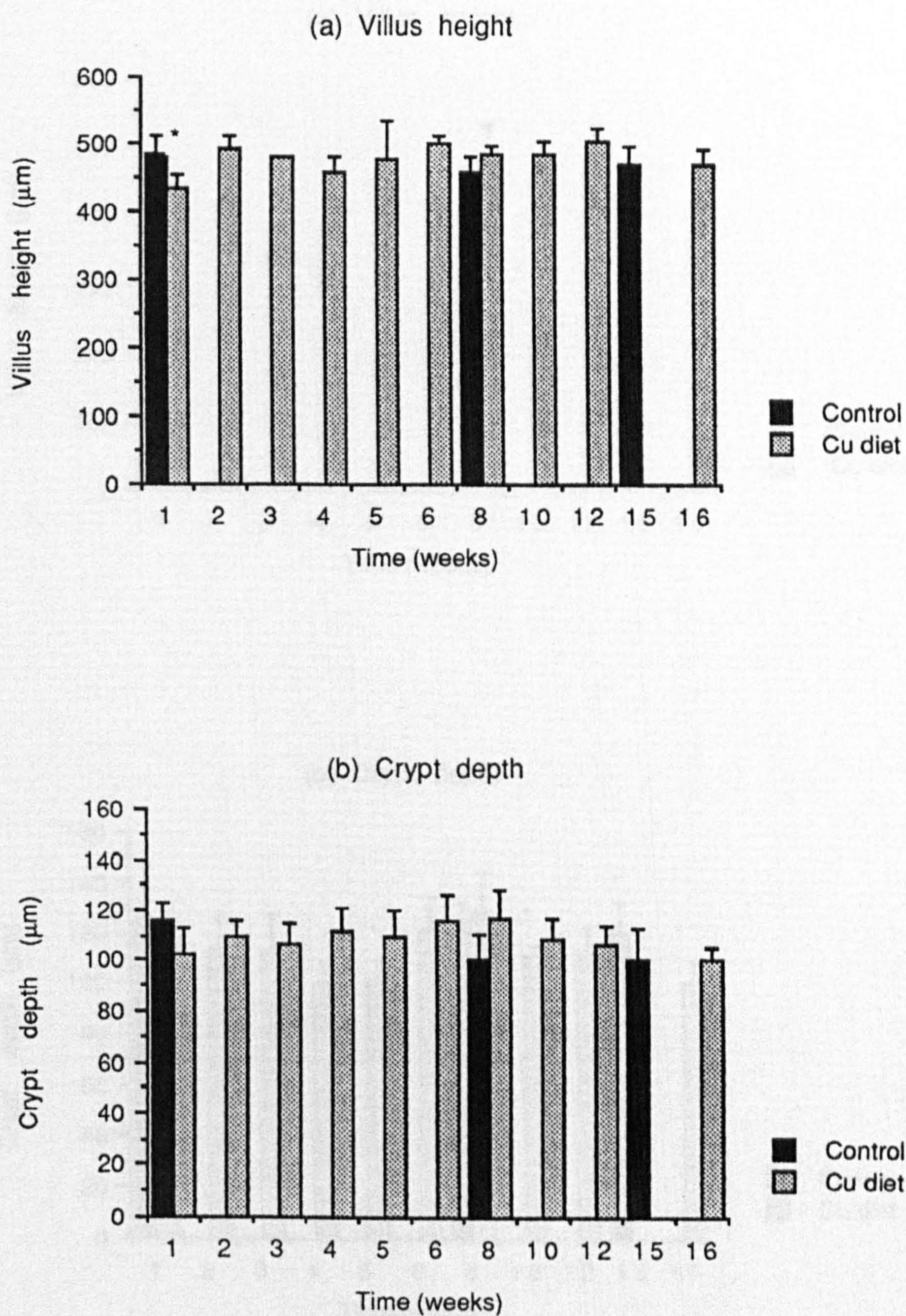


Fig. 1.9 **Proximal small intestine** : (a) Villus height and (b) Crypt depth of control and copper supplemented rats. (*) $p<0.05$.

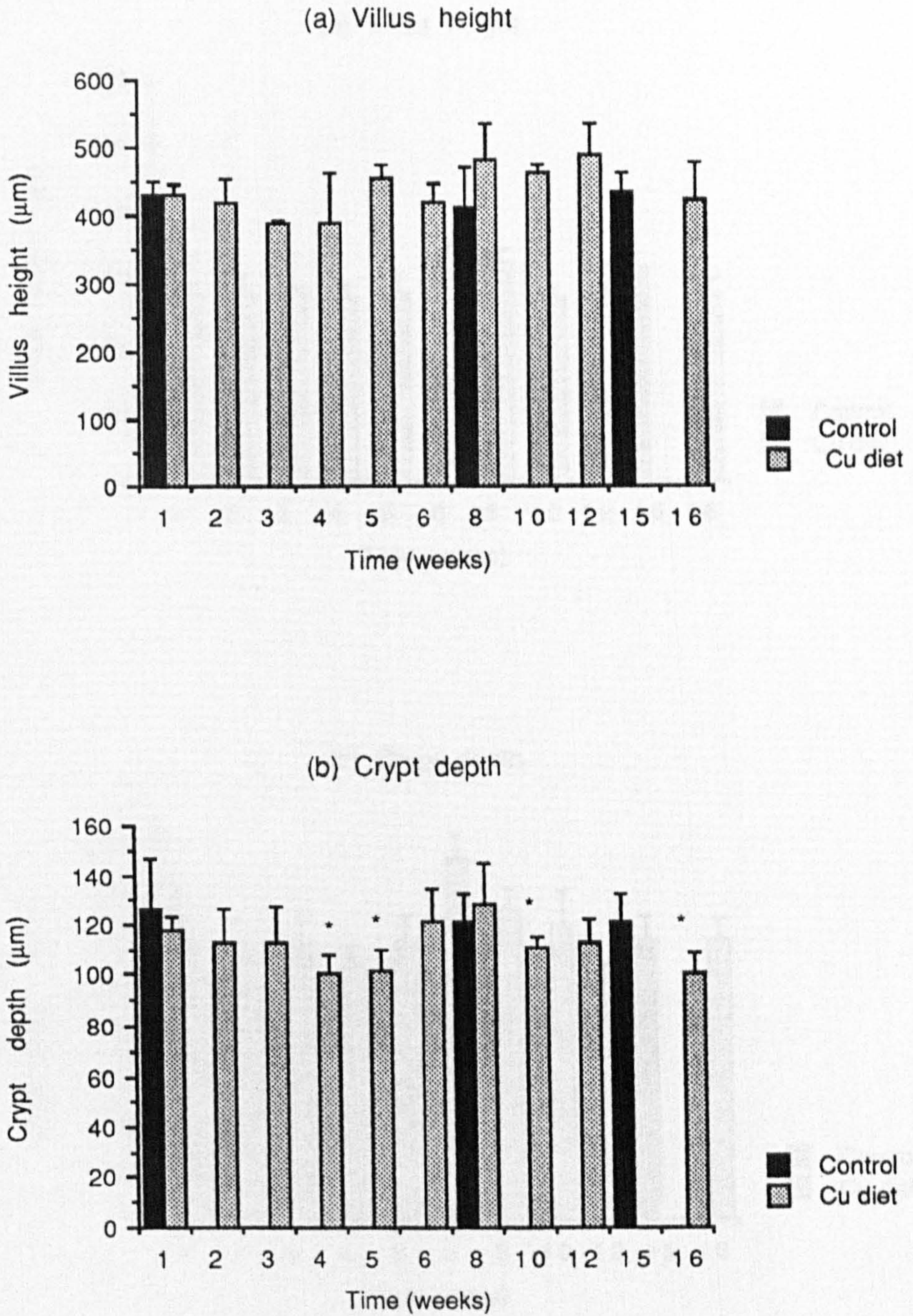


Fig. 1.10 **Middle small intestine** : (a) Villus height and (b) Crypt depth of control and copper supplemented rats. (*) $p<0.05$.

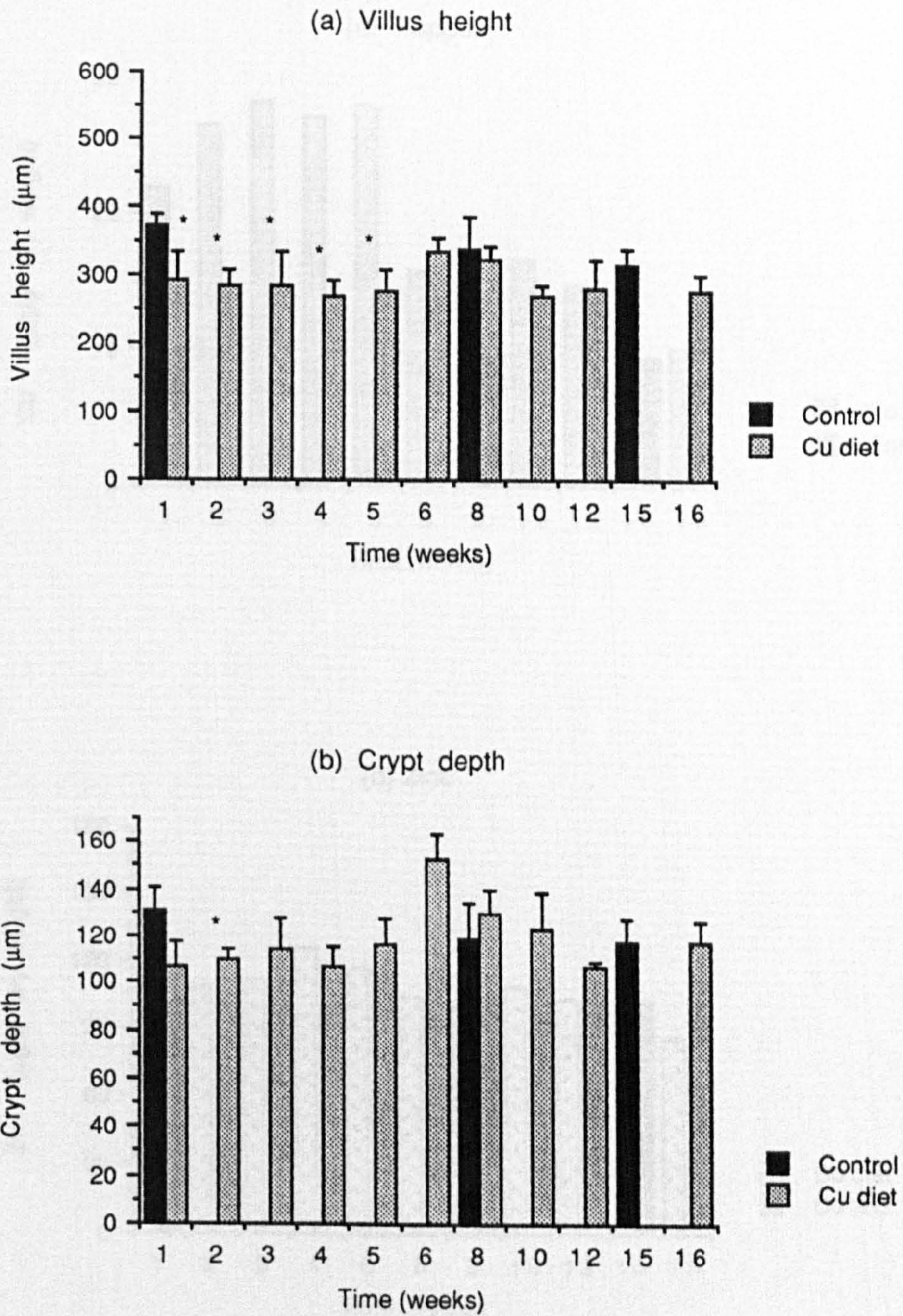
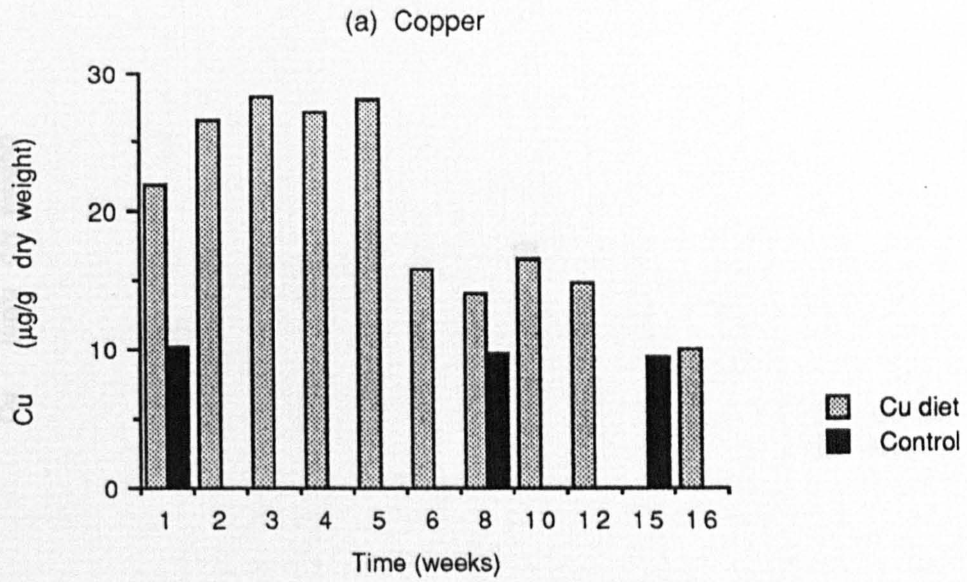


Fig. 1.11 **Distal small intestine** : (a) Villus height and (b) Crypt depth of control and copper supplemented rats. (*) $p<0.05$.



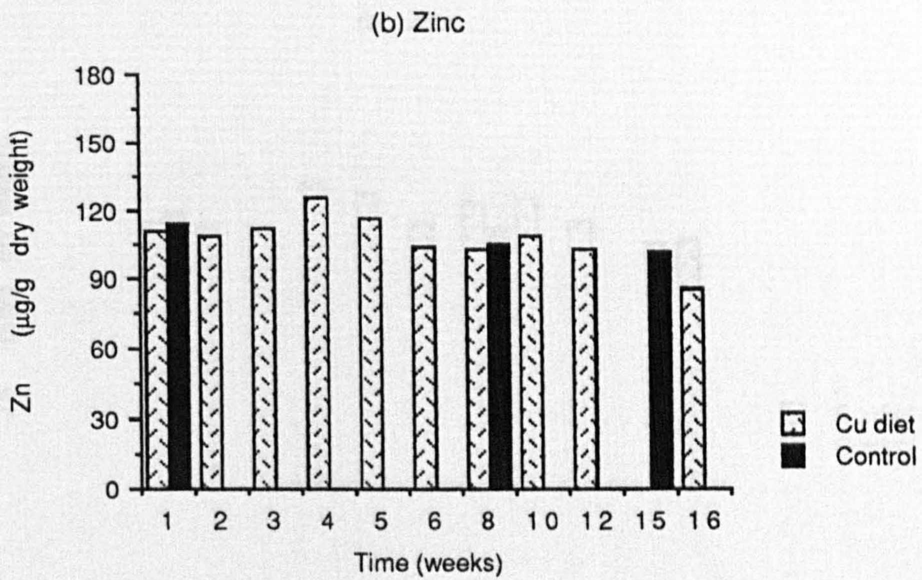
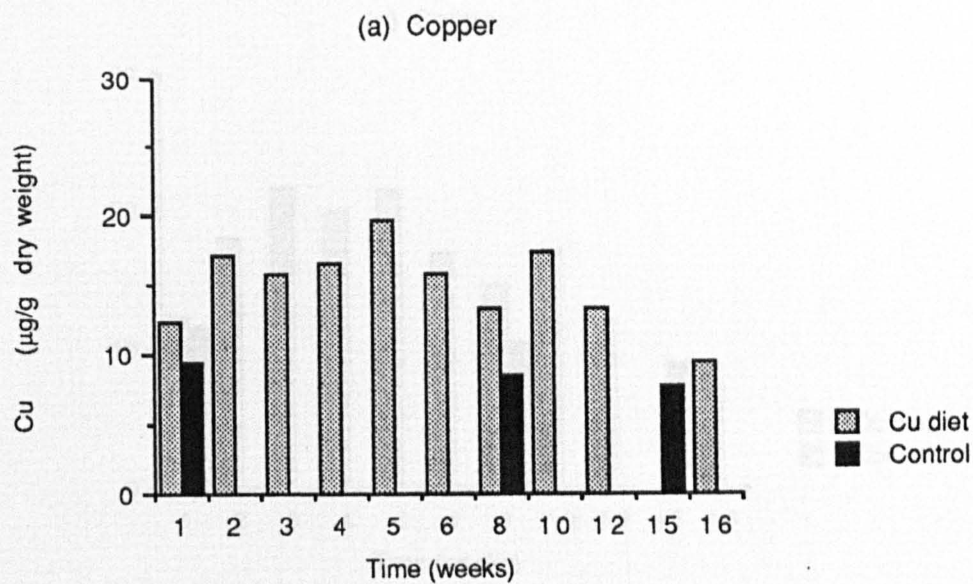


Fig. 1.12. **Stomach** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.



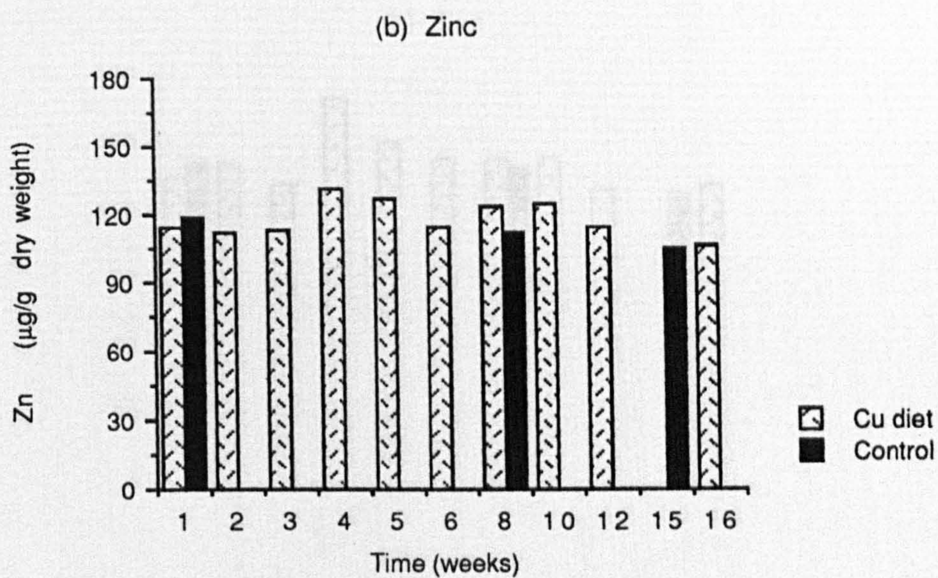


Fig. 1.13. **Proximal small intestine** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.

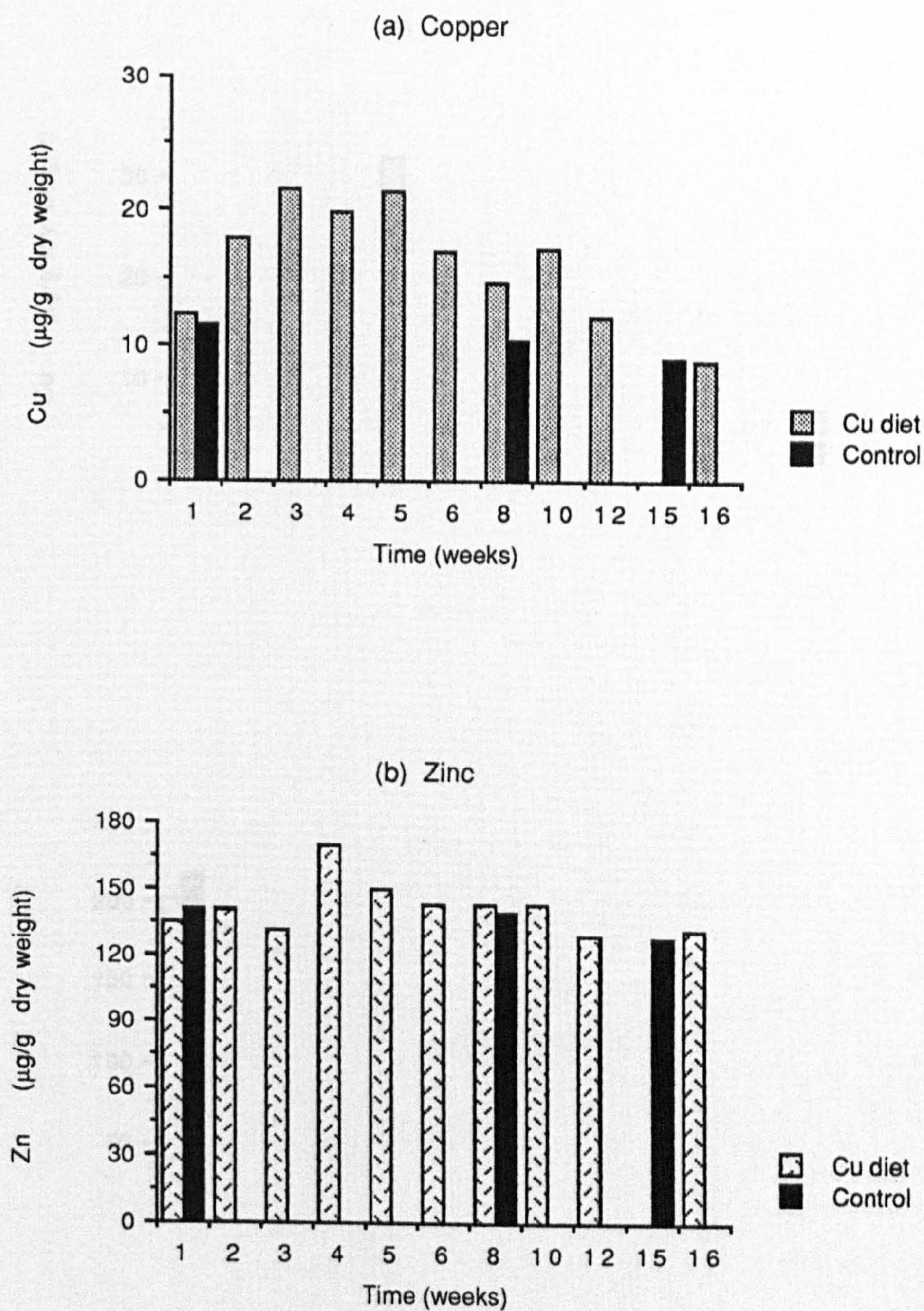


Fig. 1.14. **Middle small intestine** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.

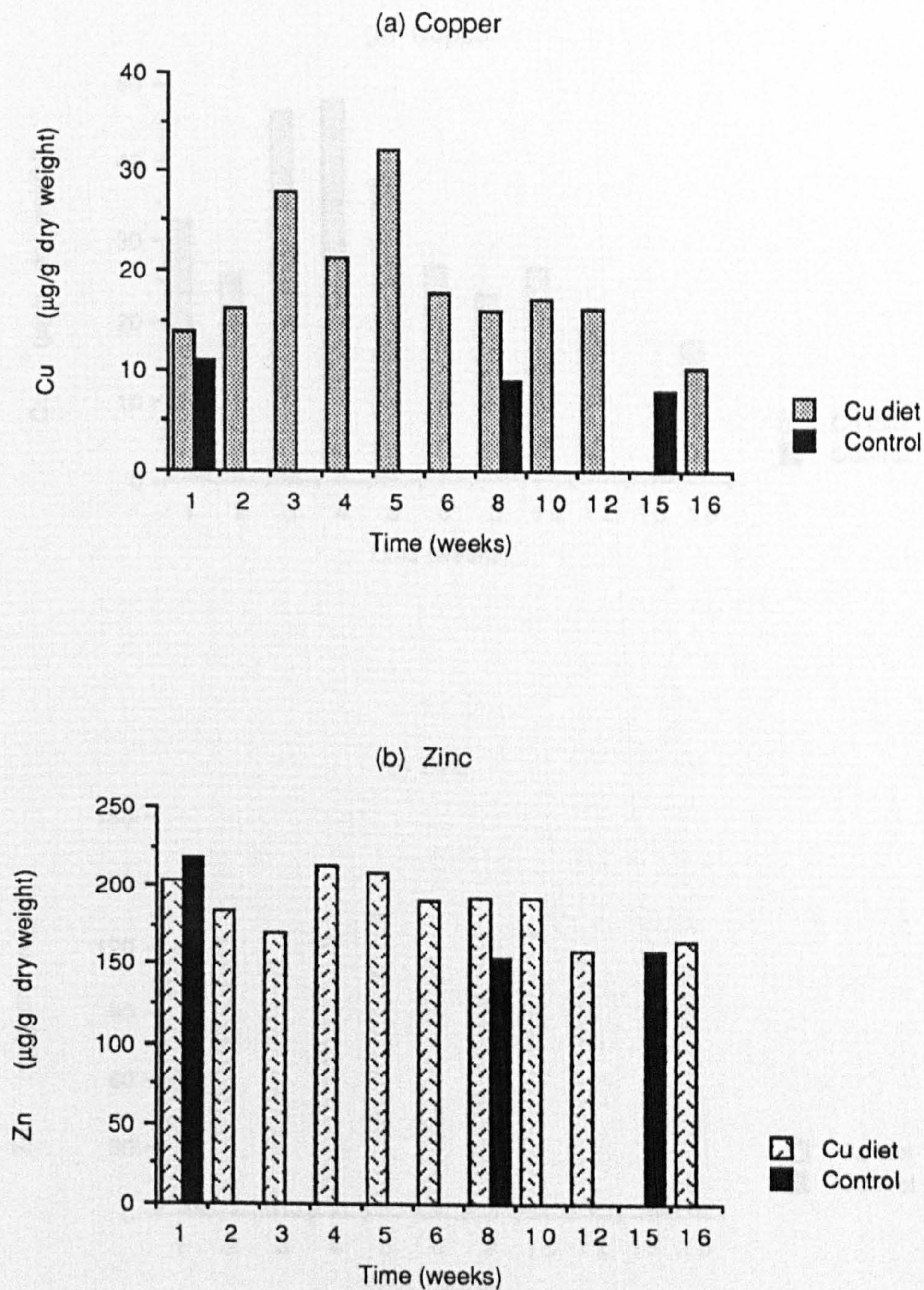


Fig. 1.15. **Distal small intestine** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.

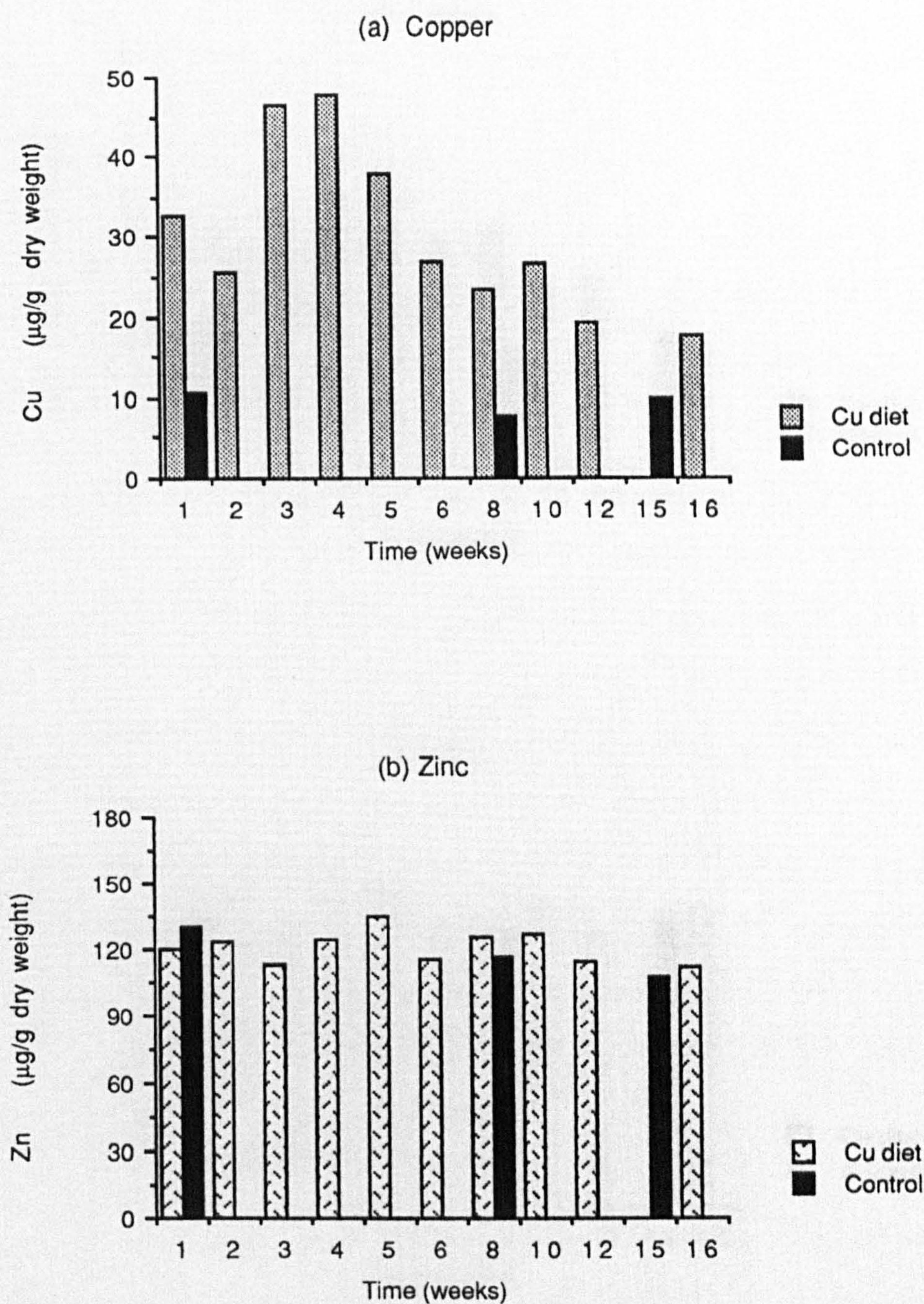
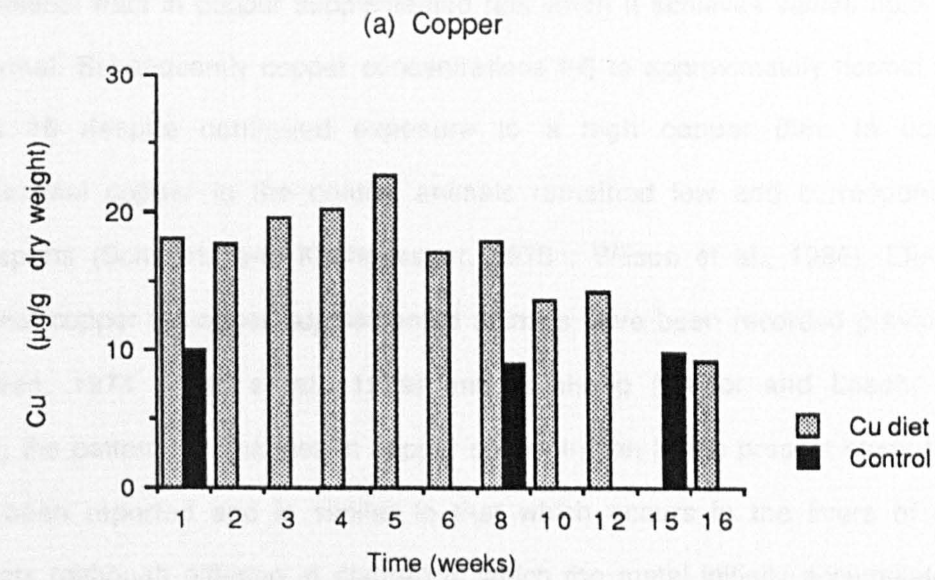


Fig. 1.16. **Caecum** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.



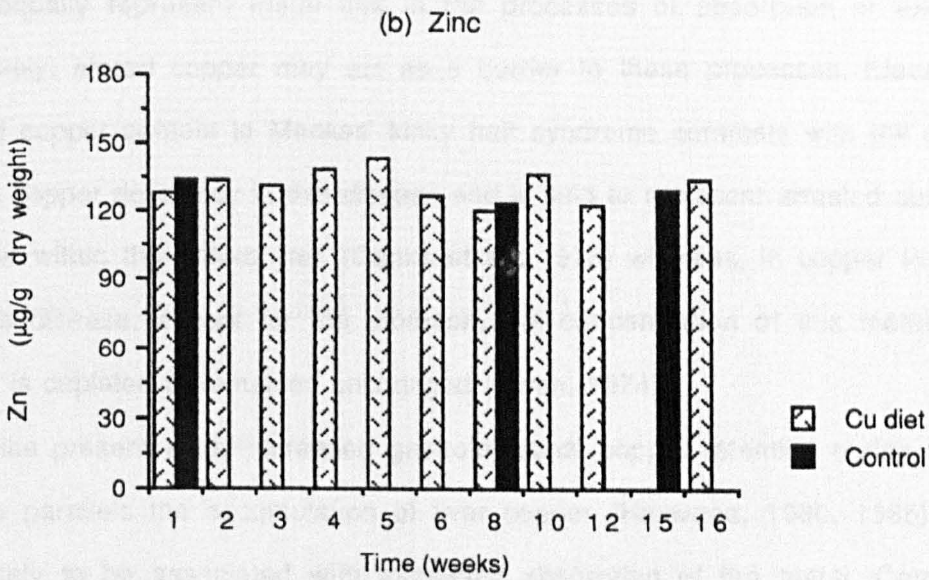


Fig. 1.17. **Colon** : (a) Copper and (b) Zinc concentrations of copper supplemented and control rats.

1.4. DISCUSSION

Increased copper retention occurs progressively upto 5 weeks throughout the gastrointestinal tract in copper supplemented rats when it achieves values upto 3 to 4 times normal. Subsequently copper concentrations fall to approximately normal values at week 16 despite continued exposure to a high copper diet. In contrast, gastrointestinal copper in the control animals remained low and corresponded to earlier reports (Schwartz and Kirchgessner, 1975 ; Wilson et al., 1986). Elevations of intestinal copper in copper supplemented animals have been recorded previously in rats (Owen, 1974 ; Hall et al., 1979) and in sheep (Saylor and Leach, 1980). However, the pattern of changes in copper concentration in the present study has not hitherto been reported and is similar to that which occurs in the livers of copper loaded rats (although differing in degree) in which the metal initially accumulates and is subsequently unloaded as the animal becomes tolerant (Haywood, 1980,1985).

Increased mural intestinal copper does not present an explanation in itself since it may equally represent metal flux in the processes of absorption or excretion; alternatively, stored copper may act as a barrier to these processes. Elevation of intestinal copper content in Menkes' kinky hair syndrome contrasts with the state of systemic copper deficiency in this disease and is said to represent arrested absorption of copper within the enterocytes (Danks et al., 1973) whereas, in copper toxicity in Wilson's disease, except for the stomach, the concentration of this metal in the intestine is depleted or remained unchanged (Owen, 1974).

In the present study increased gastrointestinal copper retention during the first 5 weeks parallels the accumulation of liver copper (Haywood, 1980, 1985) and is more likely to be associated with increased absorption of the metal. Conversely, subsequent GIT copper depletion takes place during the unloading period (Haywood, 1980, 1985) and is likely to be associated with decreased absorption of the metal. If so this particular trial situation is dissimilar to Menke's syndrome or Wilson's disease patients and might indicate different mechanisms in the regulatory control of the metal.

The absorption of copper appears to include both the uptake of the metal from the lumen across the brush border, its subsequent passage across the enterocyte and transfer via the basolateral membrane into the portal circulation (Crampton et al.,1965 ; Marceau et al., 1970). Furthermore, there is some indirect evidence to suggest that a reverse movement of copper may occur across the gastrointestinal mucosal barrier into the lumen. It has been found that fecal copper excretion from parenteral injection of copper isotopes is not prevented by either ligation of the bile duct (Mahoney et al., 1955) or duodenostomy below the pancreatic and biliary duct (Owen, 1964). Thus, the precise explanation concerning the sequence of copper retention in the present investigation could only be resolved by further study with labelled copper.

It is interesting to find that the morphological changes within the copper supplemented animals are more remarkable in the stomach in which copper is reputably mainly absorbed (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984), although, Fields et al., (1986) reported the converse. These changes were related in magnitude to overall copper content within this organ. The initial cytotoxicity displayed upto 3 weeks subsequently subsided and was replaced by a wave of regenerative activity which in turn declined by week 16. The profile of cytopathological changes are similar to those recorded in the copper loaded rat liver whereby adaptation and recovery succeeds the initial cytotoxic effect (Haywood, 1980, 1985).

In man and domestic animals gastric changes are the most commonly reported changes in the gastrointestinal tract associated with copper excess. In acute copper toxicosis in man superfical to deep erosions and ulcerations of the gastric mucosa have been reported (Chuttani et al., 1965) and intense inflammation with marked necrosis of the mucosa and submucosa of the abomasum in lambs have also been observed (Sholl, 1957). Gastric ulceration in pigs (Allen and Harding, 1962 ; Hatch et al., 1979 ; Higgins, 1981) and haemorrhage of the abomasal mucosa in goats (Adam et al., 1977) have been reported in chronic copper poisoning.

Associated adaptational gastric changes were rapidly established in the present study and persistent mucous cell hyperplasia with a corresponding depletion in the parietal cell populations suggested changes in cellular differentiation as both cell types originate from the same site, at the isthmus and neck of the gastric mucosa (Stevens and Leblond, 1953 ; Junqueira and Carnerio, 1980). However, parietal cell depletion could also be due to cell death and may be reflected in the increased apoptosis at this time. The significance of these changes is not known. A reduction in acid secreting cells (parietal cells) could elevate pH and affect the solubility of the metal which may become less available for absorption.

Mucous cell hyperplasia may be important in the protection of the organ from the toxic effect of the metal. These cells are rich in glycoprotei (mucus). Glycoprotein has many functions including the lubrication and protection of the mucosa, regulation of cell division, immunological recognition as well as being important in cell adhesion (Filipe, 1979). Furthermore, mucus may act as a medium in which macromolecules become insoluble and are prevented from contact with the cell membrane, whereas small molecules are soluble and free to diffuse (Edward, 1978). Thus, these changes may contribute to the protective or adaptive changes in copper tolerance.

By way of contrast despite broadly similar fluctuations in copper content, histological changes in the small intestine, caecum and colon corresponded less with changing patterns of copper retention than did the stomach. Thus, no observable necrotizing effect was recorded and no remarkable changes in the villus height and crypt depth were observed, other than partial villus atrophy in the distal small intestine during the first 5 weeks of copper supplementation. Mitotic activity and goblet cell populations were also depleted during these periods. However, they showed signs of recovery in the later weeks and an increased mitotic activity was sustained latterly suggesting rapid cell turnover.

A similar pattern of copper retention in the different portions of small intestine may not indicate identical processes of copper metabolism. It appears that copper is

mainly absorbed from the proximal rather than the distal part of the organ and this may suggest that the distal small intestine may be more involved in the excretion of the metal. Copper has been reported to be excreted through the intestinal wall (Mahoney et al., 1953 ; Owen, 1964). Furthermore, biliary copper is poorly absorbed (Mistilis and Farrer, 1968 ; Cikrt, 1973 ; Gollon, 1975) and pancreatic juice seems to inhibit the absorption of the metal (Jamison et al., 1983). The absence of elevation of intestinal copper in Wilson's disease (Owen, 1974) may indicate that there is a defect in the elimination of copper through this route in addition to other excretory defects that have been reported earlier (Sternlieb, 1975).

The mechanisms which may be involved in the elimination of copper from the small intestine are not known, but may be associated with characteristic features of the organ including the mean turnover time (the average time spent by the cells in the villus compartment between entry and extrusion) and the types of epithelial cell populations. Increased mitotic activity in the proximal and distal small intestine may indicate such a rapid cellular turnover. In addition, the reduction of intraepithelial leukocytes and villus goblet cell populations might be associated with these changes. Intraepithelial leukocytes are located in the intercellular spaces of villus epithelium and they are extruded into the intestinal lumen following cellular extrusion (Toner et al., 1971). Excessive extrusion of mucous goblet granules or excess immature goblet cells may cause an apparent disappearance of the cells.

The role of Paneth cells in the elimination of metals is still incompletely understood, although it has been suggested that they are concerned in the elimination of zinc, lead, mercury, calcium and cobalt (Sandow and Whitehead, 1979). However, no changes were seen in Paneth cell populations in rats in this study neither could copper be identified histochemically in this location.

Reduced cytotoxicity despite a similar intestinal assimilation of the metal compared with the stomach is suggestive of copper being present in a different form either luminally or within enterocytes. Isolation of a low molecular weight copper binding protein, identified as metallothionein from the soluble fractions of intestinal

mucosa of animals receiving copper (Evans, 1979), cadmium (Davies and Campbell, 1977) or zinc supplementation (Hall et al, 1979 ; Fischer et al.,1983) indicates that this protein is involved in copper homeostasis and may be protective.

The significance of sequential changes in copper retention in the caecum and colon is not known, although they may be involved in the excretion of the metal. Bacterial overgrowth in the caecum was mild and no other remarkable histological changes were recorded.

This study has also shown that the concentration of zinc in the gastrointestinal tract is unaffected by dietary copper supplementation. A similar finding has been observed in the liver (Fuentealba, 1988). Furthermore, semiquantitative evaluation of copper in the GIT tissues using both rhodanine and rubeanic acid were not possible; a failure most likely due to the low copper concentration in the organ, although other factors including copper binding may be involved. Copper staining in copper loaded rats (Fuentealba et al., 1987) and Wilson' s disease (Goldfisher and Sternlieb, 1968) with high liver copper concentration shows an unreliable correlation with copper concentration measured by chemical analysis. However, this technique (rhodanine) appears to give a satisfactory result in the evaluation of hepatic copper content in copper storage disease in Bedlington terriers (Johnson et al., 1984).

In conclusion this study has shown that rats can adapt to exposure to high dietary copper at the level of the gastrointestinal tract with repair of damage in much the same manner as the liver. The mechanisms may be two fold, copper may be rendered less toxic and also copper absorption may be reduced and occur both luminally and within the epithelial cell. Excess gastric mucus secretion and depletion of acid producing cells (parietal cells) could possibly change the nature of copper to become less toxic and less available for absorption Rapid cellular turnover may facilitate excretion of the metal. Furthermore, copper might be bound to metallothionein and may both protect the cell and prevent absorption.

CHAPTER : 2

Gastrointestinal Response to Copper Excess: Metallothionein (MT) Status and Metal Retention

2.1. INTRODUCTION

An accumulation of excess copper in the liver associated with copper toxicity has been recognised in both man and animals for many years. It may be of genetic origin as in Wilson's disease in man (Sternlieb, 1980) and Bedlington terriers (Johnson et al., 1980) or acquired as in sheep (Soli, 1980). The significant variation in species or even breed susceptibility to the disease has also been documented, although the reasons for this are still unclear. The sheep is a species known to be prone to chronic copper poisoning (Soli, 1980), in contrast to laboratory rats which have been shown to adapt to high dietary copper and became tolerant (Haywood, 1980, 1985).

Copper is absorbed from the gastrointestinal tract and is stored in the liver, excess being excreted mainly through the bile (Evans, 1973). A defect in biliary copper excretion has been reported in Wilson's disease patients (Sternlieb, 1980) and familial copper toxicosis in Bedlington terriers (Su et al., 1982b). Furthermore, biliary copper excretion in sheep seems to be low when compared to the monogastric animals (Soli, 1980) and the excretion of copper in the bile of sheep was not influenced by liver copper content (Caple and Heath, 1978). Conversely, the facilitated excretion of copper in bile and urine may partly explain the copper tolerance displayed by rats (Haywood et al., 1985a ; Evering, 1989).

The gastrointestinal response to copper excess suggests that excess glycoprotein secretion could possibly change the nature of copper to become less toxic and less available for absorption (Chapter, 1). Furthermore, the isolation of a copper binding protein identified as MT from the intestinal tract (Starcher, 1969 ; Evans et al., 1970 ; Evans, 1979) has strengthened the concept of metal

compartmentalisation and transfer across the absorptive cell during the process of absorption (Crampton et al., 1965; Marceau et al., 1970) and copper bound to MT at the cellular level may both protect the cell and prevent absorption. However, it appears that the role of MT at the absorptive site is still a matter of considerable conjecture and needs further clarification.

The binding of copper to MT within the intestinal mucosa of zinc (Richards and Cousins, 1977 ; Hall et al., 1979 ; Fischer et al., 1981, 1983) and cadmium (Davies and Campbell, 1977) supplemented animals corresponded with a decrease in copper absorption. A similar inhibitory mechanism with regard to copper absorption has been suggested when the dietary intake of the metal is high (Evans and Johnson, 1978). The inability of sheep to induce intestinal MT in response to copper excess may partly explain the susceptibility of sheep to copper toxicosis (Saylor et al., 1980). Furthermore, impaired copper absorption in Menkes' disease patients and brindled mice (Danks et al., 1973 ; Evans and Reis, 1978 ; Leone et al., 1985), also in oestrogen treated rats (Cohen et al., 1979) appears to be associated with excess copper bound to MT in the intestinal mucosa.

Conversely, it has been suggested that the binding of copper to MT in the intestinal mucosa can facilitate copper absorption (Starcher, 1969 ; Evans et al., 1970 ; Evans, 1979). Furthermore, it has been observed that the reduction in copper absorption in copper supplemented animals was not influenced by the binding of the metal to MT (Bremner et al., 1979). In newborn animals, increased copper uptake into the absorptive cell, possibly by pinocytosis (Mistilis and Mearrick, 1969 ; Williams and Beck, 1969), is associated with a high copper and MT content within the organ (Johnson and Evans, 1980 ; Hurley et al., 1980 ; Mason et al., 1981).

It is the aim of this study to further clarify the role of metallothionein at the absorptive site by assessing the effect of chronic exposure to high dietary copper on the gastrointestinal tract of rats in relation to metallothionein status and metal retention.

2.2. MATERIALS AND METHODS

2.2.1. Animals

Male ten-week-old Wistar rats of uniform weight were randomly allocated and caged in groups of four. They were fed a pelleted high copper diet (1000 mg/kg) (Appendix, 1.0) and a group of animals was each killed at intervals of 1, 2, 3, 4, 5, 6, 8, 10, 12, and 16 weeks. A control group was included at weeks 1, 8 and 15. Animals were deprived of food overnight prior to sampling and euthanasia was performed by carbon dioxide inhalation and subsequent cervical dislocation. The gastrointestinal tract was separated into the stomach, caecum, colon and three equal parts of small intestine namely, the proximal, middle and distal portions. The sampling procedure was as previously described in Chapter 1 and pooled group samples of each gastrointestinal division were immediately stored at -70°C until required.

2.2.2. Chromatographic separation

Gastrointestinal tissues were partially defrosted and placed on ice throughout the procedure. Tissues were scraped using a glass slide, weighed and diluted in 4 volumes (w/v) or 7 volumes (caecum) of 10 mM Tris-Cl buffer, pH 8.0, containing 0.02% (w/v) sodium azide. The buffer was prepared by adjusting approximately 1.0 M Tris-Cl (Trizma Base, Sigma Chemical Company), containing 20g/litre sodium azide (Analar, BDH Chemical Ltd.) and diluted to 10 mM in a volumetric flask.

Tissues were premixed using a Vortex Mixer and homogenised for about 1 minute in a 30 ml Dounce Tissue Grinder (Kontes Scientific) using the RW 20 DZM homogeniser (Jake and Kunkel, IKA-Labortechnik). The homogenate was then centrifuged at 16000 rpm (approximately 29900g average) for 180 minutes at 4°C using a Sorval SV 288 vertical tube rotor in a Sorval RC5B Refrigerated Superspeed centrifuge. The soluble fraction was collected, 6.5 ml of which was immediately introduced onto a column (100 X 2.5 cm) of Sephadex G-75 gel (Pharmacia AB, Laboratory Separation Division,Uppsala, Sweden) at 4°C, whilst the

remainder was stored at -70°C : for metal analysis and the detection and quantitation of MT.

The column was eluted with 10 mM Tris-Cl buffer, pH 8.0, in ascending fashion with a peristaltic pump (Pharmacia Fine Chemicals) at a flow rate of 30 ml/hour. The eluted fractions (6.5 ml each) were automatically collected using a 2111 Multirac Fraction Collector (LKB). Absorbance of the fraction was measured at 280 nm using a Beckman DU-8 UV Visible Spectrophotometer and their copper and zinc contents were determined directly using Atomic Absorption Spectrophotometry.

The chromatographic column was previously calibrated (Evering, 1989) with 30 mg each of blue dextran, bovine serum albumin (68000 daltons), ovalbumin (45000 daltons), chymotrypsin (25700 daltons), myoglobin (17200 daltons), ribonuclease A (13700 daltons), cytochrome C (12000 daltons) and sodium chloride. The profile of the column was subdivided into a high molecular weight protein band (HMW) (fractions 20 to 44, > 16000 daltons) which included the void volume of the column, a low molecular weight protein band (LMW) (fractions 45 to 60, approximately 16000 to 6000 daltons) and a very low molecular weight band (VLMW) (fractions 61 and after, < 6000 daltons) (Evering, 1989).

2.2.3. Copper and zinc analysis

Copper and zinc contents of pooled samples in the soluble fractions of the gastrointestinal tract were directly determined using Atomic Absorption Spectrophotometry. Copper and zinc concentrations are expressed as the pooled samples reading and mean $^{+}$ standard error of mean of selected groups in µg/g wet weight.

2.2.4. Enzyme linked immunosorbent assay (ELISA) for MT

The procedure established by Ghaffer et al., (1989) was performed in this experiment. The antigen and antibody were supplied by the Rowett Research Institute, Aberdeen in freeze dried form.

2.2.4.i. Preparation of MT antigen

a. MT induction

MT was induced in the liver of adult rats by intraperitoneal injection of 10 mg zinc sulphate solution or 0.5 to 2.0 mg cadmium (in 0.9% normal saline) per kg body weight, daily for 3 consecutive days. Animals were killed 24 hours after the last injection and their livers were isolated and stored at -40°C (Mehra and Bremner, 1983).

b. MT fractionation and purification

The method described by Bremner and Davies (1975) was used in this procedure. Livers were defrosted, pooled, and weighed before they were homogenised in an Ultra-Turrax homogenizer (Janke and Kunkel KG, Staufen, Germany) in 2.0 (w/v) of Tris-Cl buffer, pH 8.0. The homogenate was immediately centrifuged at 37000 rpm (100000g) for an hour, at 4°C in a MSE Superspeed 65 centrifuge. The soluble fraction was collected and immediately applied onto a column (90 X 5.5 cm) of Sephadex G-75 (Pharmacia AB, Lab. Separation Division, Uppsala, Sweden).

The chromatographic column was eluted with 10 mM Tris-Cl buffer, pH 8.0, in descending fashion using a peristaltic pump (Pharmacia Fine Chemicals) at a flow rate of 60ml/hour. The eluted fractions (18 ml per tube) were automatically collected using a 2111 Multirac Fraction Collector (LKB). MT fractions were indirectly determined using Atomic Absorption Spectrophotometry (Appendix, 2.0.i) and they were pooled and concentrated by ultrafiltration under nitrogen at 50 lbs/in^{2}, in Diflo cells using a membrane type of YMZ >1000 molecular weight (Diaflo Ultrafilters).

The concentrated MT containing fraction was then applied onto a column (25 X 1.5 cm) of DEAE Sephadex A-25 (Diethylaminoethyl Sephadex) (Sigma Chemical Company). The column was flushed with 900 ml of 10 mM Tris-Cl buffer, pH 8.0, at a flow rate of 30ml/hour before they were eluted with a linear gradient of 10 to 200 mM Tris-Cl buffer, pH 8.0 at the same flow rate. Two main fractions of MT

(MT-I and MT-II) were isolated (Appendix, 2.0.ii). They were concentrated and desalted by repeated ultrafiltration.

2.2.4. ii. Preparation of MT antibody

Antibody was obtained from sheep that had been immunized by rat Cadmium-MT-I (Mehra and Bremner, 1983). The methods involved intradermal and intramascular immunization of sheep with the conjugation mixture of Cd-MT-I (6 mg) and rabbit IgG (12 mg) emulsified with an equal volume of Freud's complete adjuvant (4ml/sheep). Booster injections were given at 6, 16 and 24 weeks and sheep were bled at 8-20 days after each booster injection. Antisera was heated at $56^{o}C$ for 30 minutes and stored at $1^{o}C$.

2.2.4.iii. Sensitization of microwell module (plate)

A solution of MT-I (100 ng per ml) was prepared in coating buffer (Appendix, 2.1.i) and dispenced at 150 µl into each microwell (Microwell Module F16, Nunc Intermed, Denmark). The plates were incubated at $4^{o}C$ overnight. They were subsequently emptied (by shaking the inverted plate with vigorous motion of the wrist) and washed 3 times with the rinsing buffer (300 µl/well) (Appendix, 2.1.ii). At the end of each washing step, residual droplets were removed by tapping the plate upside down on several layers of absorbent paper.

Bovine serum albumin (Sigma Chemical Company) prepared in distilled water (5.0% (w/v)), was added into each well (200 µl/well). The plates were then incubated at room temperature for 30 minutes before the solution was decanted and residual droplets were removed, as previously described. Plates were dried at $37^{o}C$ for 5 hours, sealed with sellotape and stored at $4^{o}C$.

2.2.4.iv. ELISA procedure (Appendix, 2.2).

MT sensitized microwell modules were allowed to reach room temperature prior to commencing the procedure. MT standard solutions were prepared in diluent buffer

(Appendix, 2.1.iii)) by first combining 1.0 μg/ml concentration of rat MT I and MT II to produce a mixture containing 1000 ng MT/ml. Doubling dilution steps were then employed to provide seven standard solutions of 1000, 500, 250, 125, 62.5, 31.25 and 15.625 ng/ml concentrations respectively. Each standard solution was pipetted accordingly into the microwell (50 μl/well) in triplicate (Appendix, 2.3). The maximum binding wells had 50 μl each of diluent buffer instead of standard (Appendix, 2.3). Test samples were then pipetted (50 μl/well) into other wells either in 2 or 4 replications (Appendix, 2.3). Test samples were further diluted in the diluent buffer if required.

Antibody (Rowett Research Institute, Aberdeen) of 1:1000 dilution was subsequently added into each well (50 μl/well). The microwells were then placed on the orbital shaker (Titertex) for 30 minutes. At the end of the incubation period the solution was decanted by shaking the inverted plate with vigorous motion of the wrist and they were washed 5 times with wash solution (300 μl/well in each step) (Appendix, 2.1.iv). Residual droplets were removed at the end of each washing procedure by the tapping the plate upside down on several layers of absorbent paper.

Peroxidase conjugate (Appendix, 2.1.v) was added into each well (100 μl/well) and the plate were incubated for 30 minutes on the orbital shaker. The solution was then decanted and the wells were washed 5 times with wash solution (300 μl/well in each step), and at the end of each washing sequence residual droplets were removed as previously described.

A working substrate solution (Appendix, 2.1.vi) was subsequently added into each well (100 μl/well) and they were incubated for 15 minutes on the orbital shaker. The absorbance of the solution was then determined using Titertex Multiscan (MCC/340) fitted with 405 nm filter. A standard graph was plotted and the amount of MT in the tested samples was obtained by using this graph (Appendix, 2.3.ii). MT concentration was expressed as the average data from 2 or 3 assays (Appendix, 2.4) and the mean ± standard error of mean of selected groups in μg/g wet weight.

2.2.5. Immunocytochemistry

This procedure was carried out at The University of Wales College of Medicine using the method described by Jasani et al., (1981, 1983) and Clarkson et al., (1985). Formalin fixed, paraffin embedded stomach and small intestine subdivisions were deparaffinised and dehydrated before they were stained for immunoreactive MT using the Dinitrophenyl (DNP)-Localisation System kit (Bioclinical Services Ltd., Unit 9, Willow Brook Laboratories, St. Mellons, Cardiff, CF3 6EF, UK) applied in conjunction with a mouse IgG monoclonal antibody to MT. This antibody was prepared using horse MT I and MT II (Sigma) as the immunogen and was applied to the sections at a dilution of 1 : 50000 (of its ascites preparation) overnight at 4°C in a moist chamber. This was followed by sequential incubation of sections in the ready-to-apply reagents 1 to 4 of the DNP-Localisation System kit, each for a 30 minute duration at room temperature. The reagents 1 to 4 consisted of:

1. DNP-labelled 'universal' (anti-mouse Ig/anti-rabbit Ig) secondary antibody solution.
2. Monoclonal, DNP-specific, bridge antibody solution.
3. DNP-labelled peroxidase conjugate solution.
4. DNP- labelled glucose oxidase solution.

The colour reaction of tissue-bound peroxidase was then developed using diaminobenzidine solution (0.5 mg/ml of phosphate-bound saline, 0.1M, pH 7.1) containing BD(+) glucose (15 mg/ml) (for 5 minutes). Excess reagents were removed after each incubation step using three washes (1 minute each) in PBS. All sections were finally counter-stained with haematoxylin, dehydrated in series of alcohol and xylene baths and mounted in Terpene. Blanks were also included in each procedure. Tissue sections of the blanks were incubated with non-immune serum in placed of anti-MT serum. The intensity and distribution of MT immunoreactivity was assessed under X10 and X40 magnifications.

2.2.6. Statistical analysis

Statistical analysis was performed using Student's t-test in cricket software (version 1.1) for Macintosh (Rafferty et al., 1985).

2.3. RESULTS

2.3.1. Copper and zinc concentrations in the gastrointestinal soluble fractions

a. Stomach

Copper concentrations increased ($p<0.05$) in copper supplemented groups (0.64 ± 0.02 µg/g), when compared with the controls (0.45 ± 0.00 µg/g), although in each group the concentration remained unchanged thoughout the trial (Table, 2.0 ; Fig, 2.0.a). Zinc concentrations remained constant in both controls (7.0 ± 0.2 µg/g) and copper supplemented groups throughout the periods (6.8 ± 0.0 µg/g), (Table, 2.1 ; Fig, 2.1.a).

b. Small intestine

Proximal

Copper concentrations in the copper supplemented groups (0.84 ± 0.06 µg/ml) were elevated ($p<0.05$) when compared with the controls (0.45 ± 0.03 µg/ml) with the greatest rise occuring during the first 10 weeks and subsequently declined by week 16 (0.70 µg/ml) (Table, 2.0 ; Fig, 2.0.b). Zinc concentrations remained constant (8.4 ± 0.6 µg/g) in copper supplemented groups when compared with the controls (8.3 ± 0.5 ug/g), although in each group the concentration was slightly higher in the earlier than the later weeks (Table, 2.1 ; Fig, 2.1.b).

Middle

Copper concentrations in the copper supplemented groups (0.66 ± 0.09 µg/g) were elevated ($p<0.05$) throughout the trial when compared to the controls (0.32 ± 0.02 µg/g) with peak copper content occurring at week 5 (0.90 µg/g) which partially regressed thereafter (Table, 2.0 ; Fig, 2.0.c). Zinc concentrations in copper supplemented groups (9.0 ± 0.8 µg/g) were elevated ($p<0.05$) when compared to the controls (6.3 ± 0.3 µg/g) (Table, 2.1 ; Fig, 2.1.c).

Distal

Copper concentrations in the copper supplemented groups (0.96 ± 0.20 µg/g) were increased ($p<0.05$) when compared to the controls (0.42 ± 0.02 µg/g) with peak copper content occurring at week 5 (1.50 µg/g) and declined by week 16 (0.60 µg/g) (Table, 2.0 ; Fig, 2.0.d). Zinc concentrations in the copper supplemented groups (12.4 ± 0.4 µg/g) were elevated ($p<0.05$) when compared to the controls (9.73 ± 0.8 µg/g) (Table, 2.1, Fig, 2.1.d).

c. Caecum

Copper concentrations in the copper supplemented groups (1.25 ± 0.13 µg/g) were elevated ($p<0.05$) when compared to the controls (0.60 ± 0.03 µg/g) with the greatest rise to about 3X the controls occurring in the first 5 weeks (1.46 ± 0.06 µg/g), which was reduced (0.96 µg/ml) by week 16 (Table, 2.0 ; Fig, 2.0.e). Zinc contents in both the controls (8.0 ± 0.0 µg/g) and copper supplemented groups (9.0 ± 0.5 µg/g) remained unchanged ($p>0.05$) throughout the periods (Table, 2.1 ; Fig, 2.1.e).

d. Colon

Copper contents in the copper supplemented groups (0.91 ± 0.07 µg/g) were elevated ($p<0.05$) when compared to the controls (0.62 ± 0.02 µg/g) with peak copper concentration occurring at week 5 (1.10 µg/g) which declined by week 16 (0.80 µg/g) (Table, 2.0 ; Fig, 2.1.f). Zinc contents in both the controls (8.8 ± 0.6 µg/g) and copper supplemented groups (8.9 ± 0.2 µg/g) remained unchanged ($p>0.05$) throughout the trial (Table, 2.1, 2.1.f).

2.3.2. MT concentration in the gastrointestinal soluble fractions

a. Stomach

MT concentrations in the copper supplemented groups (28.2 ± 2.8 µg/g) were greater ($p<0.05$) than the controls (9.6 ± 1.0 µg/g) with maximum concentration occurring at week 10 (35.6 µg/g) (Table, 2.2 ; Fig, 2.2.a).

b. Small intestine

Proximal

MT concentrations in the copper supplemented groups (72.7 ± 8.0 µg/g) were raised ($p<0.05$) when compared to the controls (52.8 ± 1.0 µg/g) with peak concentration occurring at week 10 (90.9 µg/g) (Table, 2.2 ; Fig, 2.2.b).

Middle

MT contents in the copper supplemented groups (96.6 ± 8.2 µg/g) were elevated ($p<0.05$) when compared to the controls (57.1 ± 0.8 µg/g) with the greatest rise occurring in the first 5 weeks (110.6 ± 0.4 µg/g) and subsequently reduced by week 16 (86.3 µg/g) (Table, 2.2 ; Fig, 2.2.c).

Distal

MT concentrations in the copper supplemented groups (68.2 ± 8.5 µg/g) were slightly increased, but statistically insignificant ($p>0.05$), when compared to the controls (55.2 ± 1.0 µg/g) with the greatest rise occurring in the first 5 weeks (81.8 ± 12.4 µg/g), but was reduced thereafter to the normal levels (54.9 ± 1.2 µg/g) (Table, 2.2 ; Fig 2.2.d).

c. Caecum

MT concentrations in the copper supplemented groups (17.4 ± 3.0 µg/g) were increased ($p<0.05$) when compared to the controls (9.9 ± 0.8 µg/g) with the

greatest rise occurring in the first 5 weeks (22.1 ± 2.7 μg/g), decreasing by week 16 (12.6 μg/g) (Table, 2.2 ; Fig, 2.2.e).

d. Colon

MT concentrations in both the copper supplemented (20.1 ± 1.1 μg/g) and control groups (19.1 ± 0.1 μg/g) remained unchanged throughout the trial, although in the former group mild elevation was observed at week 5 (23.1 μg/g) (Table, 2.2 ; Fig, 2.2.f).

2.3.3. Chromatographic separation of the gastrointestinal soluble fractions

The distribution of zinc (copper) and absorbance of the eluant fractions from the stomach, proximal, middle, and distal small intestinal subdivisions, caecum and colon of control and copper supplemented groups are as shown in figures 2.3, 2.4, 2.5, 2.6, 2.7 and 2.8 respectively.

a. Control

A similar pattern of absorbance and zinc (copper) distribution was observed in all gastrointestinal soluble fractions throughout the trial. Absorbance was high in HMW protein region and the peak reduced constantly and remained low in LMW protein (MT) band. Zinc was isolated at HMW band, but not of the LMW band, whilst copper was not detected either in the HMW or LMW region.

b. Copper supplementation

The distribution of zinc (copper) and absorbance of the gastrointestinal soluble fractions remained unchanged in copper supplemented groups throughout the trial from those observed in the controls. Absorbance was high in HMW band and the peak reduced thereafter and remained low in LMW band. Zinc was only isolated in the HMW region, whilst copper was not detected either in the HMW or LMW band.

2.3.4. Immunocytochemistry

a. Stomach

The distribution and intensity of immunoreactivity in copper supplemented groups remained unchanged compared to the controls throughout the trial. Intense reticulate staining was observed in the cytoplasm of the peptic cells, whilst a variable diffuse reaction was present in the cytoplasm of the parietal cells. The mucous secreting cells and the nuclei of gastric mucosal cell populations remained unstained (Figs, 2.9a, b).

b. Small intestine

Proximal

Villus epithelial cells remained unstained in the control groups, whilst the cytoplasm of the Paneth cells at the base of the crypt was postively stained (Fig, 2.10.a). In the copper supplemented groups moderate to marked intracytoplasmic reticulate and diffuse immunostaining of the enterocytes occupying the lower third of the villi were observed at week 1 which extended to involve the lower half of the villi by week 5 (Figs, 2.10b, c). However, the nuclei of the enterocytes within this area were only mildly immunoreactive or remained unstained (Fig, 2.13 a). Immunoreactivity was reduced to slightly above normal at week 16 (Fig, 2.10d). The Paneth cells were always markedly stained throughout the trial, compared to those of the controls.

Middle

A mild reticulate and diffuse intracytoplasmic staining was observed in the enterocytes occupying the base of the villi of the controls (Fig, 2.11a). In the copper supplementation groups moderate to marked intracytoplasmic staining of the enterocytes extending upto involve half of the villus were observed at at week 1 and 5 (Figs, 2.11b, c). The staining intensity was reduced to about the control at week

16 (Fig, 2.11d). Paneth cells remained more intensely stained in the copper supplemented groups than the controls.

Distal

The villus epithelial cells of the controls remained unstained, whilst Paneth cells were moderately stained. An intense reticulate pattern superimposed on a mild diffuse intracytopasmic staining of the enterocytes was observed within the lower villus extending to half at week 5, which had regressed by week 16 to about the controls (Figs, 2.12a, b, c, d). Intense staining was marked in the cytoplasm of the Paneth cells of copper supplemented groups (Fig, 2.13b).

c. Caecum

The distribution and intensity of immunoreactivity in the copper supplemented groups remained unchanged when compared to the controls throughout the trial. Intense staining was observed in both the cytoplasm and nuclei of the enterocytes occupying within the middle and upper regions of the mucosa (Fig, 2.14a).

d. Colon

Intense immunostaining was observed in the cytoplasm and nuclei of the enterocytes occupying within the upper half of the mucosa in both the controls and copper supplemented animals (Fig, 2.14b).

Table 2.0

Copper concentration in the soluble fractions of gastrointestinal tract of the control and copper supplemented rats

Time (weeks)	Copper concentration in the soluble fractions of gastrointestinal tract (µg/g wet weight)					
	Stomach	Small intestine			Caecum	Colon
		Proximal	Middle	Distal		
			Control			
1	-	0.50	0.35	0.40	0.60	0.60
8	0.45	0.40	0.30	0.45	0.65	0.60
15	0.45	0.45	0.30	0.40	0.56	0.65
Mean	0.45	0.45	0.32	0.42	0.60	0.62
± SEM	0.00	0.03	0.02	0.02	0.03	0.02
			Copper supplementation			
1	0.65	0.80	0.60	0.80	1.52	0.90
5	0.65	0.90	0.90	1.50	1.40	1.10
10	0.65	0.95	0.65	0.95	1.10	0.85
16	0.60	0.70	0.50	0.60	0.96	0.80
Mean	0.64*	0.84*	0.66*	0.96*	1.25*	0.91*
± SEM	0.02	0.06	0.09	0.20	0.13	0.07

Values are the mean of pooled samples of 4 rats. (*) $p<0.05$. (-) sample missing.

Table 2.1

Zinc concentration in the soluble fractions of gastrointestinal tract of the control and copper supplemented rats

Time (weeks)	Zinc concentration in the soluble fractions of gastrointestinal tract (μg/g wet weight)					
	Stomach	Small intestine			Caecum	Colon
		Proximal	Middle	Distal		
Control						
1	-	9.2	6.8	8.4	8.0	8.0
8	6.8	8.0	6.0	11.2	8.0	8.4
15	7.2	7.6	6.0	9.6	8.0	10.0
Mean	7.0	8.3	6.3	9.7	8.0	8.8
± SEM	0.2	0.5	0.3	0.8	0.0	0.6
Copper supplementation						
1	6.8	10.0	11.2	12.4	9.2	8.8
5	6.8	8.4	8.4	13.6	10.4	9.2
10	6.8	8.0	8.8	11.6	8.4	9.2
16	6.8	7.2	7.6	12.0	8.0	8.4
Mean	6.8	8.4	9.0*	12.4*	9.0	8.9
± SEM	0.0	0.6	0.8	0.4	0.5	0.2

Values are the mean of pooled samples of 4 rats. (*) $p<0.05$. (-) sample missing.

Table 2.2

Metallothionein concentration in the soluble fractions of gastrointestinal tract of the control and copper supplemented rats

Time (weeks)	Stomach	Small intestine: Proximal	Small intestine: Middle	Small intestine: Distal	Caecum	Colon
	Metallothionein concentration in the soluble fractions of gastrointestinal tract (µg/g wet weight)					
			Control			
1	-	53.2	56.3	56.9	11.3	19.1
8	8.6	54.2	56.6	53.5	8.9	19.0
15	10.6	50.9	58.4	55.1	9.5	19.2
Mean	9.6	52.8	57.1	55.2	9.9	19.1
± SEM	1.0	1.0	0.8	1.0	0.8	0.1
			Copper supplementation			
1	22.1	55.0	110.3	72.6	24.8	19.8
5	27.4	64.9	110.8	90.2	19.4	23.1
10	35.6	90.9	79.1	56.1	12.7	19.5
16	27.7	79.8	86.3	53.7	12.6	18.1
Mean	28.2*	72.7*	96.6*	68.2	17.4*	20.1
± SEM	2.8	8.0	8.2	8.5	3.0	1.1

Values are the mean of pooled samples of 4 rats. (*) $p<0.05$. (-) sample missing.

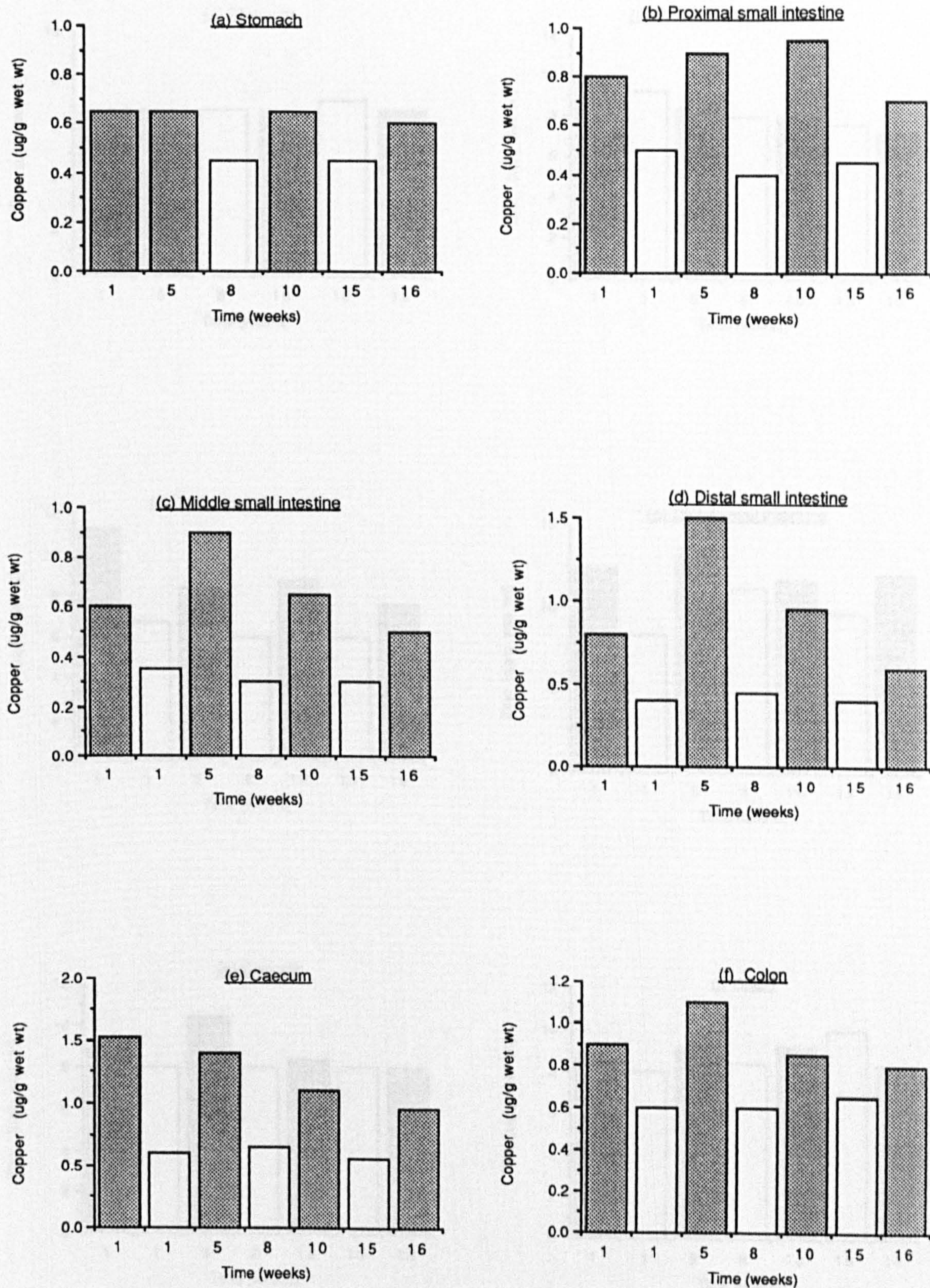


Fig. 2.0. **Copper concentration.** Gastrointestinal soluble fraction of the control ( ) and copper supplemented ( ) rats.

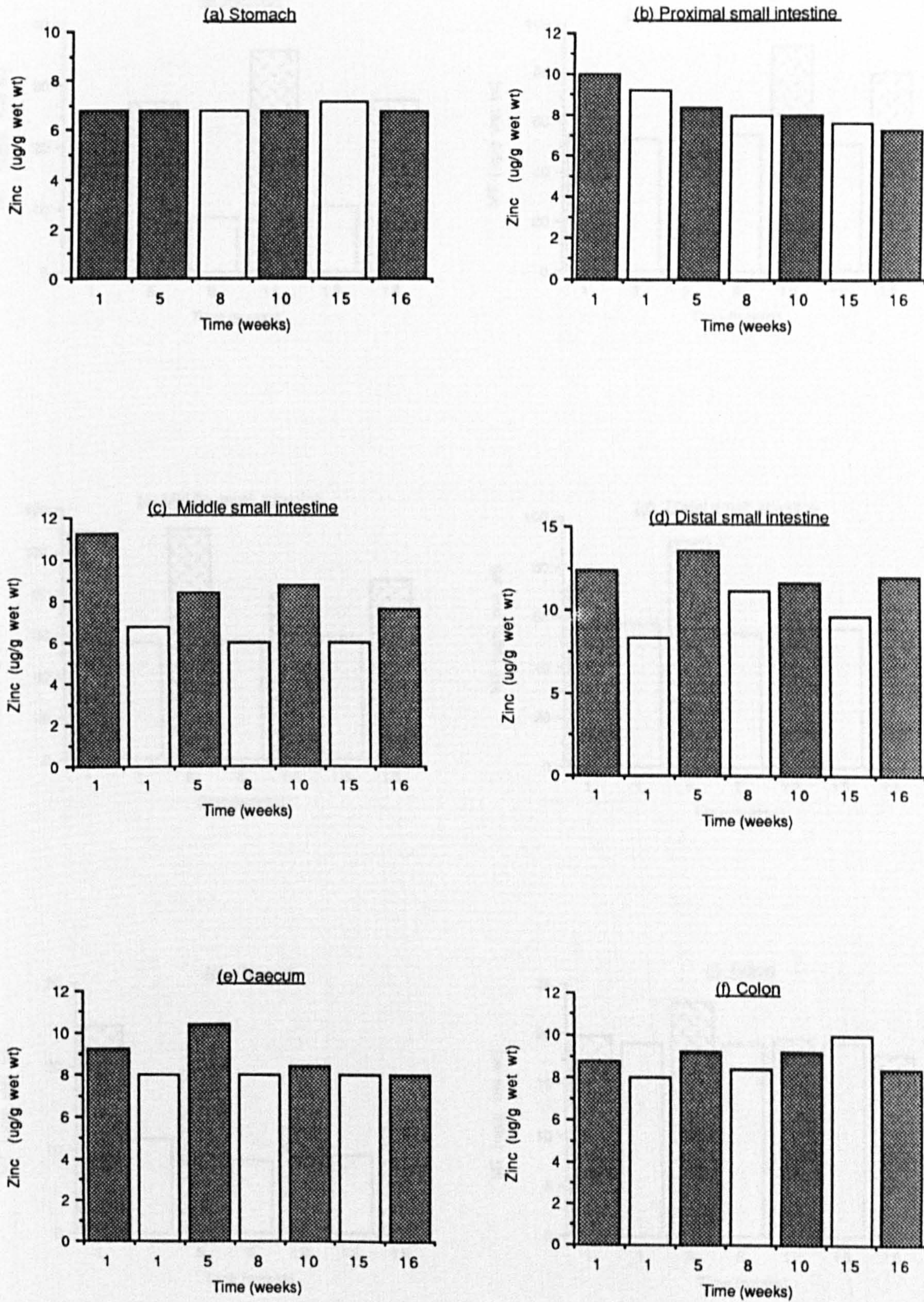


Fig. 2.1. **Zinc concentration.** Gastrointestinal soluble fraction of the control ( ) and copper supplemented ( ) rats.

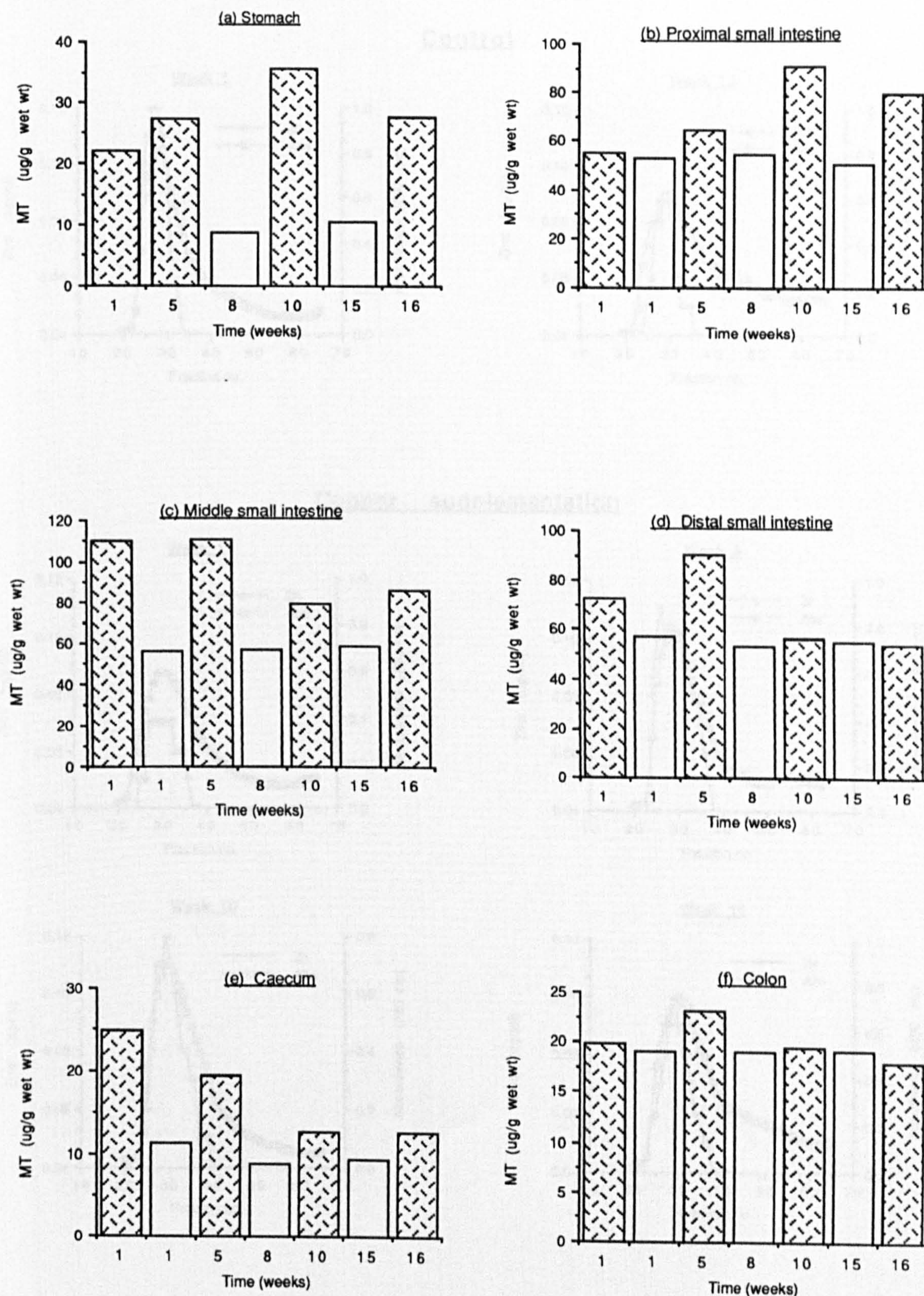
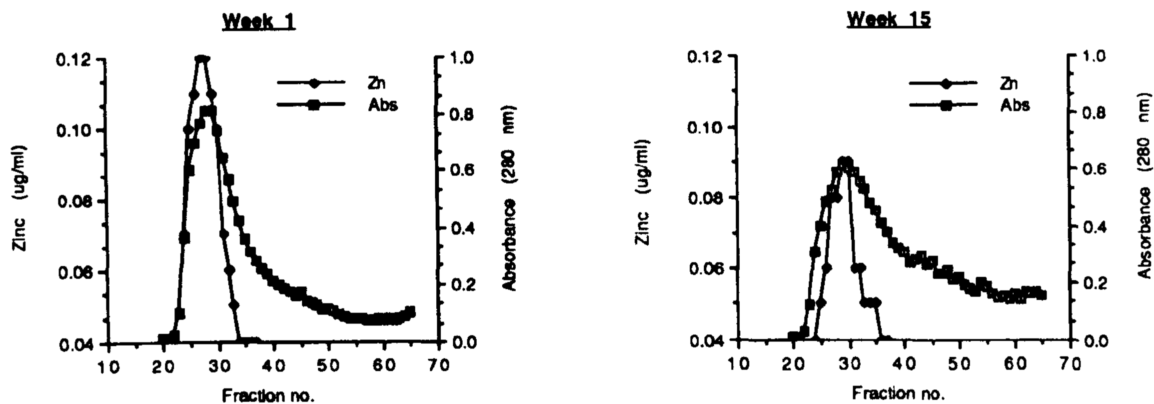


Fig. 2.2. **Metallothionein concentration.** Gastrointestinal soluble fraction of the control (☐) and copper supplemented (▨) rats.

Control



Copper supplementation

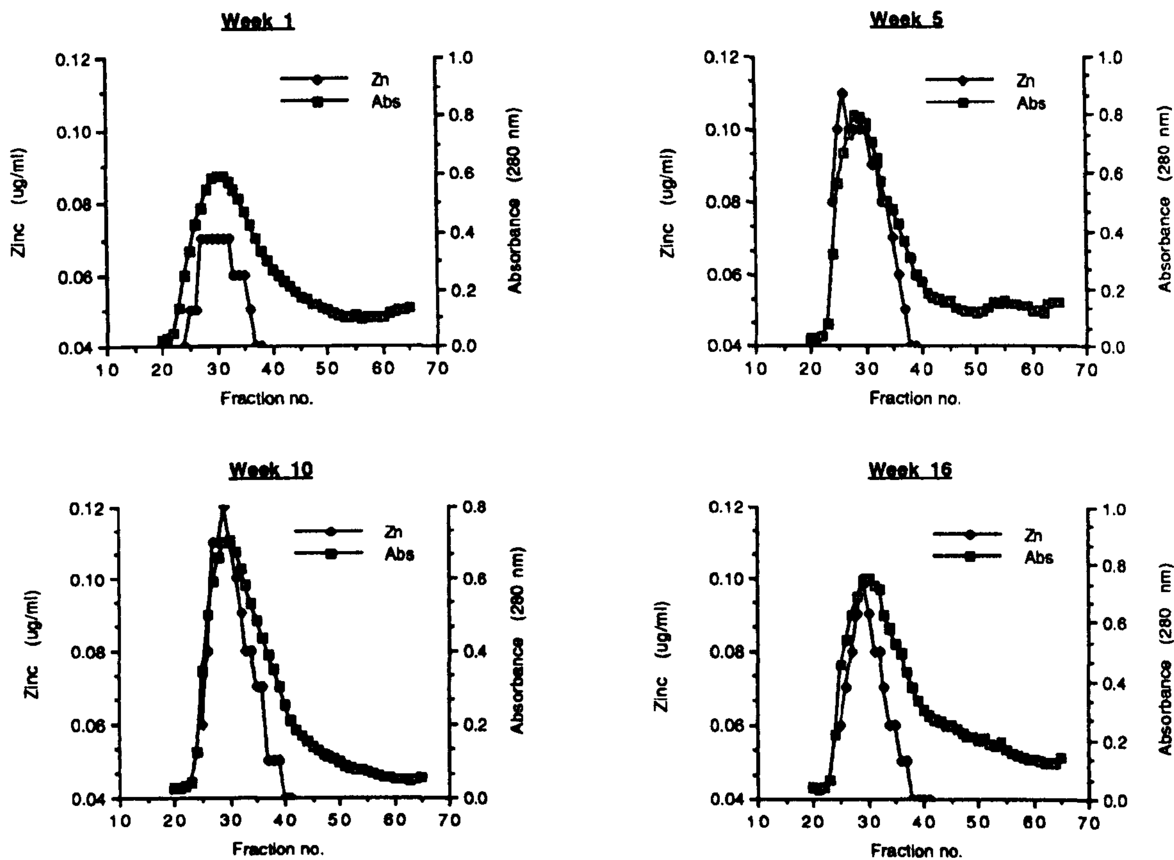
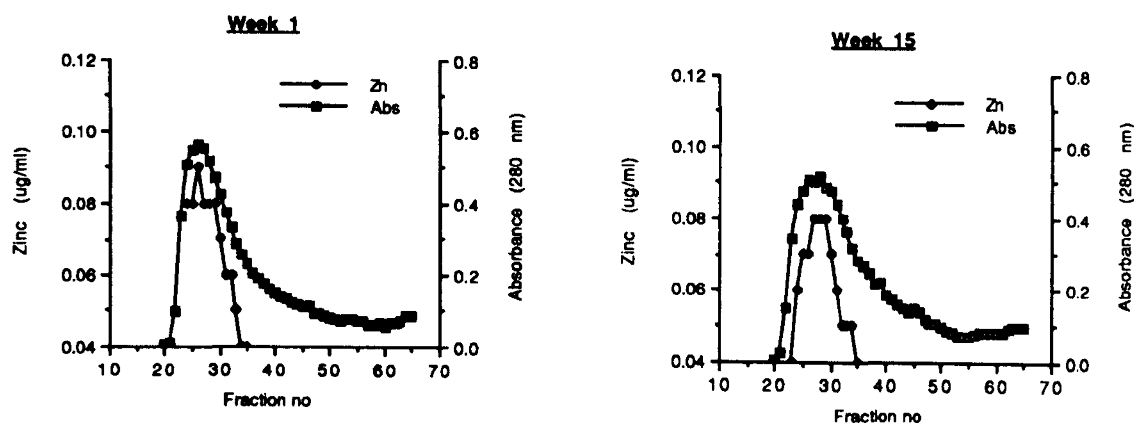


Fig. 2.3. **Stomach.** Zinc (copper) distribution in the soluble fraction.

Control



Copper supplementation

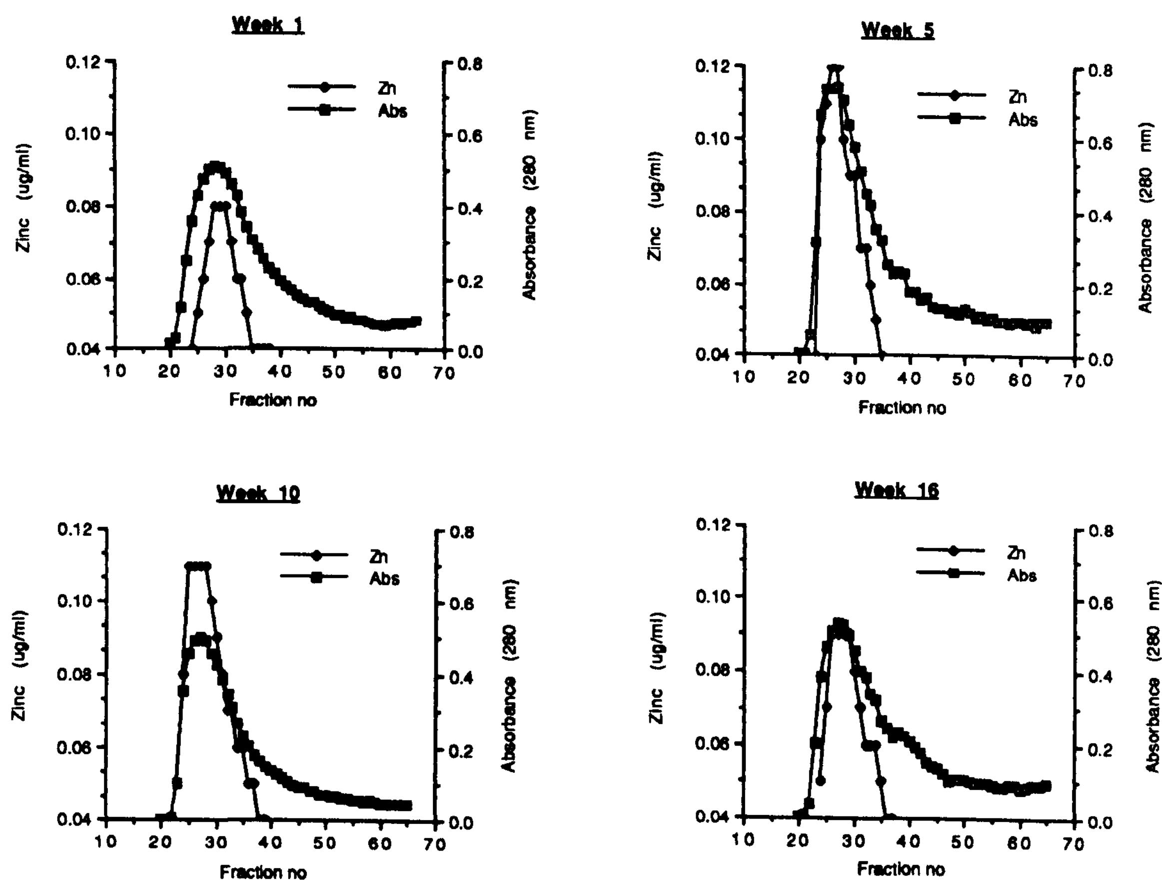
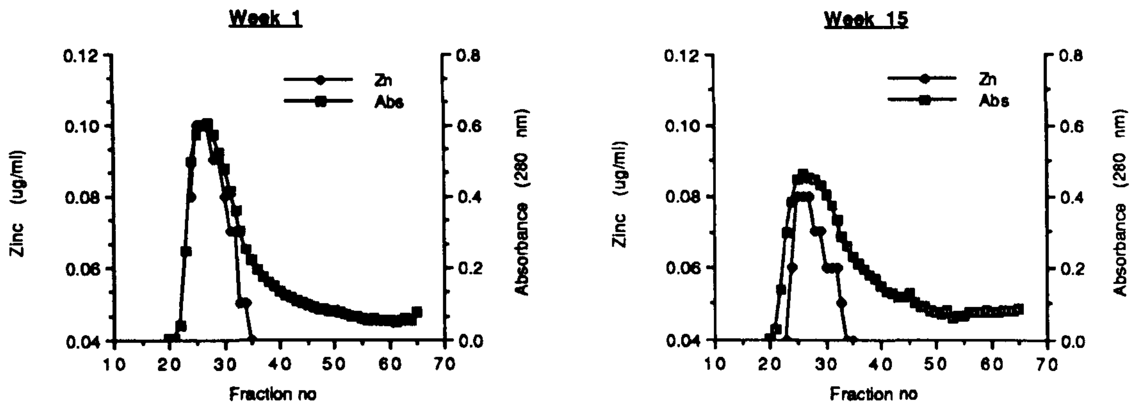


Fig. 2.4. **Proximal small intestine.** Zinc (copper) distribution in the soluble fraction.

Control



Copper supplementation

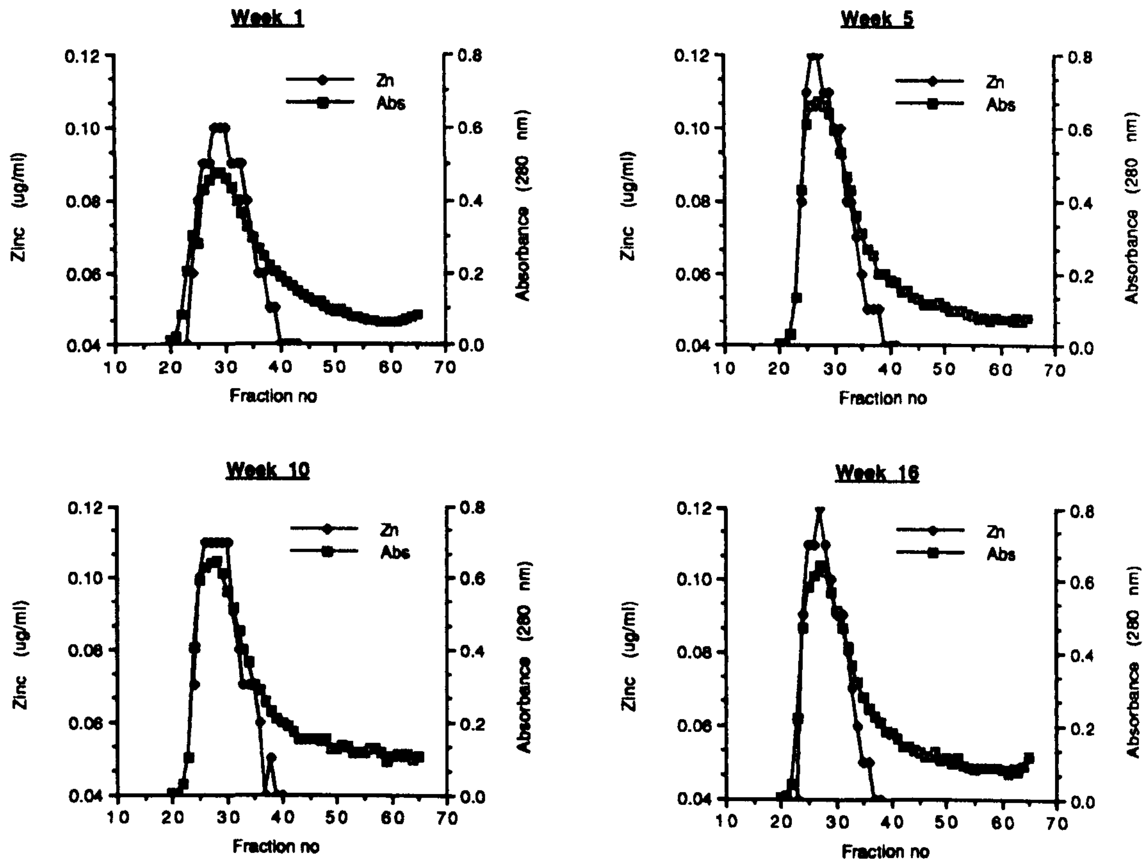
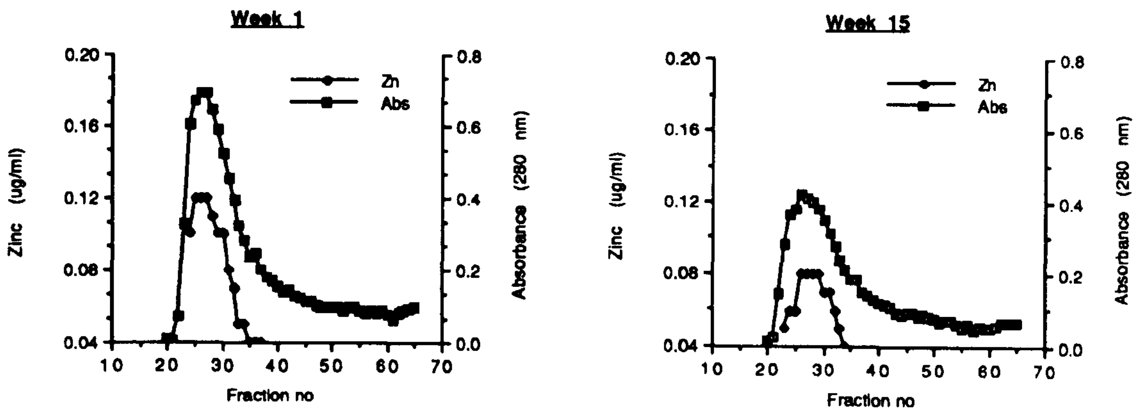


Fig. 2.5. **Middle small intestine.** Zinc (copper) distribution in the soluble fraction.

Control



Copper supplementation

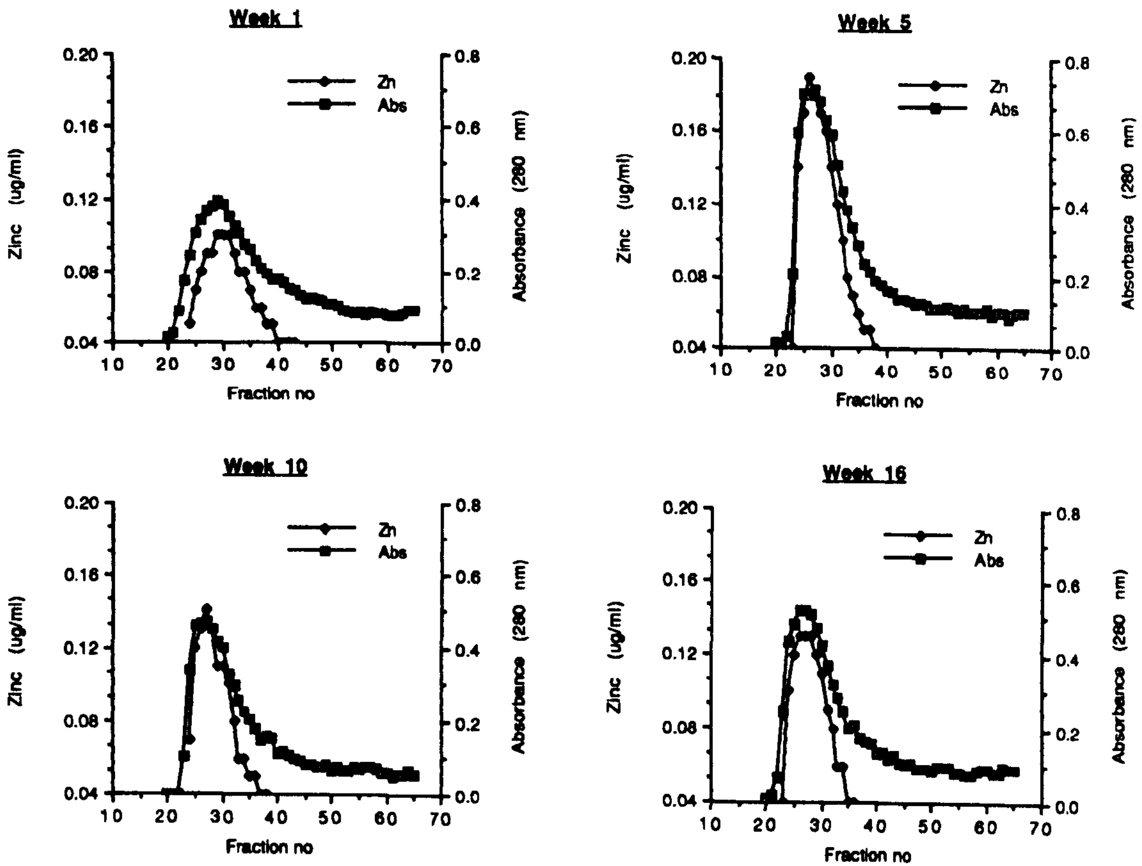
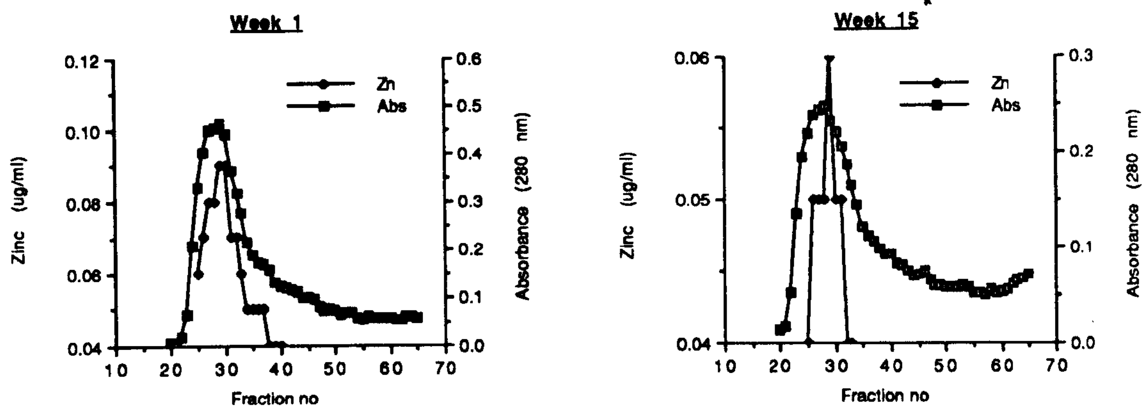


Fig. 2.6. **Distal small intestine.** Zinc (copper) distribution in the soluble fraction.



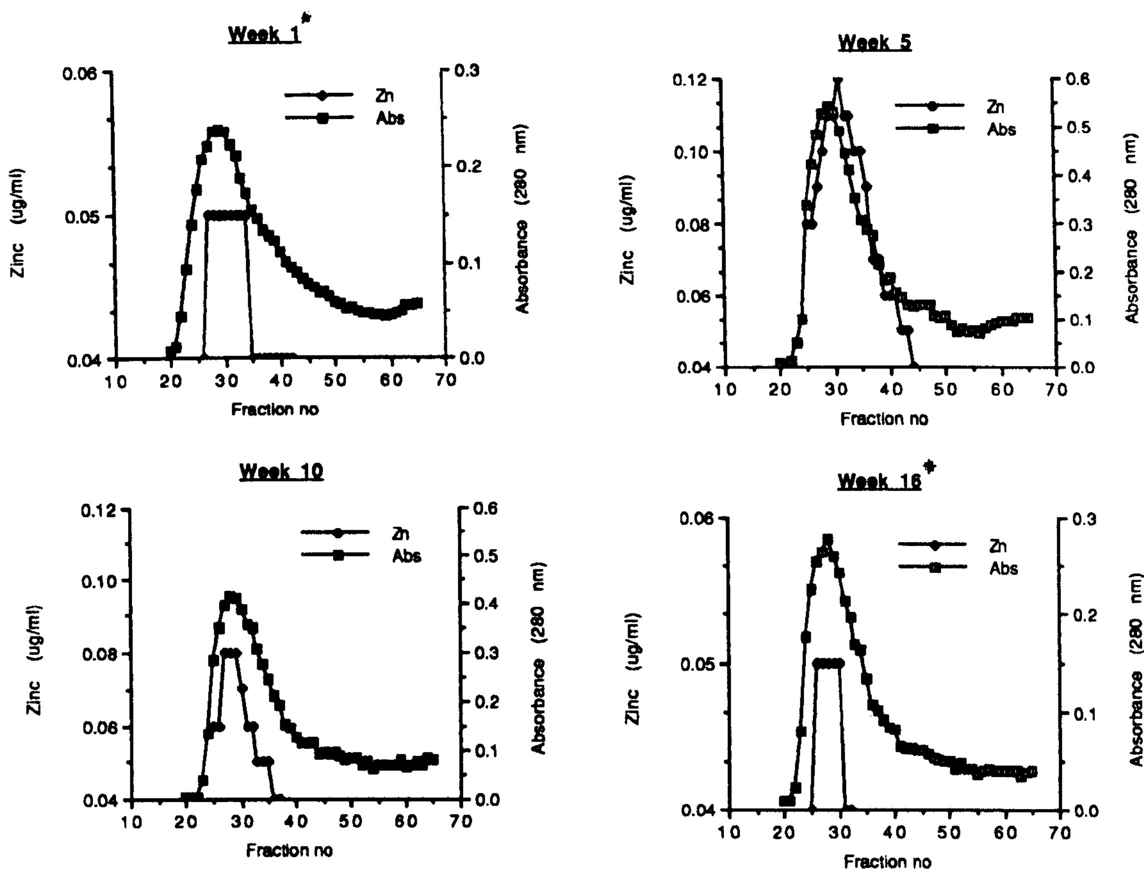
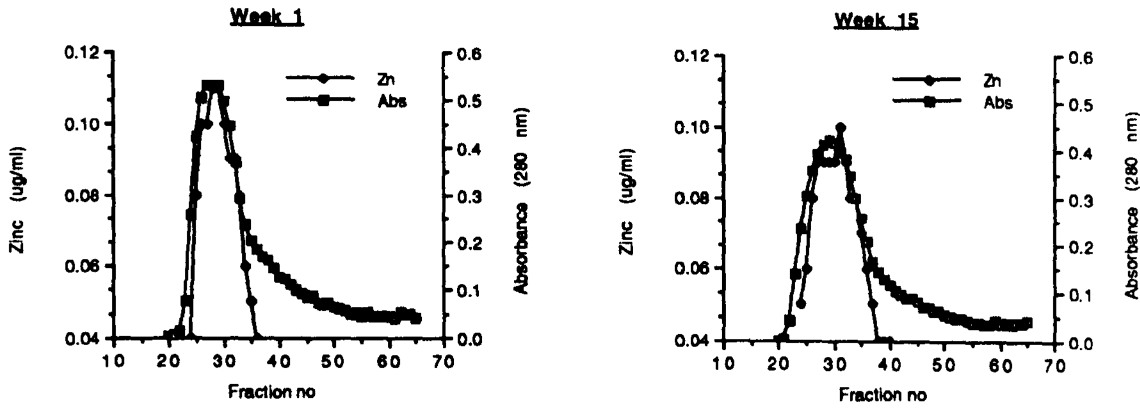


Fig. 2.7. **Caecum.** Zinc (copper) distribution in the soluble fraction.
(*) Diluted in 7 vol (w/v).

Control



Copper supplementation

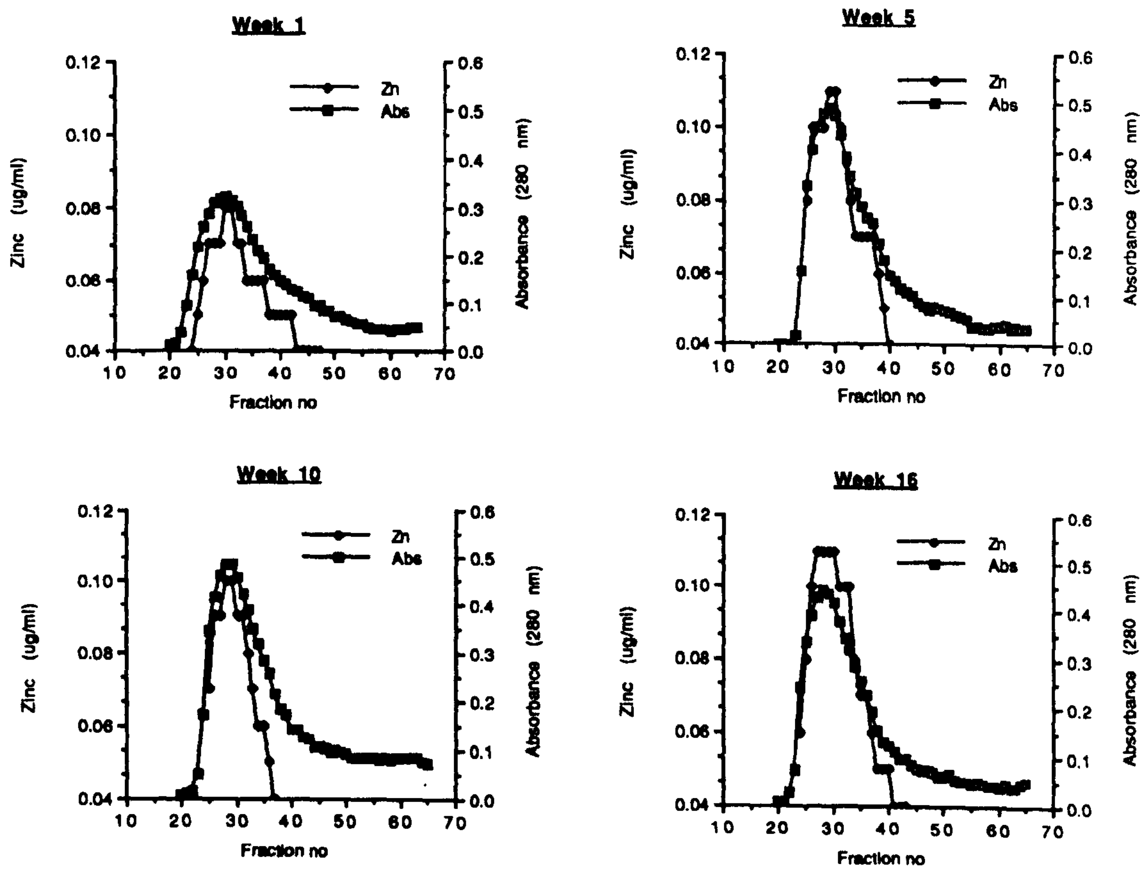
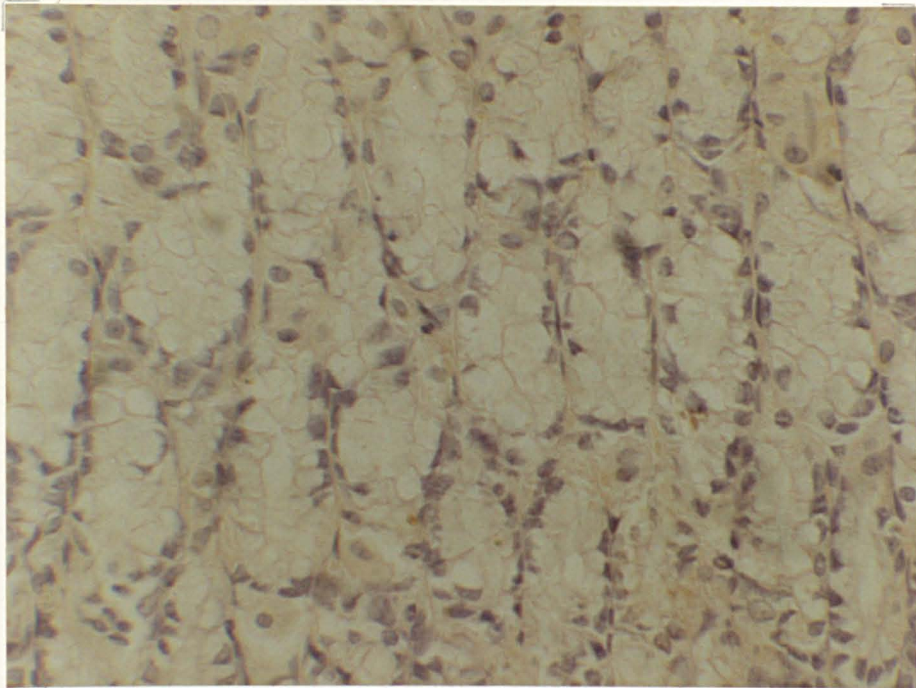
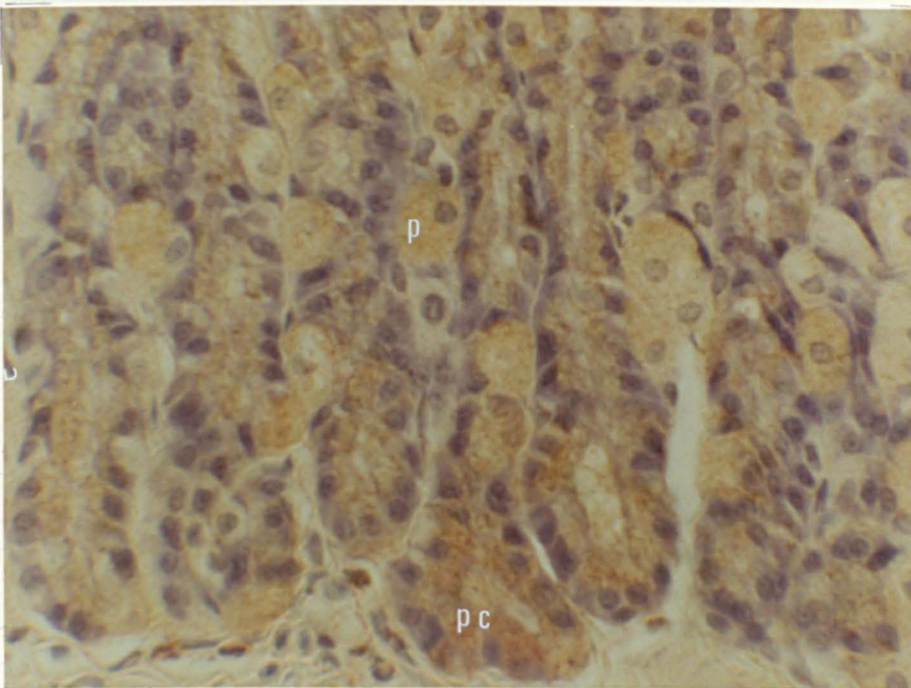


Fig. 2.8. **Colon.** Zinc (copper) distribution in the soluble fraction.

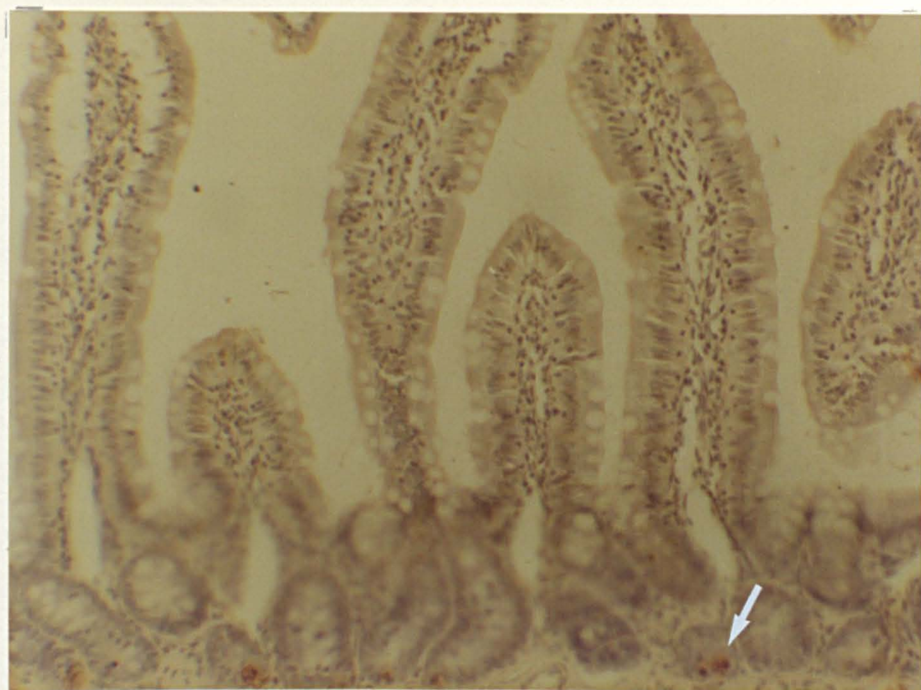


(a)

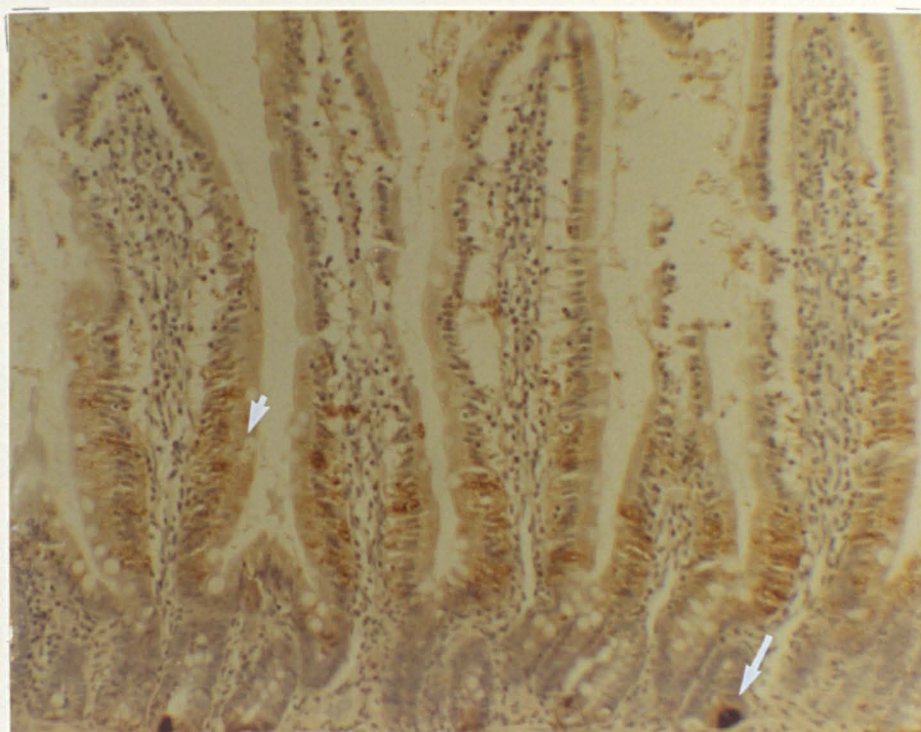


(b)

Fig. 2.9. **Gastric mucosa.** (a) **Copper supplementation.** Week 3. Mucous secreting cell remained unstained (X 310) (b) **Control.** Parietal (p) and peptic (pc) cells showing positively staining cytoplasm (X 400). DNP-peroxidase.

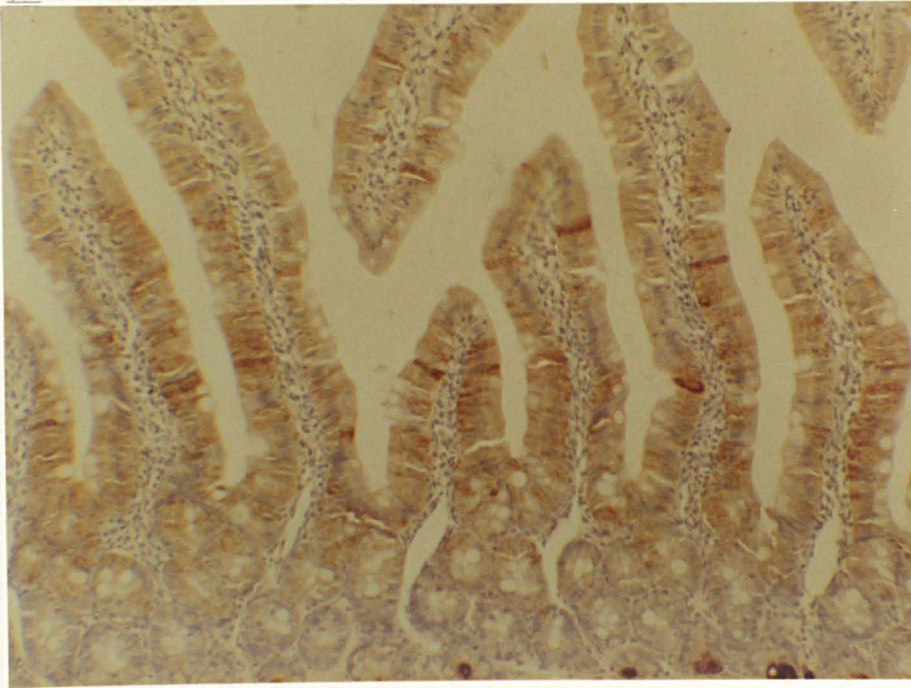


(a)

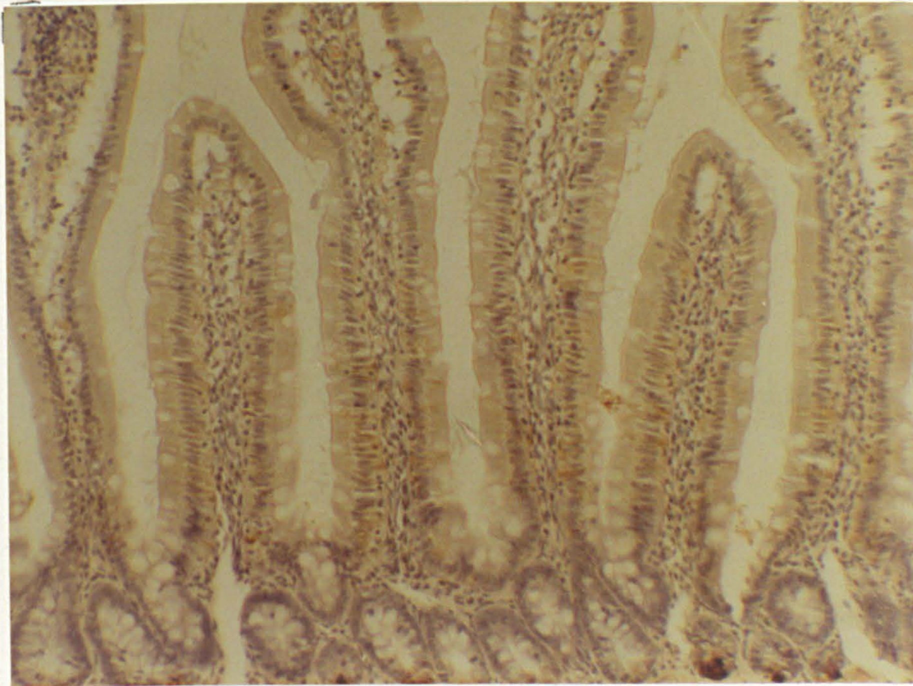


(b)

Fig. 2.10. **Proximal small intestine.** (a) **Control.** Paneth cells were moderately stained (X 125) (arrow) (b) **Copper supplementation. Week 1.** Enterocyte cytoplasm occupying the lower third of the villi and the Paneth cells were moderate to intensely stained (arrow) (X 100). DNP-peroxidase.

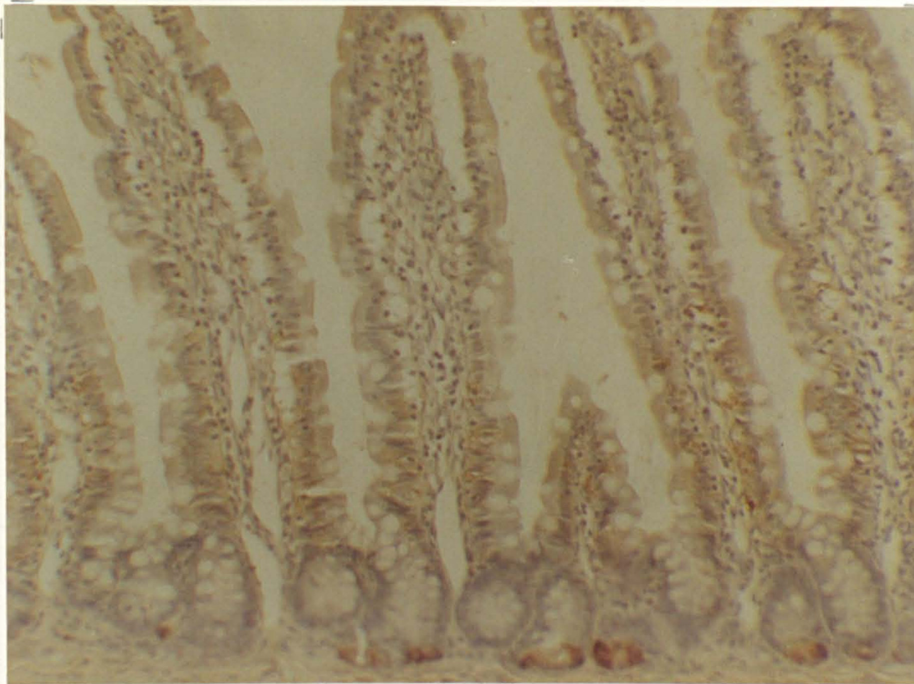


(c)

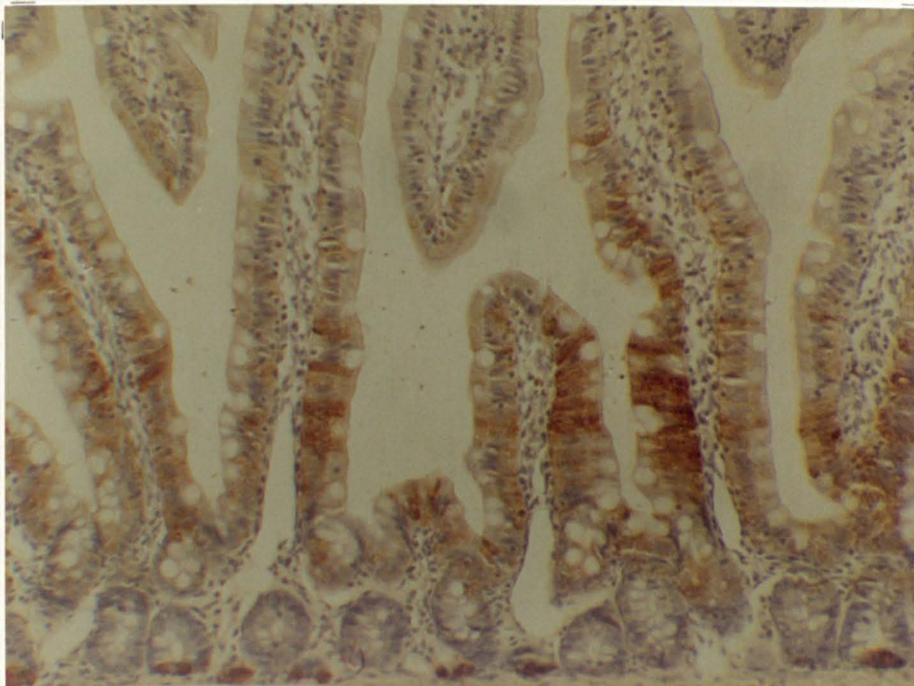


(d)

Fig. 2.10. **Proximal small intestine. Copper supplementation.** (c) **Week 5.** Immunostaining of the enterocyte cytoplasm extended to involve the lower half of the villi, but reduced to about control by (d) **Week 16.** Paneth cells remained intensely stained. DNP-peroxidase (X125).

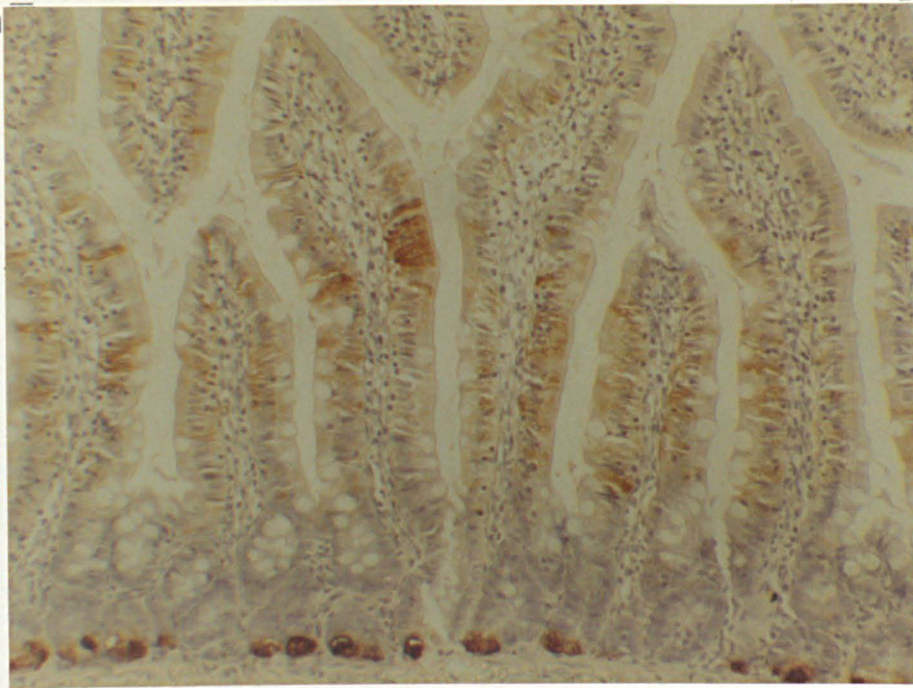


(a)

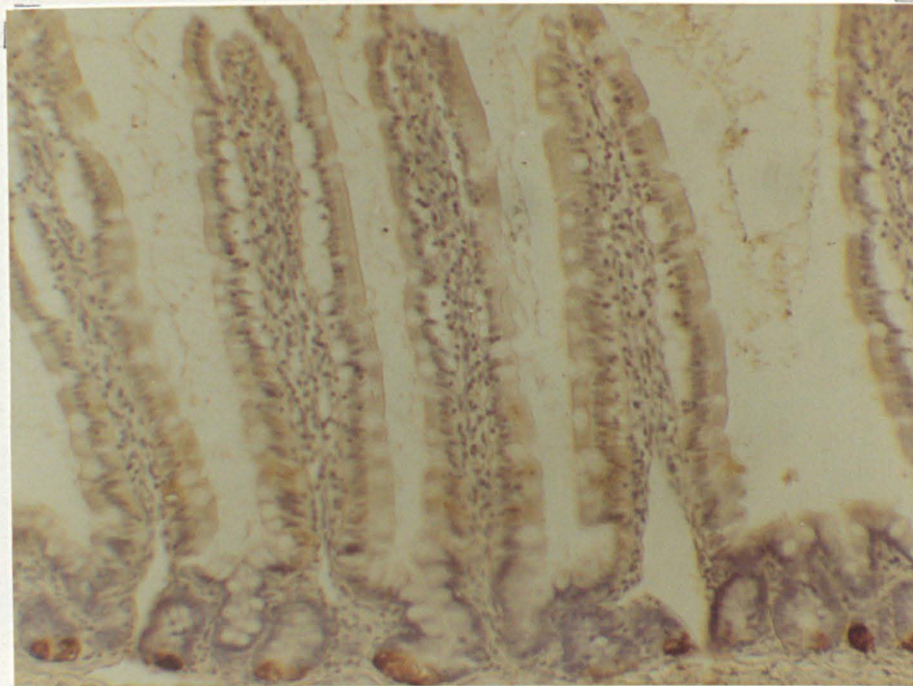


(b)

Fig. 2.11. **Middle small intestine.** (a) **Control.** Paneth cells were moderately stained. (b) **Copper supplementation. Week 1.** Intracytoplasmic staining of the enterocytes extended upto involve half of the villus. Paneth cells were intensely stained. DNP-peroxidase (X 125).

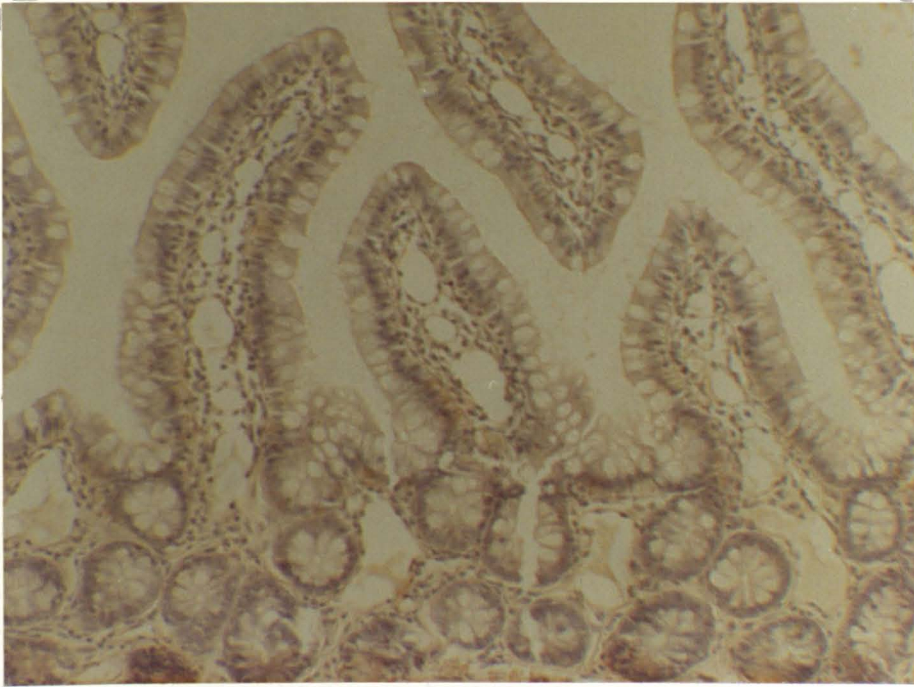


(c)

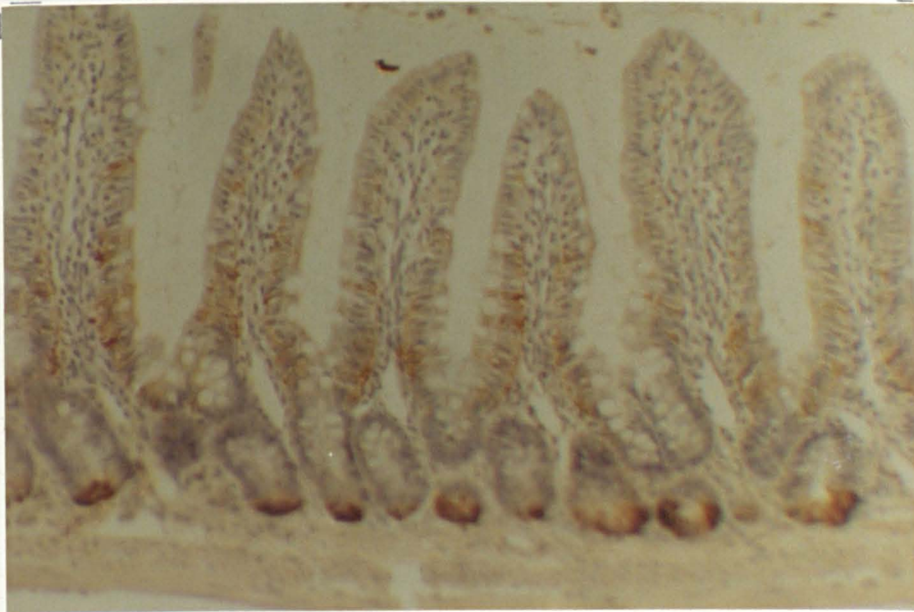


(d)

Fig. 2.11. **Middle small intestine. Copper supplementation.** (c) **Week 5.** Intracytoplasmic staining of the enterocytes extended upto involve half of the villus, but regressed to about control by (d) **Week 16.** Paneth cells remained intensely stained. DNP-peroxidase (X 125).

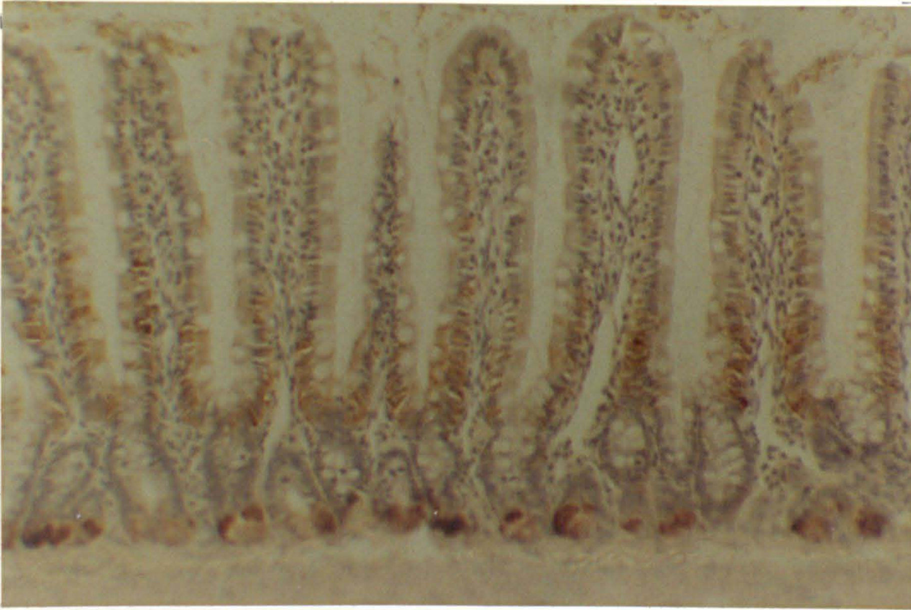


(a)



(b)

Fig. 2.12. **Distal small intestine.** (a) **Control**. Paneth cells were moderately stained. (b) **Copper supplementation. Week 1.** Intracytoplasmic staining of the enterocytes extended within the lower half of the villi. DNP-peroxidase (X 125).



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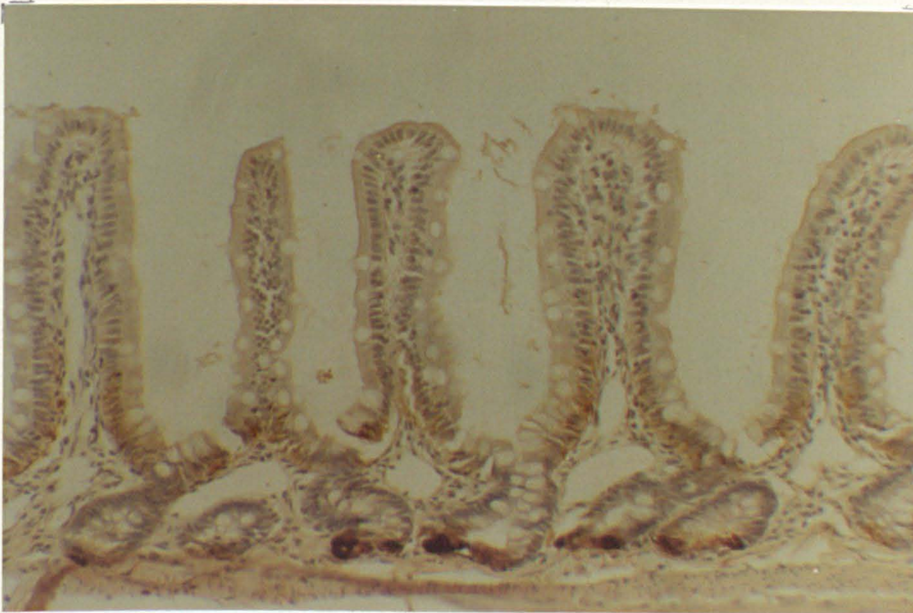
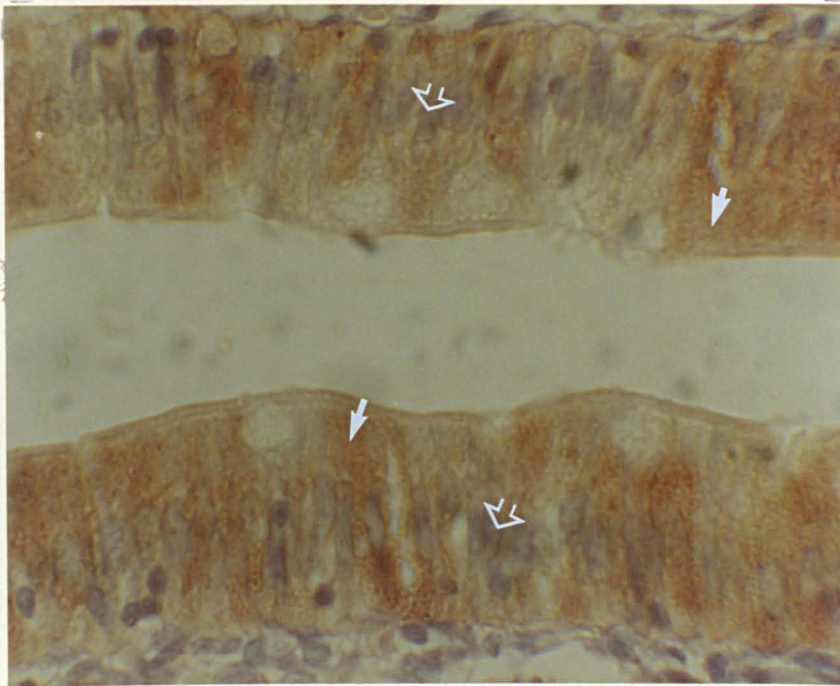
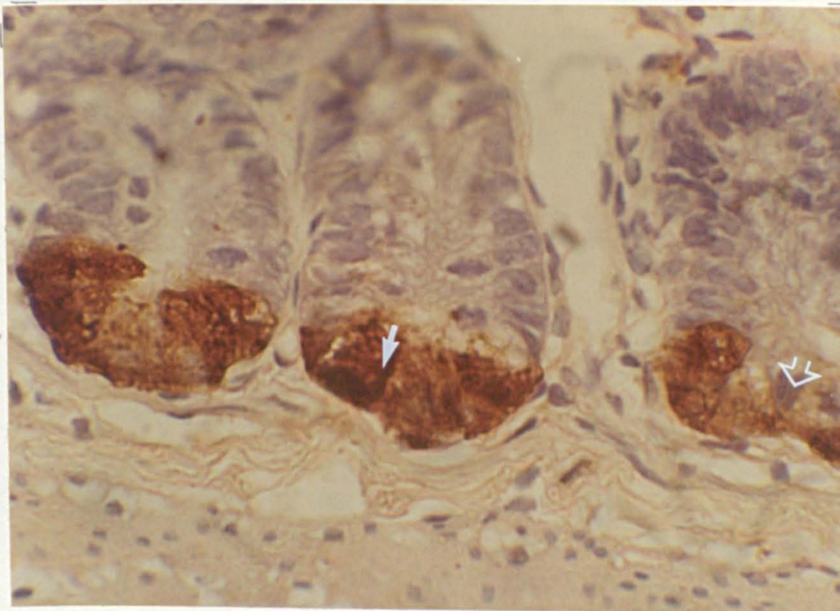


(d)

Fig. 2.12. **Distal small intestine. Copper supplementation.** (c) **Week 5.** Intracytoplasmic staining of the enterocytes extended to involve the lower half of the villi, but reduced to about control by (d) **Week 16.** Paneth cells remained intensely stained. DNP-peroxidase (X 125).

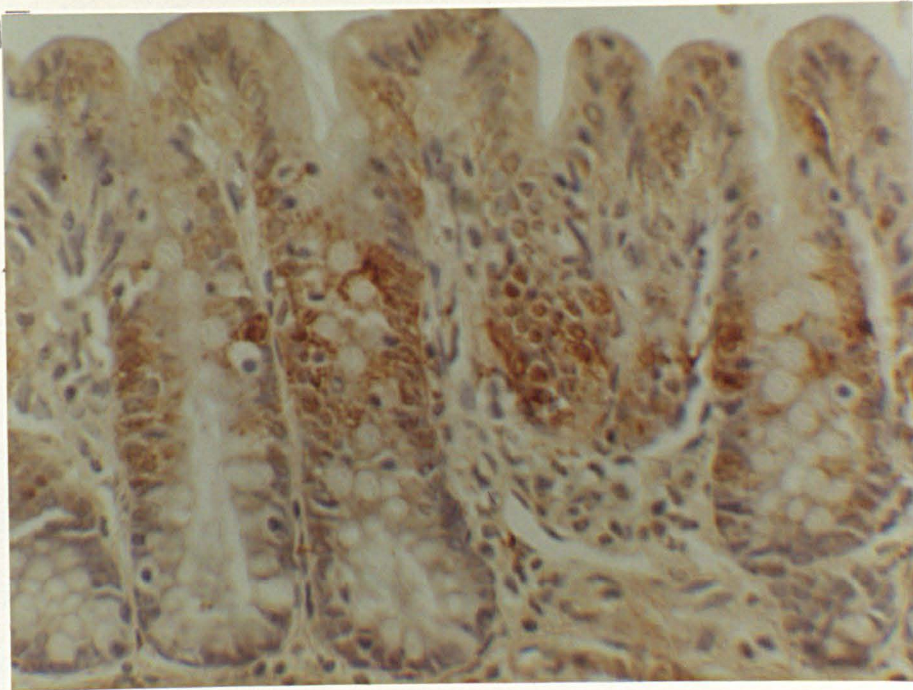


(a)

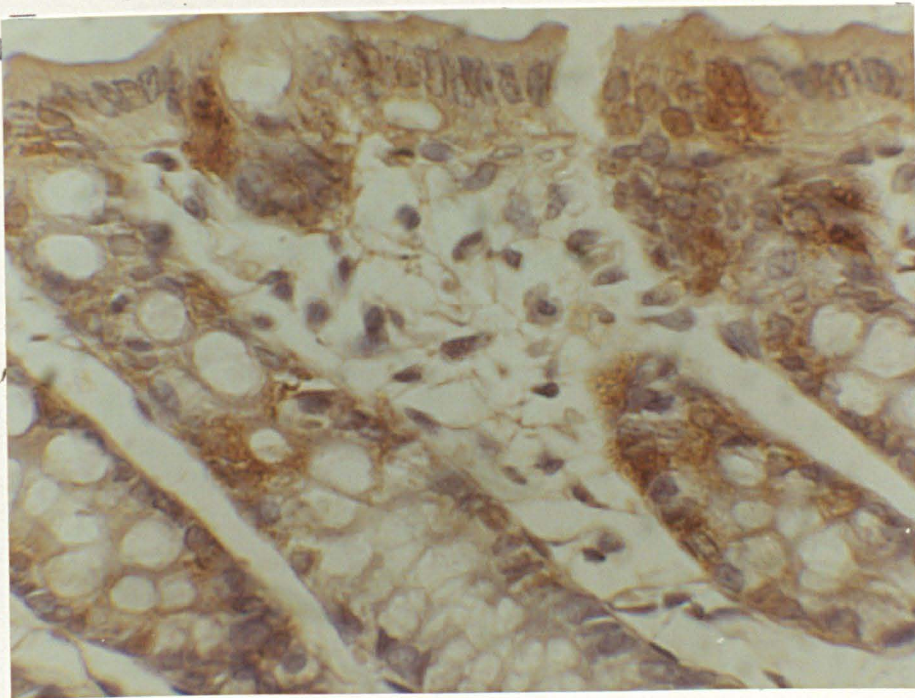


(b)

Fig. 2.13. (a) **Copper supplementation. Week 5.** Proximal small intestine. Base of the villi. Intense reticulate and diffuse staining occupying the enterocyte cytoplasm (arrow). The nuclei remained unstained. (b) **Paneth cells.** Distal small intestine. Intense reticulate and diffuse staining occupying the cytoplasm. The nuclei remained unstained. DNP-peroxidase (X 500).



(a)



(b)

Fig. 2.14. (a) **Caecum.** Control. Intense reticulate and diffuse staining in the cytoplasm and nuclei within the middle and upper regions of the mucosa. (b) **Colon.** Control. Intense reticulate and diffuse staining occupying in the cytoplasm and nuclei at the upper half of the mucosa. DNP-peroxidase (X310).

2.4. DISCUSSION

The pattern of response of gastrointestinal MT and copper in copper supplemented rats, elevated initially and declining with time, in the present study is similar to that occurring in both the intestinal tract and liver of newborn rats (Johnson and Evans, 1980 ; Hurley et al., 1980 ; Mason et al., 1981). It is also similar to that of the rat liver during the development of copper tolerance (Evering, 1989). However, the changes in both the gastrointestinal copper and MT in the present study were quantitatively much less and this might explain the failure to identify MT chromatographically. The ability to identify MT using immunocytochemical techniques, compared with the failure of histochemical methods for copper (Chapter, 1) confirmed the sensitivity of the former technique over the latter for the identification of copper, as reported previously in the liver (Elmes et al., 1989 ; Evering et al., 1990). Immunoreactive stains for MT seem to correspond better with the analytical measurement of copper content (Chapter, 1) in the intestinal subdivisions.

The elevation of zinc concentration in soluble fractions of the middle and distal small intestine may suggest some intracellular redistribution of the metal since the whole tissue zinc concentration remained unchanged (Chapter, 1) and may influence the induction of MT. However, the association between elevated copper and MT is far more constant suggesting that MT synthesis in the gastrointestinal tract of copper supplemented rats in the present study is much more likely induced by the copper than the zinc. This agrees with the previous reports concerning the ability of copper to induce intestinal MT either from in vitro (Scarino et al, 1988) or in vivo studies (Evans, 1979 ; Hall et al., 1979 ; Blalock et al., 1988).

It is interesting to find that the immunoreactivity within the enterocytes in the intestinal subdivisions was located mainly within the lower third of the villi, suggesting a concentration of copper within these cells. Reasons for this are not known, although it may indicate a similar phenomenon of high copper concentration in both the small intestine and liver of neonatal animals (Hurley et al., 1980 ;

Johnson and Evans, 1980 ; Mason et al., 1981 ; Dinsdale et al., 1986). The epithelial lining of a villus consists of a continously renewable layer of cells, having its progenitor cells in the crypts, these cells migrate upwards along the side of the villus accompanied by various changes reflecting the onset of functional maturity (Leblond and Stevens, 1948 ; Toner, 1968). The enterocyte population at the base of the villus mainly consists of nonfully functional absorptive cells, whilst those at the middle or dorsal part of the villus are fully developed for absorption. However, as the cells approach the tip of the villus signs of natural aging phenomenona such as changes in the cellular organelles and microvilli occur prior to cellular extrusion into the intestinal lumen (Toner, 1968 ; Phillips et al., 1979).

Intestinal copper concentration in the neonatal rats parallels MT content which increases rapidly following birth, but declines to about the adult levels by the third week (Hurley et al., 1980 ; Johnson and Evans, 1980 ; Mason et al., 1981). These changes seem to be associated with the gradual process of cellular maturation within this period and the ability of neonatal animals to regulate copper uptake by pinocytosis (Mistilis and Mearrick, 1969 ; Williams and Beck, 1969 ; Dinsdale et al., 1986). Furthermore, the time taken for cellular turnover which was about ten fold longer in the neonatal than the adults (2.1 days) (Altmann and Enesco, 1967) could also effect the retention of copper within the organ.

The distribution of immunostaining to MT in the present study suggests that high copper concentration is localized at the base of the villi in nonfully functional absorptive cells which may resemble those of enterocytes in neonatal animals. Although copper is probably taken up by enterocytes throughout the length of the villus, it may be retained longer in the less mature enterocytes possibly due to a defect or undeveloped mechanisms that may involve the transfer of copper to the portal circulation with synthesis of intestinal MT (Hurley et al., 1980). Evidence for increased cell immaturity as a consequence of rapid turnover was identified earlier (Chapter, 1) and may act as a temporary barrier to absorption. Persistence

of enterocyte immaturity seems to occur with excess intestinal copper retention in Menkes' disease patients and brindled mice (Holtzman, 1976 ; Bremner, 1987a).

The failure to maintain mucosal copper and MT elevation in rats at week 16 suggests that copper tolerance in rats does not ultimately depend on a mucosal barrier to copper absorption as may occur in Menkes' disease patients and brindled mice (Danks et al., 1973 ; Evans and Reis, 1978 ; Leone et al., 1985) or zinc (Hall et al., 1979 ; Fischer et al., 1981, 1983) and cadmium supplemented animals (Davies and Campbell, 1977). MT may rather represent a temporary binding of copper in transport across the enterocyte.

The localization of immunoreactivity to MT in the Paneth cells in both copper supplemented and control rats suggests that Paneth cells may play an important role in metal metabolism. It has been argued that Paneth cells contribute to the elimination of zinc, lead, mercury, calcium and colbalt (Sandow and Whitehead, 1979) and thus, copper may also be excreted through this route.

The absence of obvious differences in the staining of immunoreactive MT within the stomach, caecum and colon in both the control and copper supplemented groups is in accordance with the lower levels of MT in these organs when compared to the intestine. MT content in the soluble fraction of the gastrointestinal subdivisions varies in the normal animals with a 3 to 6 fold concentration in the intestine compared with the stomach, caecum and colon, although the protein was elevated proportionately during copper supplementation.

It is interesting to find that MT immunostaining was mainly localized within the cytoplasm of the intestinal enterocytes, Paneth cells and gastric peptic and parietal cells, but intense staining was demonstrated in both the cytoplasm and nuclei of the enterocytes in the caecum and colon. Absence of nuclear MT staining within the enterocyte has been reported previously (Danielson et al., 1982), although positive staining has also been observed (Clarkson et al., 1985). Both cytoplasmic and nuclear MT immunoreactivity have been demonstrated in the hepatic and renal tubular cells (Banerjee et al., 1982 ; Evering et al., 1990).

In conclusion the evidence from this study suggests that copper induced MT in the gastrointestinal tract constitutes neither a barrier to copper absorption nor does it facilitate this process. Rather copper bound to MT appears to represent a temporary binding of the metal in transport across the absorptive cell.

CHAPTER : 3

Gastrointestinal Response to Copper Excess: Ultrastractural Changes and Metal Localisation

3.1. INTRODUCTION

Copper associated disease is well recognised in both man and animals. It may be genetic in origin as in Wilson's disease in man (Scheinberg and Sternlieb, 1976) and Bedlington terriers (Twedt et al., 1979) or acquired as in sheep (Soli, 1980). Paradoxically, rats can adapt to copper excess and became tolerant (Haywood, 1980, 1985). Reasons for this are still incompletely understood, although it has been demonstrated that copper overload can be reduced by biliary and urinary excretion of excess copper (Evering, 1989). Furthermore, adaptation also seems to be associated with the gastrointestinal changes in response to the metal which may serve to inhibit copper absorption or facilitate excretion (Chapter, 1).

The gastrointestinal response to copper excess has not been extensively studied, although in copper poisoned pigs (Allen and Harding, 1962 ; Hatch et al., 1979 ; Higgins, 1981), lambs (Sholl, 1957) and man (Chuttani et al., 1965) changes mainly occured at the proximal part of the tract, particularly the stomach, the site from which copper appears to be mainly absorbed (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984). Likewise, histological changes were most marked in the gastric mucosa of rats in parallel with the copper content on the organ (Chapter, 1). An elevation of mural gastric copper during the first 5 weeks of copper supplementation was associated with cell death (apoptosis) and parietal cell deletion and was counterbalanced by mucous cell hyperplasia and increased mitotic activity. As the copper content fell thereafter to about the normal level at week 16, apoptosis was rarely observed, whilst mucous cell hyperplasia and mitotic activity persisted (Chapter, 1). Changes in the remaining intestinal tract were more subtle and less obviously related to changes in

copper content; they included however early villus atrophy (distal small intestine) followed by a rapid cell turnover latterly (Chapter, 1).

Changes in immunoreactive MT were identified in the gastrointestinal tract of copper supplemented rats that closely corresponded to copper retention. It was concluded however that MT was an indicator of copper transport rather than a regulator of copper absorption in the copper loaded rat and probably served to bind copper in a non toxic form (Chapter, 2).

Immunostaining for MT was more intense in the Paneth cell and enterocytes at the base of the villi of copper supplemented rats (Chapter, 2). The role of Paneth cells in metal metabolism is little understood, although it has been suggested that this cell could play some important role in the elimination of zinc, lead, mercury, calcium and cobalt (Sandow and Whitehead, 1979). Paneth cells may play a role likewise in the excretion of copper.

The aim of this study is to further clarify the copper induced cytotoxic and adaptive changes of the gastrointestinal tract at the ultrastructural level and explore their relation to the intracellular localisation of copper.

3.2. MATERIALS AND METHODS

3.2.1. Animals

Eight groups of four ten-week-old male Wistar rats of uniform weight (366 ± 3 (mean ± SE)) were randomly allocated into plastic cages with woodchips bedding. Five groups of them were fed a copper supplemented diet (1000 mg/kg), whilst the remainder were fed the unsupplemented diet (Appendix, 1.0). Food and water were always available and the animals were regularly observed and weighed at weekly intervals (Appendix, 3.0). The food was also weighed and dietary intake per group was recorded weekly (Appendix, 3.1). Groups of copper supplemented and control rats were killed at intervals of 1, 3, 5, 10 and 16 weeks and 1, 5 and 16 weeks respectively. Animals were deprived overnight of food prior to killing and euthanasia was performed by carbon dioxide inhalation and subsequent cervical dislocation. A midline incision was made into the abdominal cavity and samples were taken from the stomach, middle small intestine and caecum for ultramicroscopy. Triplicate samples from the right middle lobe of the liver (Haywood, 1981) were also collected for copper and zinc analysis (Appendix, 3.2) using a similar technique as described in Chapter 1. Stastical analysis was perfomed using Student's t-test (Rafferty et al., 1985).

3.2.2. Transmission Electron Microscopy (TEM)

3.2.2.i. Preparation of samples

Gastrointestinal samples were immediately fixed in situ with freshly prepared chilled (4^oC) 2.5% cacodylate buffered glutaraldehyde (Appendix, 3.3.i) starting from the middle small intestine, stomach and the caecum. Samples were then isolated and prepared as those described in Chapter 1 and fixed for about another 15 minutes before they were blocked into a longitudinal piece of 2 X 4 mm and further fixed at 4^oC for 3 hours. Tissues were then rinsed (3X) with sucrose cacodylate wash (Appendix, 3.3.ii) and kept overnight in the same buffer. They were further trimmed longitudinally into a piece of 1.5 X 3 mm prior to secondary fixation with

1% Zetterquist's buffered isotonic osmic acid (Appendix, 3.3.iii), stained with 2% uranyl acetate in 0.69% maleic acid (Appendix, 3.3.v.c), dehydrated in a graded series of ethanol, cleared in acetone and embedded in epon resin (Appendix, 3.3.iv) at 20°C using an Automatic Lynx Microscopy Tissue Processor (EM Scope) (Appendix, 3.3.vi). Samples were finally embedded in fresh resin and polymerised at 60°C for 24 hours.

3.2.2.ii. Ultrathin sections and TEM examination

Tissue sectioning was performed using a glass knife on a Reichert-Jung Ultramicrotome (Reichert Ltd., Austria). Tissue blocks were first trimmed and cut for semithin sections (0.3 to 0.5 µm), mounted on glass slides and were stained with 0.1% alkaline toluidine blue (Appendix, 3.3.v.a). They were examined under X10 and X40 magnifications to identify their morphological locations and histological changes. The blocks were then retrimmed and cut for ultrathin sections (gold or silver colour sections). The sections were placed on 150 or 200 mesh copper grids and were stained with lead citrate (Appendix, 3.3.v.c) prior to TEM (Hitachi H-600) examination at 75 KV. Ultrastructural changes were recorded and micrographs were taken using Ilford EM film and they were printed on Ilford Multigrade III photographic paper (Ilford Ltd., Mobberley Cheshire, UK).

3.2.3. X-ray electron probe microanalysis

3.2.3.i. Preparation of samples

A similar procedure was used in the preparation of the samples and ultrathin sectioning for X-ray probe microanalysis as those for transmission electron microscopy, except omitting osmication and uranyl acetate and lead citrate staining. They were placed on 150 or 200 mesh aluminium grids.

3.2.3.ii. Examination and analysis of samples

Metal analysis was performed using energy dispersive X-ray microanalysis at an accelerating voltage of 80 KV and a beam current of 30 mA and a spot diameter of 0.1 nm in a JEOL JEM 100 CX transmission electron microscope. The microscope was fitted with a KEVEX detector integrated to a microplus plus multichannel analyser system (Dapple System, Sunnyvale, California, USA). The specimen was orientated at an angle of 45^{0} and the metal content was analysed for 50 seconds.

3.3. RESULTS

3.3.1. Stomach

a. Surface mucous cells

The mucus of the control rats mainly consisted of homogenous finely granular material (type I) with occasionally a highly electron dense core granule (type II) (Figs, 3.1a, b). The apical surface of the cell was covered by relatively thin mucosubstance and a prominent junction complex fused the adjacent lateral membrane of the cell (Fig, 3.1b).

X-ray emission spectra of the type II granules failed to demonstrate any metal peaks of significance (Fig, 3.1c).

In contrast, in copper supplemented rats, the type II granules were observed to be very much more numerous throughout the trial (Figs, 3.2a, b). Furthermore, at week 16 the type II granules consisted of more than one single electron dense core and the apical surface of the cell was covered by a thickened electron dense mucosubstance (Figs, 3.2c, d).

X-ray emission spectra of the type II granules now demonstrated markedly elevated copper peaks. Sulphur, phosphorus, chlorine and silica peaks were also detected (Fig, 3.2e).

b. Mucous neck cells

Despite an increase in the population of the mucous neck cells in copper supplemented groups when compared with the controls the ultrastractural features in either group showed no striking dissimilarities (Figs, 3.3a, b). Uniformly electron lucent mucous granules with a distinct thin electron dense limiting membrane mainly occupied a supranuclear position within the cell. Occasionally in the both groups, a pin point highly electron dense core substance was observed within the granule (Fig, 3.3.b).

c. Parietal cells

There was some variation in the appearance of these cells in both the control and copper supplemented groups which may suggest differences in the physiological stages of the cell (i.e. secreting and nonsecreting cells). These distinctions were not studied.

In the copper supplemented groups, at the isthmus and neck of the gastric mucosa, degenerate, dying or dead parietal cells were observed. The nuclei of these cells were condensed, the mitochondria swollen and the cytoplasmic vacuoles and intracellular canaliculi were less prominent. Such cells were rarely observed in the control rats. In copper supplemented groups such cells were most commonly identified during the first 5 weeks and later the incidence regressed, although at week 16 it was still above the control (Figs, 3.4a, b).

d. Peptic cells

The ultrastractural appearance of these cells in both the control and copper supplemented rats was indistinguishable. The apical cytoplasm was mainly occupied by zymogen granules, whilst at the base of the cell granular endoplasmic reticulum was marked. Mitochondria were lodged between the cristae of the granular endoplasmic reticulum at the base of the cell and were found rarely in the apical cytoplasm. Electron dense bodies (lysosomes) were also rarely observed (Fig, 3.5b).

e. Mitotic figure

Mitotic cells were observed in greater numbers at the isthmus and neck of the gastric gland of the copper supplemented groups (Fig, 3.5a).

3.3.2. Small intestine

a. Enterocyte

Upper villi

Intracytoplasmic electron dense granules were more obvious in the supranuclear portion of the cells in copper supplemented rats than the controls throughout the trial, although at week 16 there was some decline in numbers (Figs, 3.6a, b). These granules were enclosed by well defined membranes and identified as lysosomes. The contents of these organelles were morphologically homogenous or consisted of materials of various densities (Fig, 3.7.c). Electron dense materials were also ocasionally found in the " intercellular space " at the middle or lower half of the enterocyte, but the membranous structure of these granules were less defined (Figs, 3.7a, b). Search was made for these structures in the unstained sections for microanalysis without success and it proved not possible to determine an X-ray elemental profile.

The apical surfaces of the enterocytes were covered by numerous finger like projecting microvilli. During copper supplementation morphological changes of the microvilli were observed which include swelling, vesiculation and irregularity of the surface membrane of the microvill, although these changes had regressed by week 16. The microvilli appeared to be shorter in copper supplemented groups than those of the controls at all this points.(Figs, 3.8a, b, c).

X-ray emission spectra of the microvill failed to detect any copper (Fig, 3.8d).

Base villi

There were no remarkable ultrastructural changes within the enterocytes as a result of copper supplementation throughout the trial. Enterocytes at the base of the villi were closely packed together. Mitochondria were scattered throughout the cytoplasm and electron translucent vacuoles were found at the apical pole of the nuclei (Fig, 3.9a).

X-ray emission spectra of the microvilli failed to demonstrate any copper peak (Fig, 3.9.b).

b. Goblet cell

The ultrastructure of these cells remained unchanged during copper supplementation throughout the trial when compared to the controls. The cytoplasm of the cell was often distorted by the accumulation of compacted less electron dense mucous granules at the apical region of the cell. The nuclei were basally located and golgi complex was mainly found within this region. Mitochondria and endoplasmic reticulum were scattered throughout the cell.

c. Intraepithelial leukocytes (IEL)

Intraepithelial leukocytes were commonly found within the intercellular space of the villus epithelium, at lower half of the villus in both copper supplemented and control animals. These cells were mainly characterised as lymphocytes, although macrophages and eosinophils were also identified. The migration of IEL from the lamina propria across the basement membrane into the intercellular space or vice versa was occasionally demonstrated (Fig, 3.10).

d. Crypt epithelial cells

The crypt of the villi was lined by undifferentiated cells (Fig, 3.11). Mitotic figures were more numerous in copper supplemented groups than the controls, otherwise there were no other remarkable changes observed.

e. Paneth cells

The ultrastructure of these cells throughout the trial remained essentially unchanged, although electron dense bodies (lysosomes) appeared to be increased in copper supplemented groups when compared to the controls (Figs, 3.12a, b). The apical cytoplasm of the cell was mainly occupied with mass of large secretory

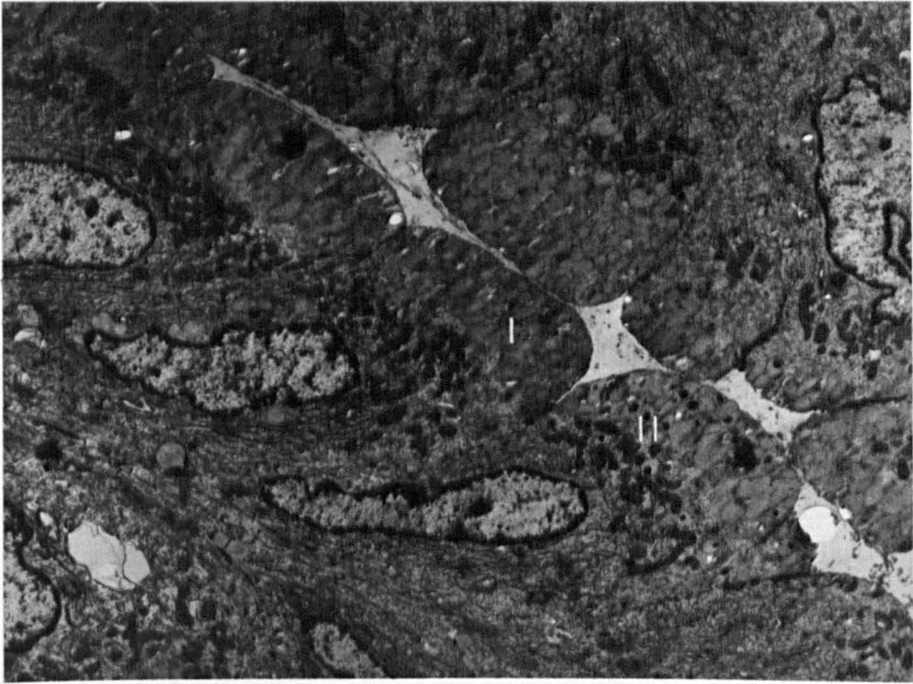
granules of various sizes and electron densities. The nucleus of the cell was located basally and the rough endoplasmic reticulum mainly occupied the lower cytoplasm.

X-ray electron spectra of the large oval electron dense granules at the apical cytoplasm of the control failed to demonstrate any metal peak (Fig, 3.13a). However, copper was detected within the granules of copper supplemented rats (Fig, 3.13b). By contrast, X-ray emission spectra to the electron dense eosinophil granules in the lamina propria within the area failed to identify any metal (Fig, 3.13c).

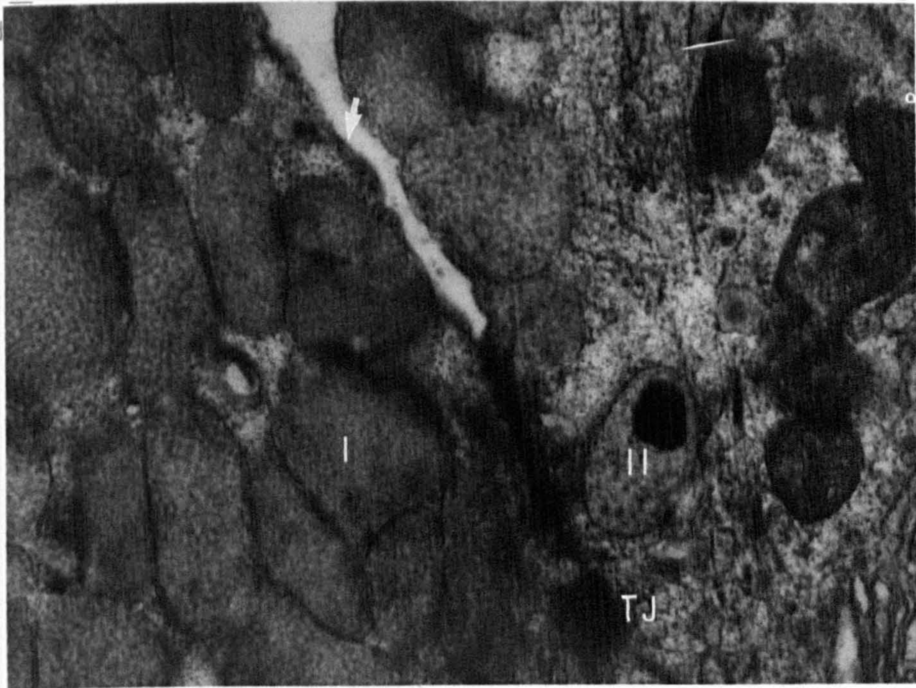
At week 16 of copper supplementation some variations on the copper peaks were recorded from the granules ranging from extremely high to nil. Furthermore, sulphur, chlorine, phosphorus and silica peaks were also demonstrated (Figs, 3.13d, e, f).

3.3.3. Caecum.

Electron dense granules (lysosomes) were more obvious in the supranuclear cytoplasm of copper supplemented groups (Figs, 3.14 a, b). Apoptosis was observed in the epithelium of the caecum in both the control and copper supplemented rats (Figs, 3.14c, d). The crypt of the caecum remained constant in both groups, although the appearance of bacterial colonies was slightly increased in the copper supplemented groups. Invasion of the organisms into the apical cytoplasm of the crypt epithelial cells was occasionally observed (Fig, 3.14 e, f).



(a)



(b)

Fig. 3.1. **Control**. Surface mucous cell of rat stomach. Type I mucous granule (I), type II mucous granule (II), apical surface (arrow) and tight junction (TJ). (a) X 3750. (b) X 37500.

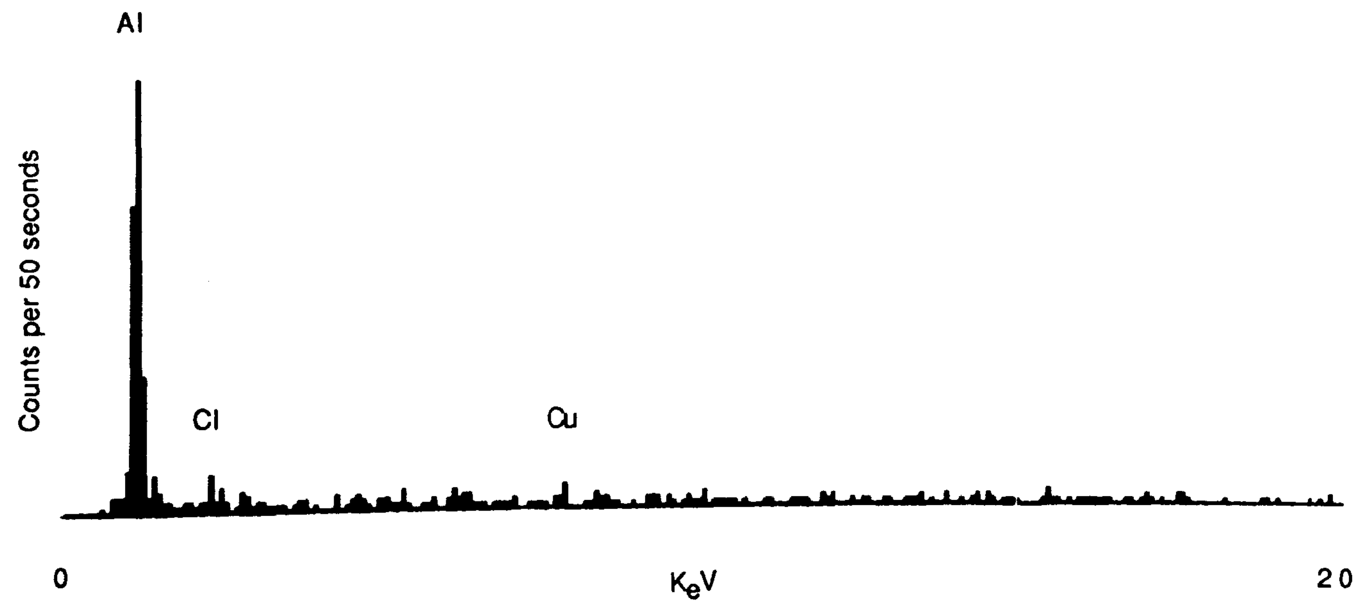
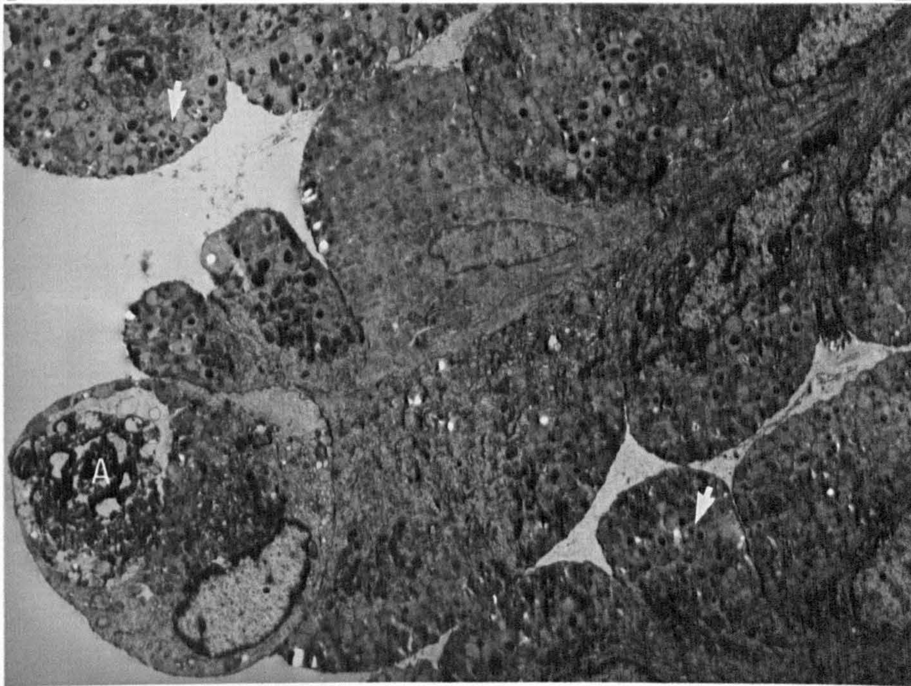


Fig 3.1.c. : **Control**. X-ray emission spectra of type II surface mucous cell granules of rat stomach. The aluminium (Al) peak was derived from the grid material. Chlorine (Cl) and copper (Cu).



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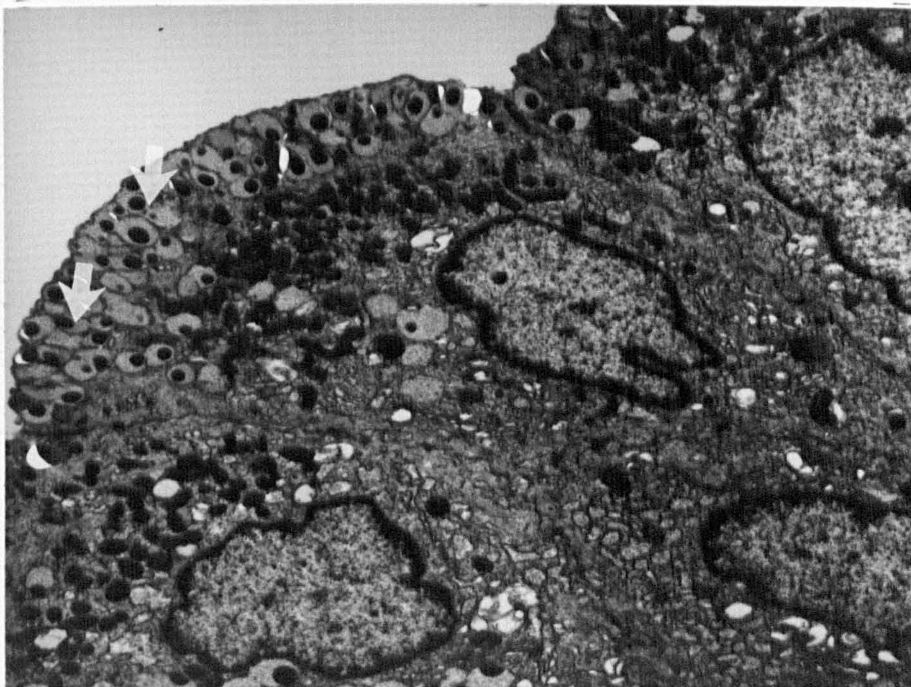
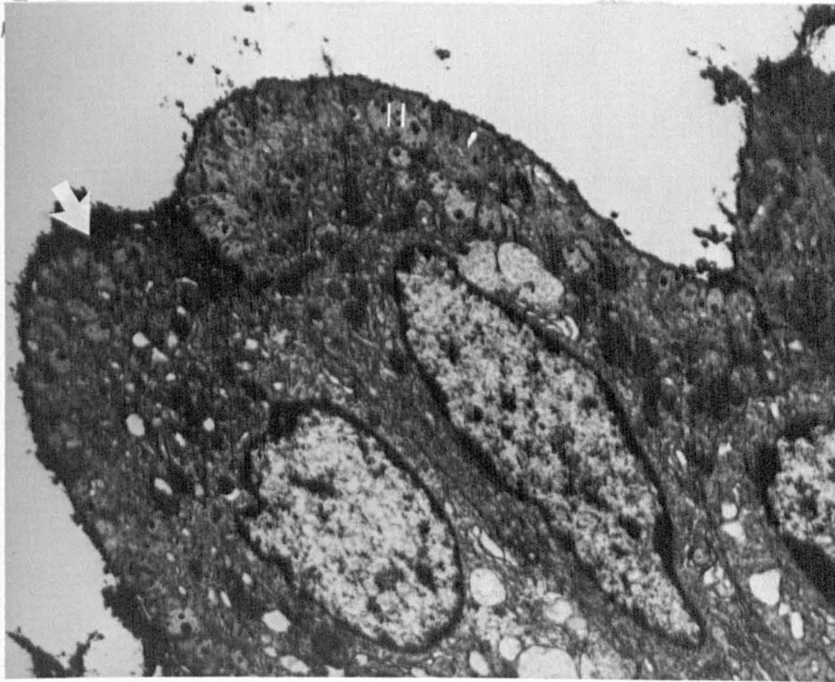


(b)

Fig. 3.2. **Copper supplementation. Week 3.** Surface mucous cell of rat stomach. Type II mucous granules were prominent (arrow). Apoptosis (A). (a) X 3000. (b) X 6000.



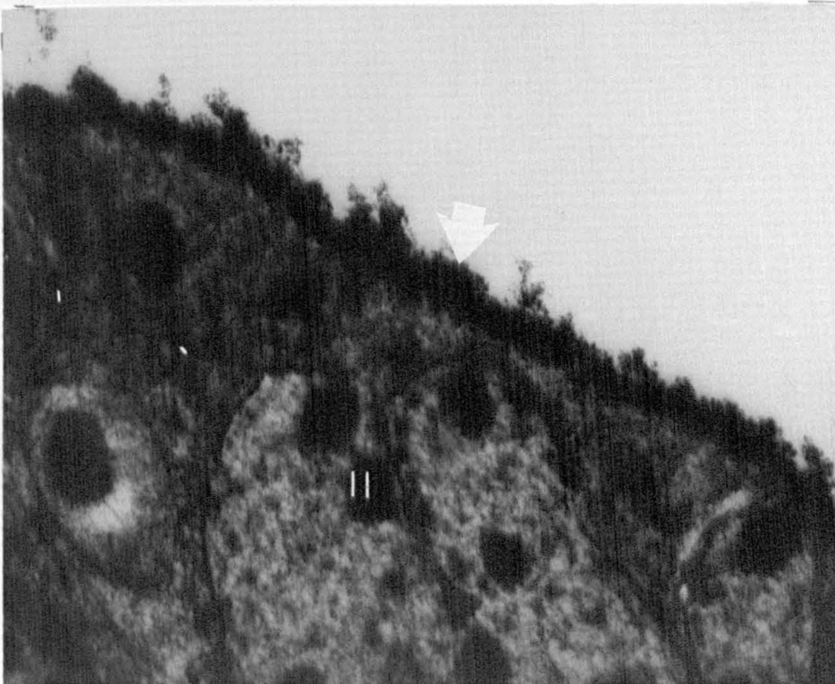


Fig. 3.2. **Copper supplementation. Week 16.** Surface mucous cell of rat stomach. Type II mucous granules were prominent. Multiple electron dense core found in the granule (II). Electron dense mucosubstance covered the apical surface of the cell (arrow). (c) X 7500. (d) X 75000.

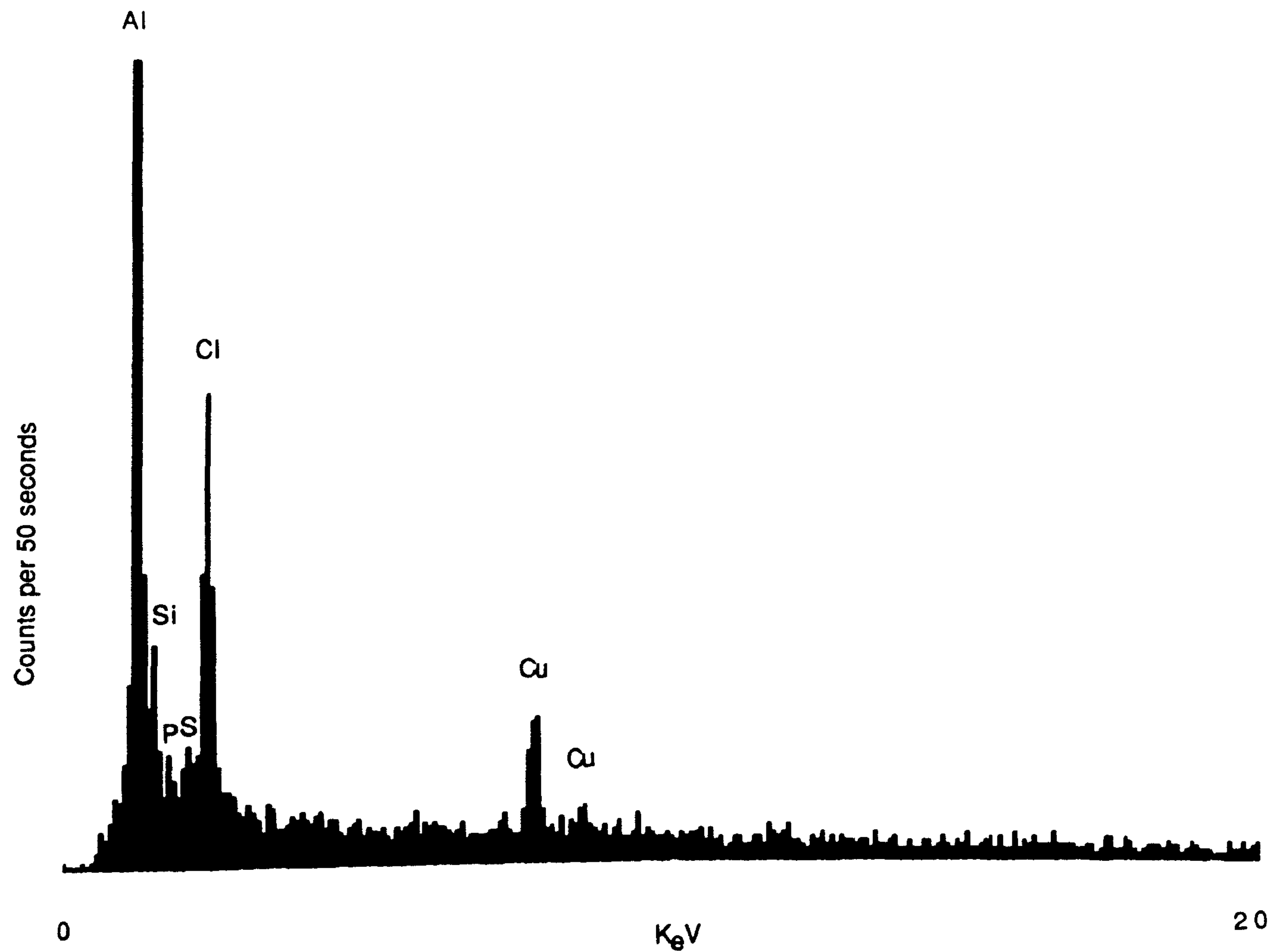
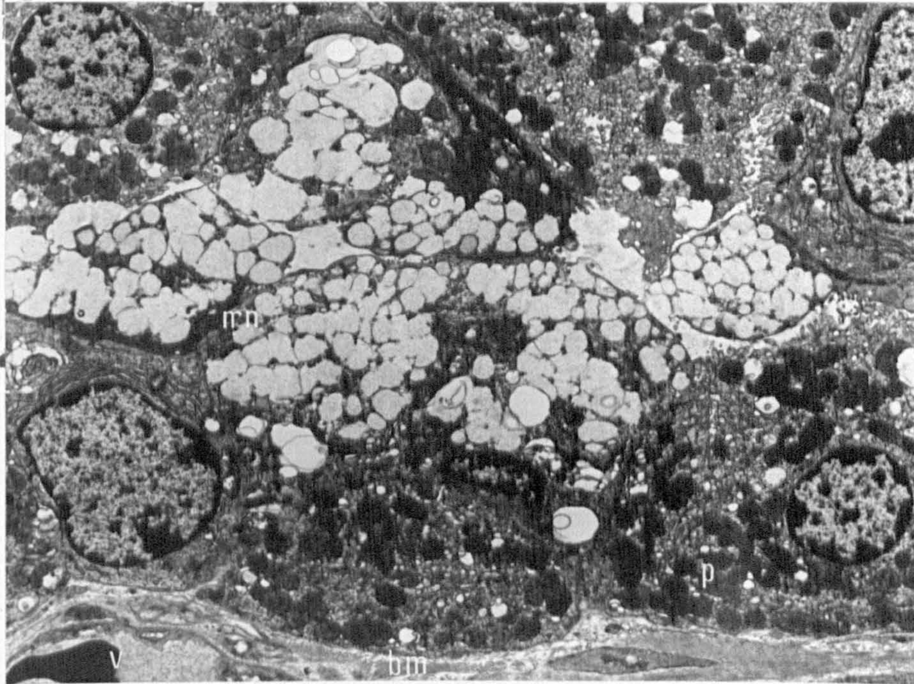


Fig. 3.2.e. : **Copper supplementation. Week 3.** X-ray emission spectra of the type II surface mucous cell granules of rat stomach. Copper (Cu), sulphur (S), chlorine (Cl), phosphorus (P) and silica (Si).The aluminium (Al) peak was derived from the grid material.



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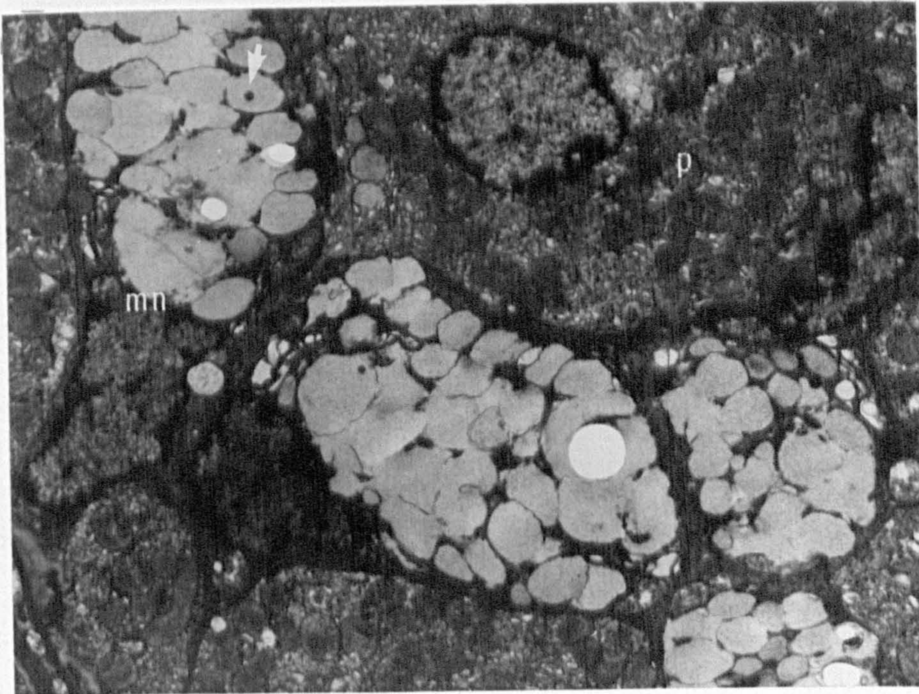
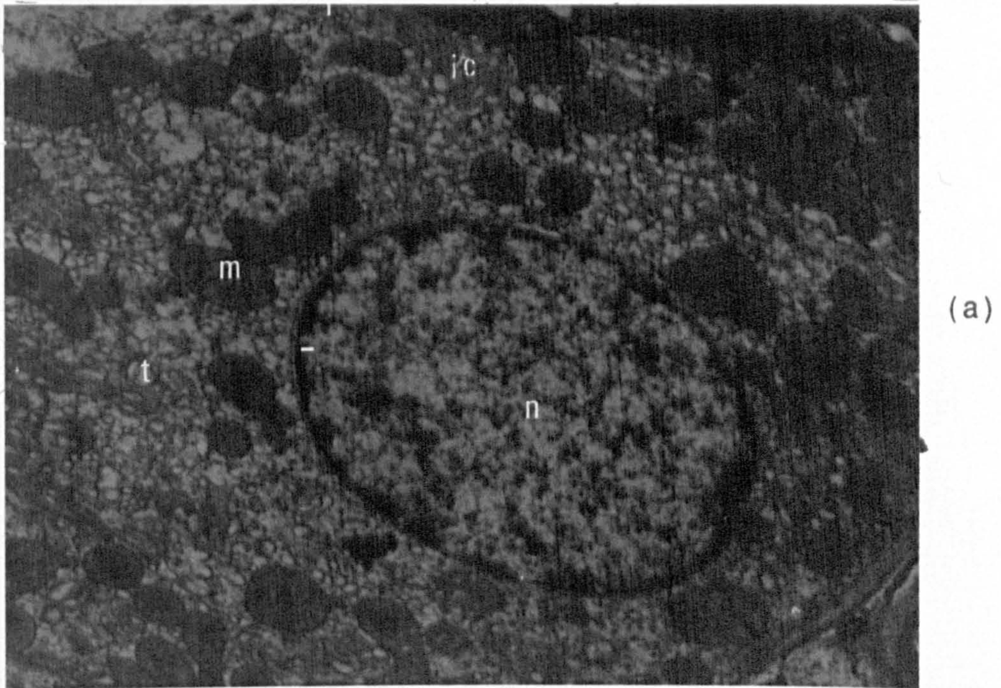


(b)

Fig. 3.3. **Neck of gastric gland of rat.** Clusters of mucous neck cells (mn) and parietal cell (p). Mucous granules with homogenous low electron density and occasionally pin point electron dense core (arrow). Basement membrane (bm) and blood vessel (v). (a) Control (X 3750). (b) Copper supplementation. Week 3 (X 5 250).



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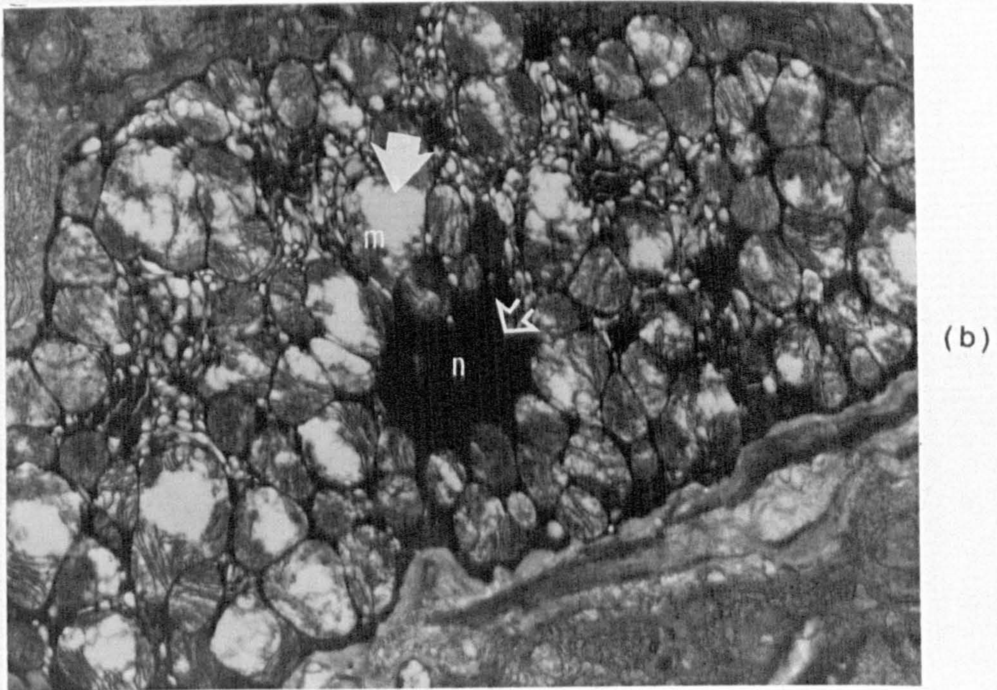
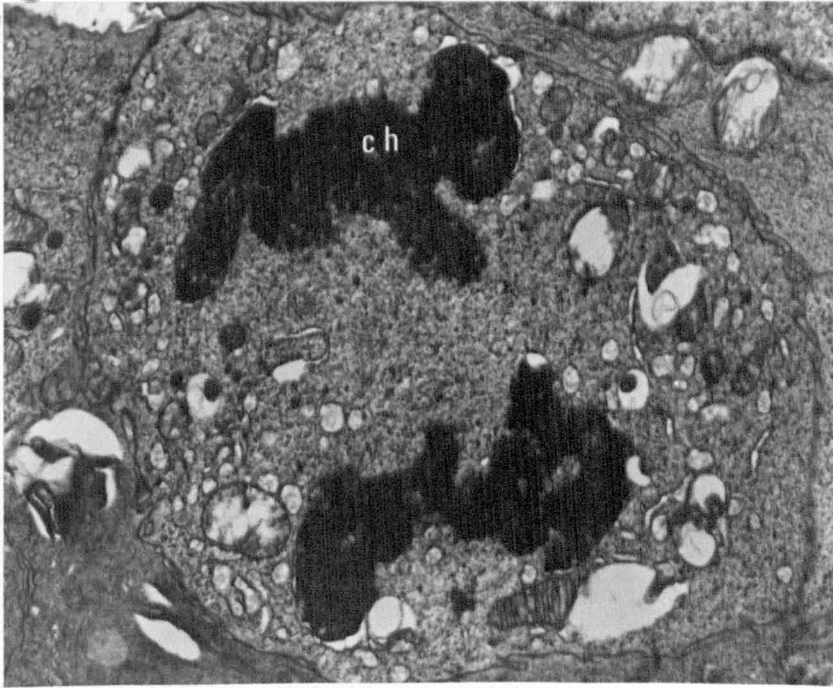


(b)

Fig. 3.4. **Parietal cell of rat stomach.** (a) **Control**. (b) **Copper supplementation.** Week 3 **(Cell death).** The mitochondria were markedly swollen and nucleus was condensed (arrow). Mitochondria (m), nuclei (n), intracellular canaliculus (ic) and tubulovesicular system (t) (X 9000).



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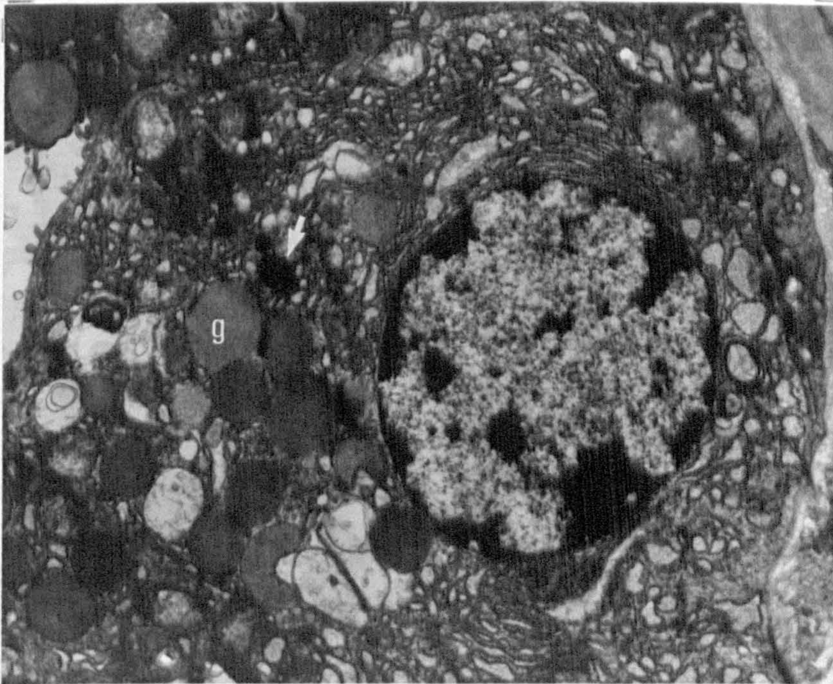
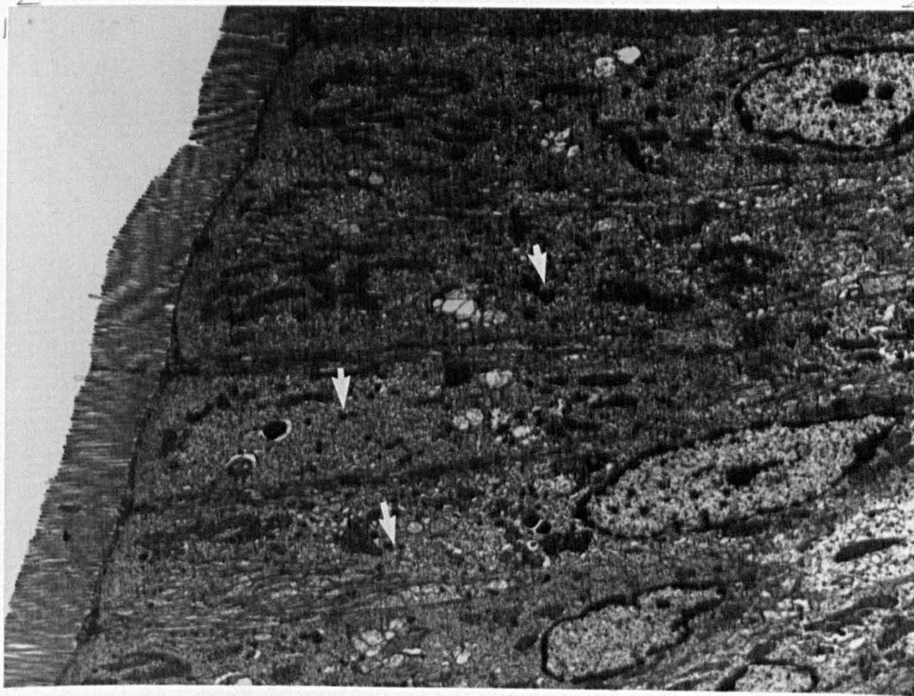


(b)

Fig. 3.5. (a) **Mitotic cell. Copper supplementation. Week 5.** Upper neck of gastric gland of rat. Clumps of chromatin (ch) (X 9000). (b) **Peptic cell of rat stomach.** Apical cytoplasm contained zymogen granules (g). Electron dense granules (lysosomes) (arrow) (X 7500).



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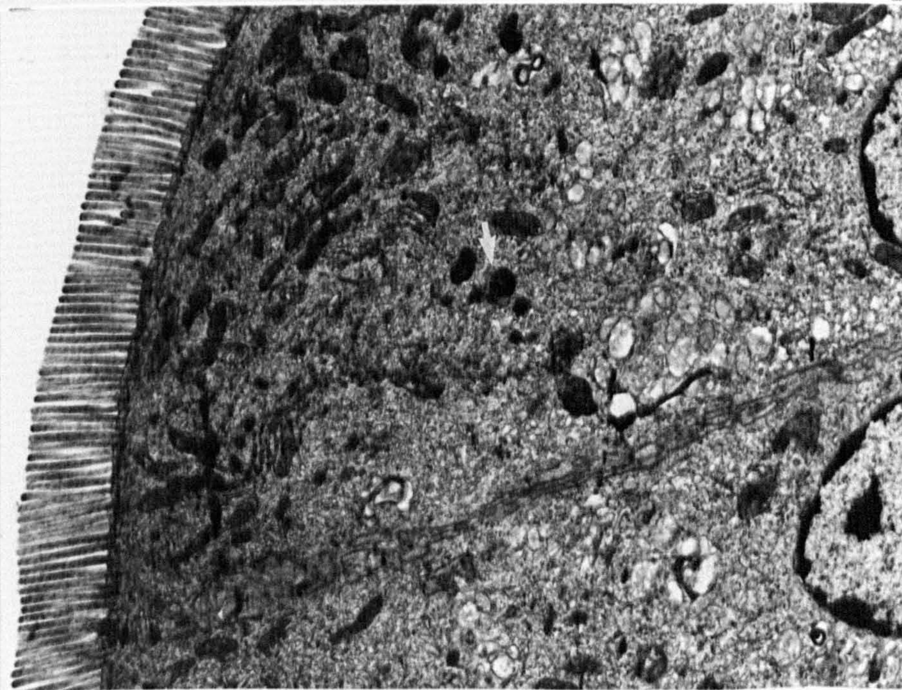
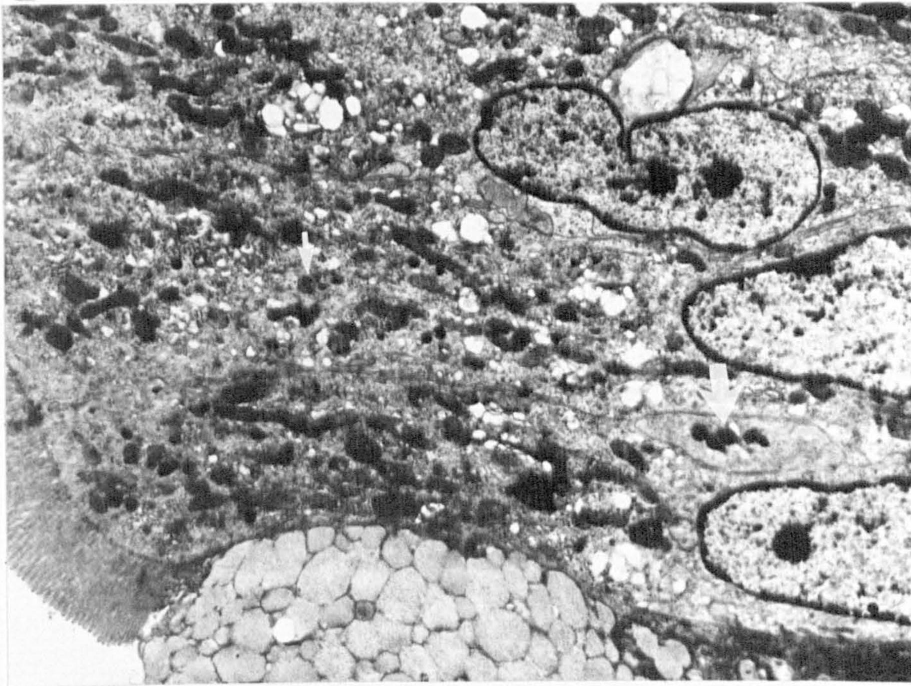


(b)

Fig. 3.6. **Copper supplementation. Enterocyte.** (a) **Week 1** (X3750). Upper villus of rat small intestine. Electron dense granules (lysosomes) were prominent at the apical cytoplasm (arrow), but reduced by (b) **Week 16** (X9000).



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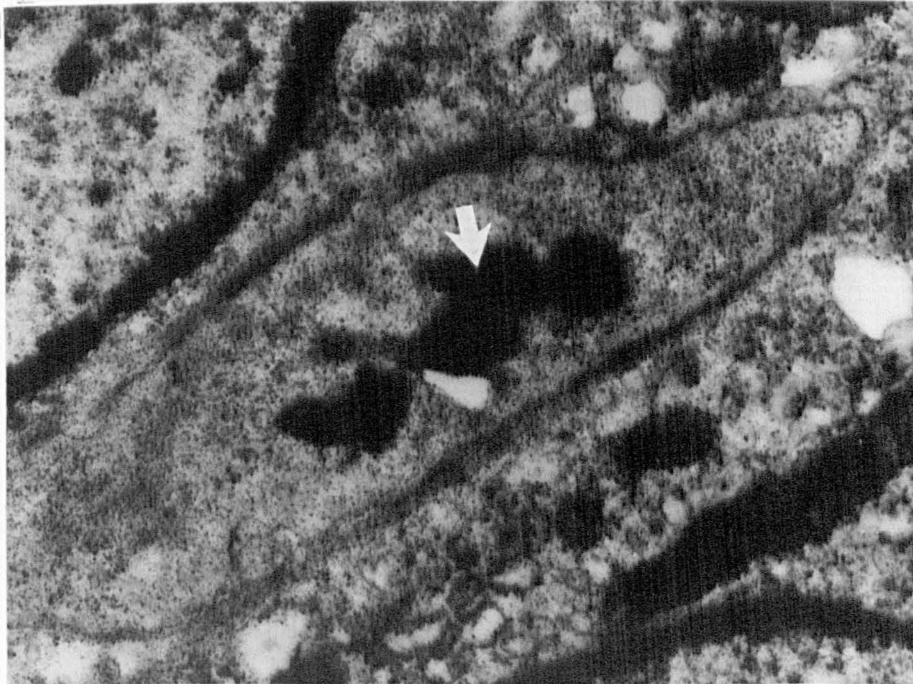


(b)

Fig. 3.7. **Copper supplementation. Week 5. Enterocyte.** Upper villus of rat small intestine. Electron dense granules were prominent within apical cytoplasm (lysosomes). Electron dense materials were also demonstrated within the "intercellular space" (arrow). (a) X 4500. (b) X 22500.

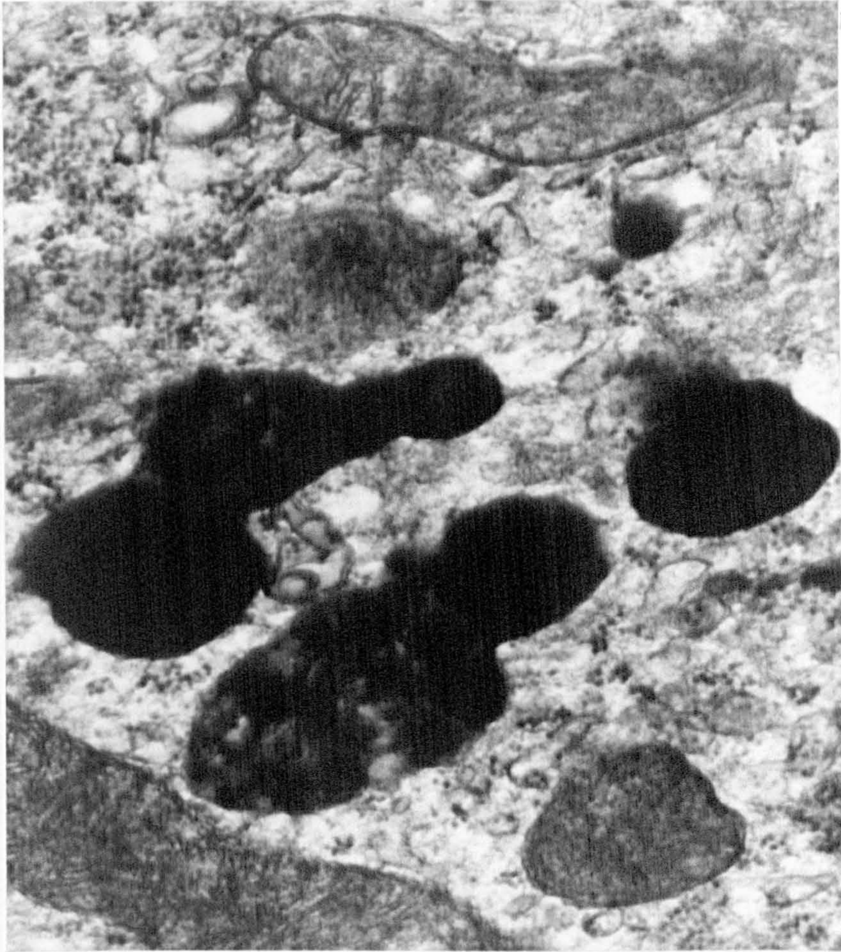


Fig. 3.7.c. **Copper supplementation. Week 5. Enterocyte, apical cytoplasm.** Upper villus of rat small intestine. Electron dense granules enclosed by well defined membranes (lysosomes) (X 45000).

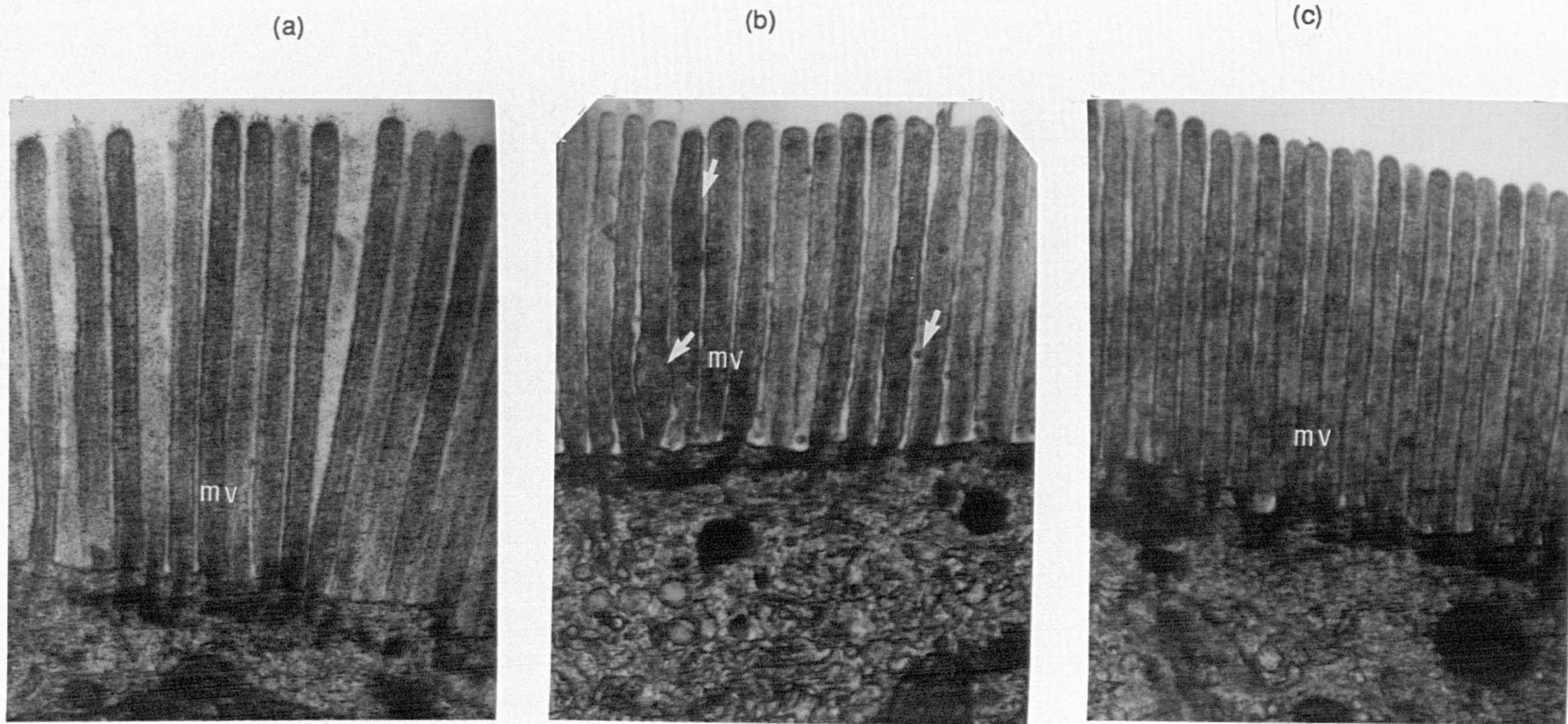


Fig. 3.8. **Microvilli of the enterocyte.** Upper villus of rat small intestine. (a) **Control.** (b) **Copper supplementation Week 3.** Swollen, vesiculation and irregularity of the surface membrane of the microvilli (mv) (arrow), recovery at (c) **Week 16.** (X 30000).

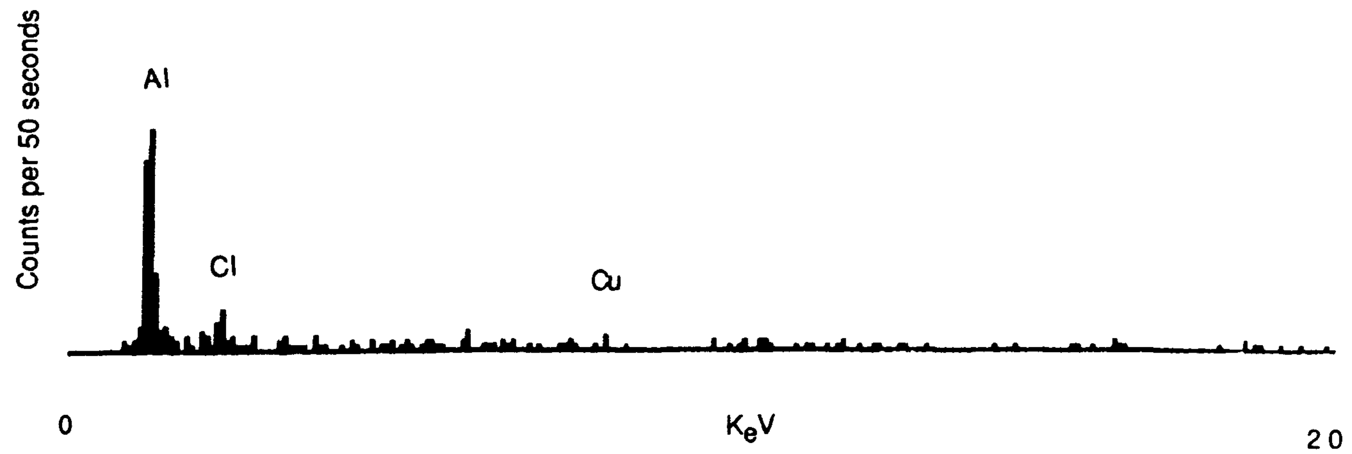


Fig. 3.8.d. **Copper supplementation. Week 5.** X-ray emission spectra of the enterocyte microvilli. Upper villi of rat small intestine. The aluminium (Al) peak was derived from the grid material. Chlorine (Cl) and copper (Cu).

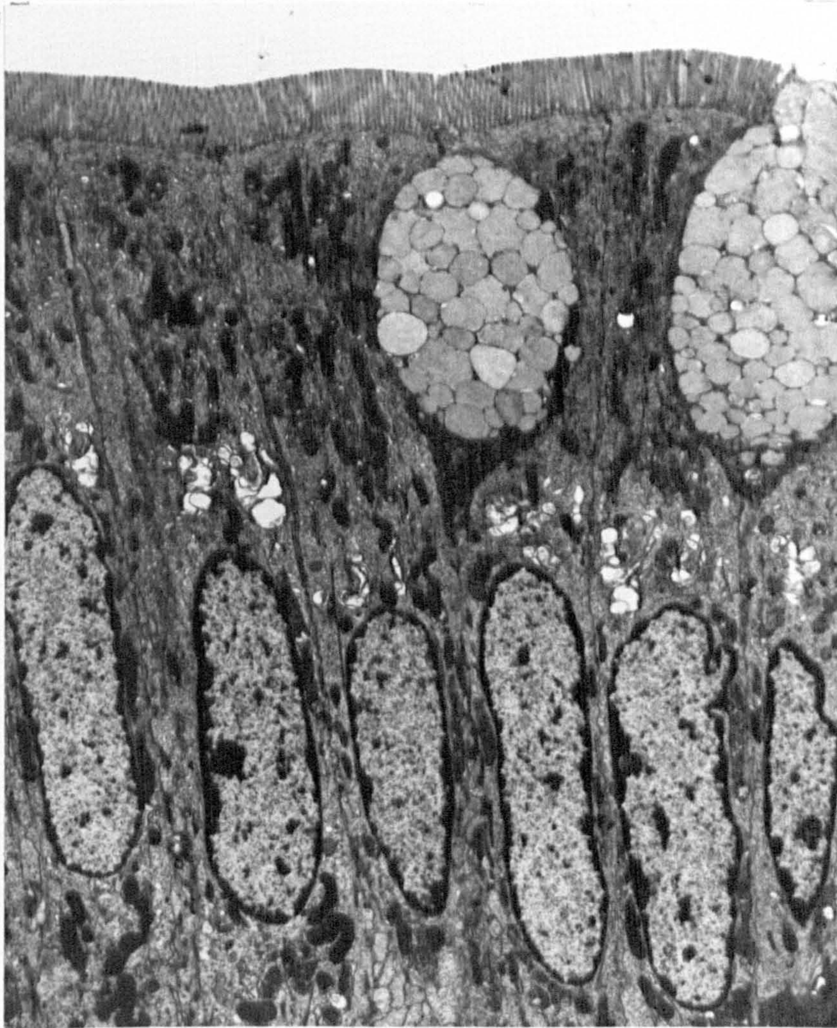


Fig. 3.9.a. **Base villus of rat small intestine.** Control (X 3750).

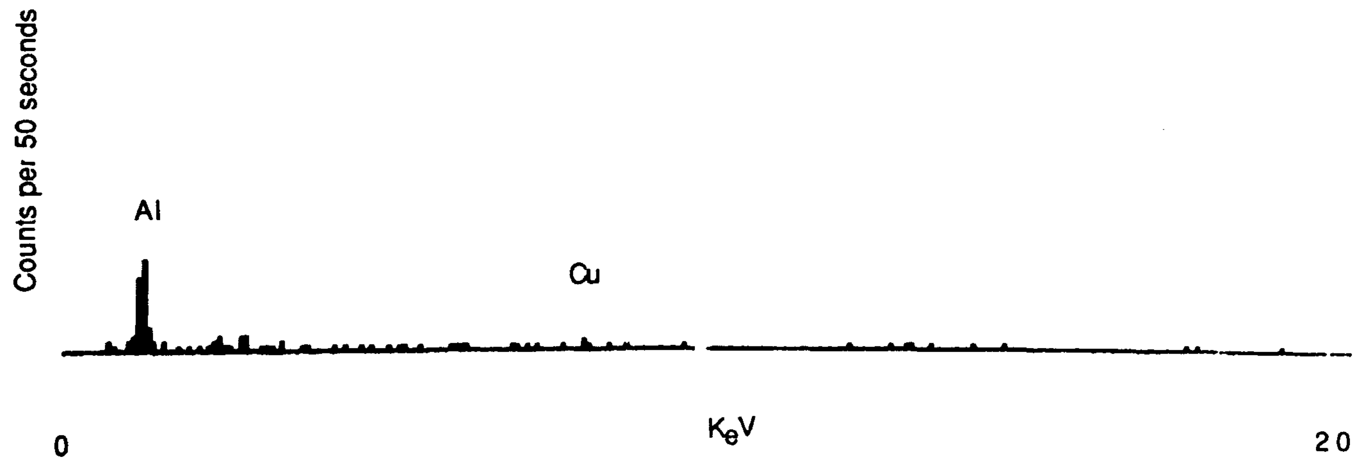


Fig. 3.9.b. **Copper supplementation. Week 5.** X-ray emission spectra of enterocyte microvilli. Base villus of rat small intestine. The aluminium (Al) peak was derived from the grid material. Copper (Cu).

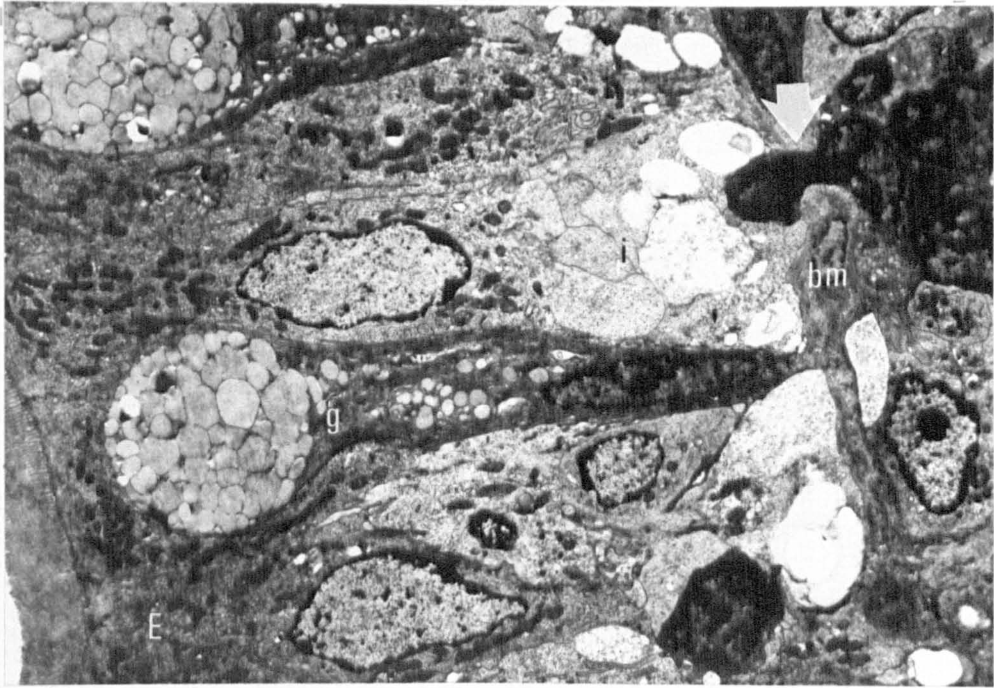


Fig. 3.10. **Migration of intraepithelial lymphocyte (IEL)** (arrow). Copper supplementation. Week 5. Base villus of rat small intestine. Enterocyte (E), goblet cell (g), intercellular space (i) and basement membrane (bm) (X 3 000).

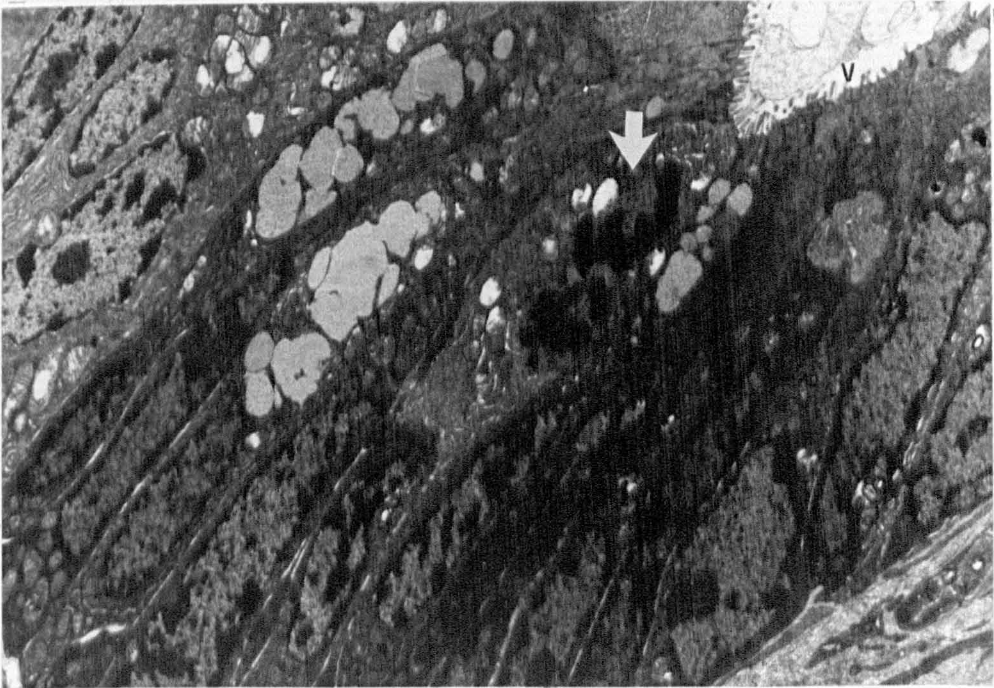
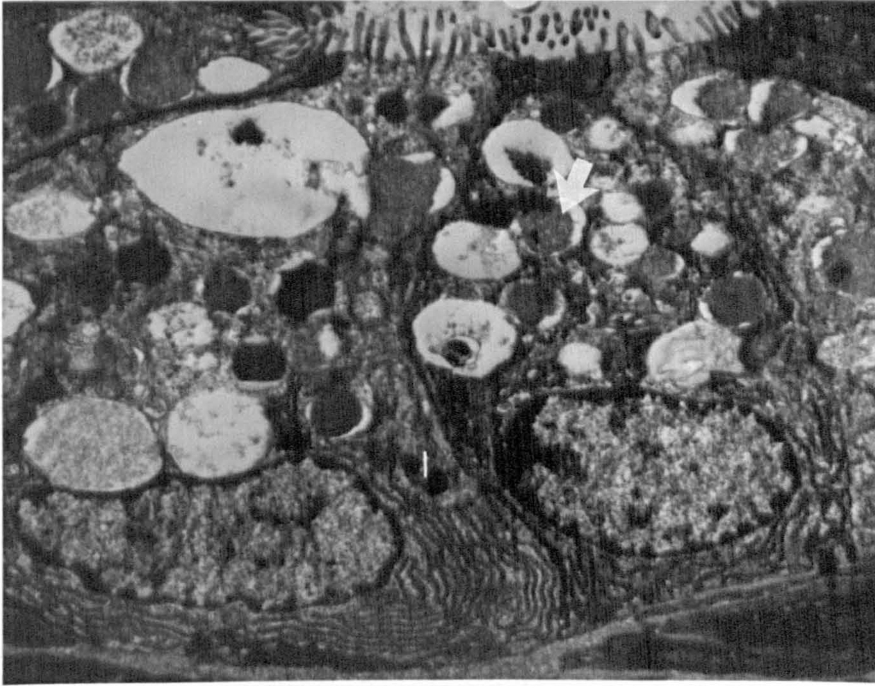
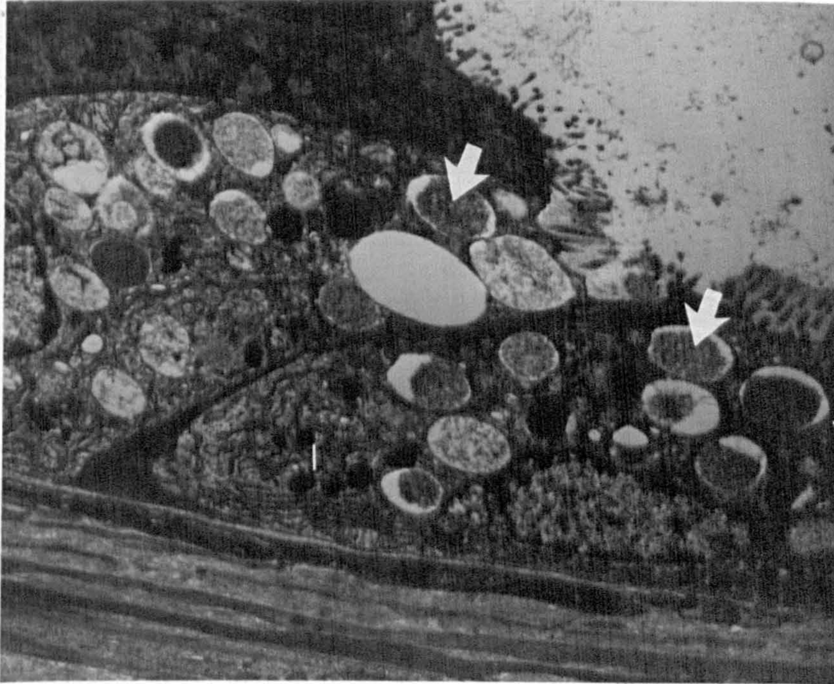


Fig. 3.11. **Undifferentiated cells.** Copper supplementation. Week 1. Crypt of rat small intestine. Mitotic cell (arrow) and irregular short microvilli (v) (X 5 250).



(a)



(b)

Fig. 3.12. **Paneth cells of rat small intestine.** (a) Control and (b) Copper supplementation. Week 5. Apical cytoplasmic granules (arrow) and lysosomes (l) (X 6000).

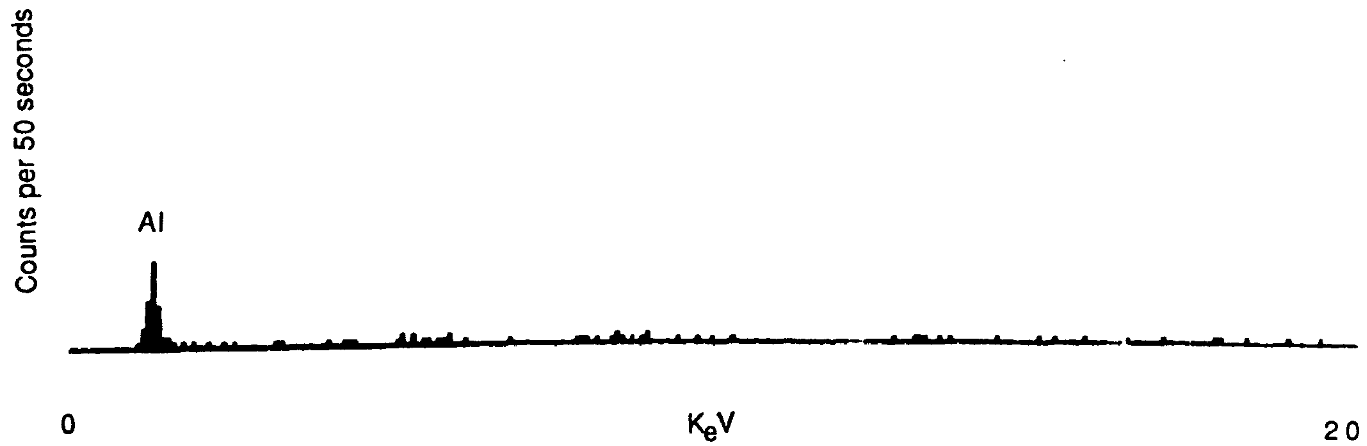


Fig. 3.13.a. **Control**. Paneth cell of rat small intestine. X-ray emission spectra of the apical cytoplasm of the homogenous oval electron dense granules. The aluminium (Al) peak was derived from the grid material.

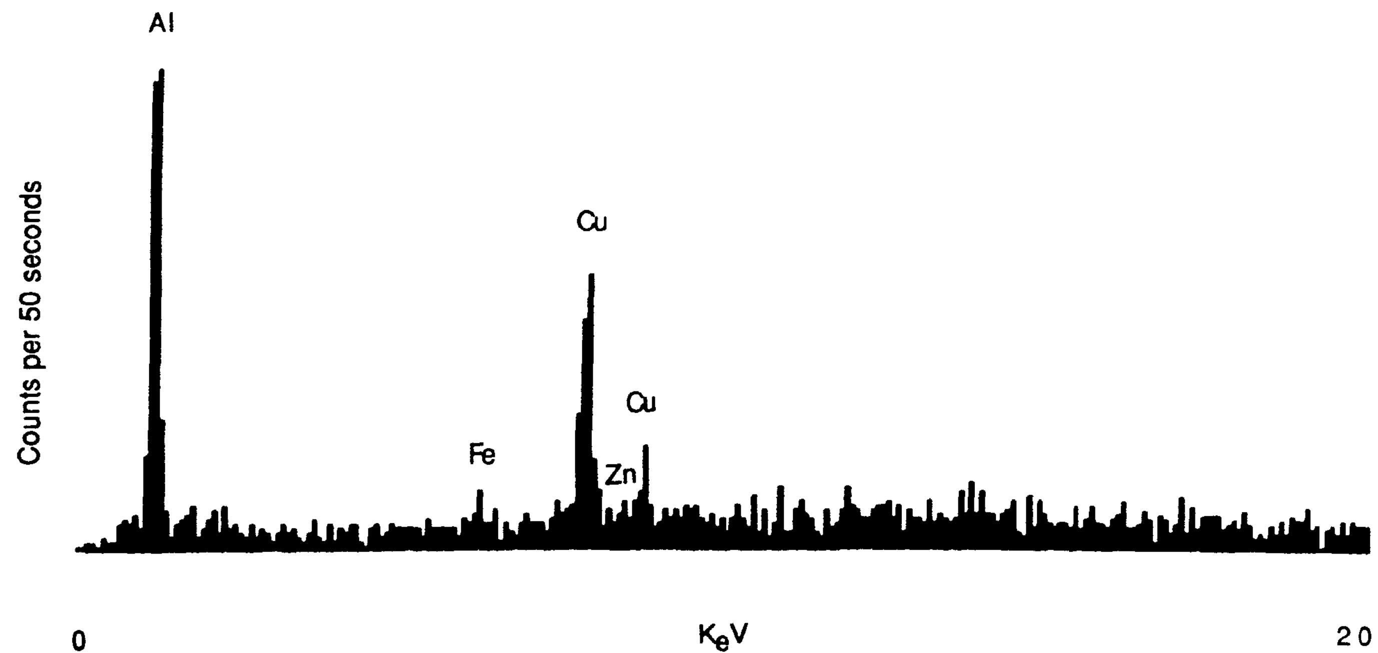


Fig. 3.13.b. **Copper supplementation. Week 5.** Paneth cell of rat small intestine. X-ray emission spectra of the apical cytoplasm of the homogenous oval electron dense granules. Copper (Cu) peaks were demonstrated. The aluminium (Al) peak was derived from the grid material. Iron (Fe) and zinc (Zn).

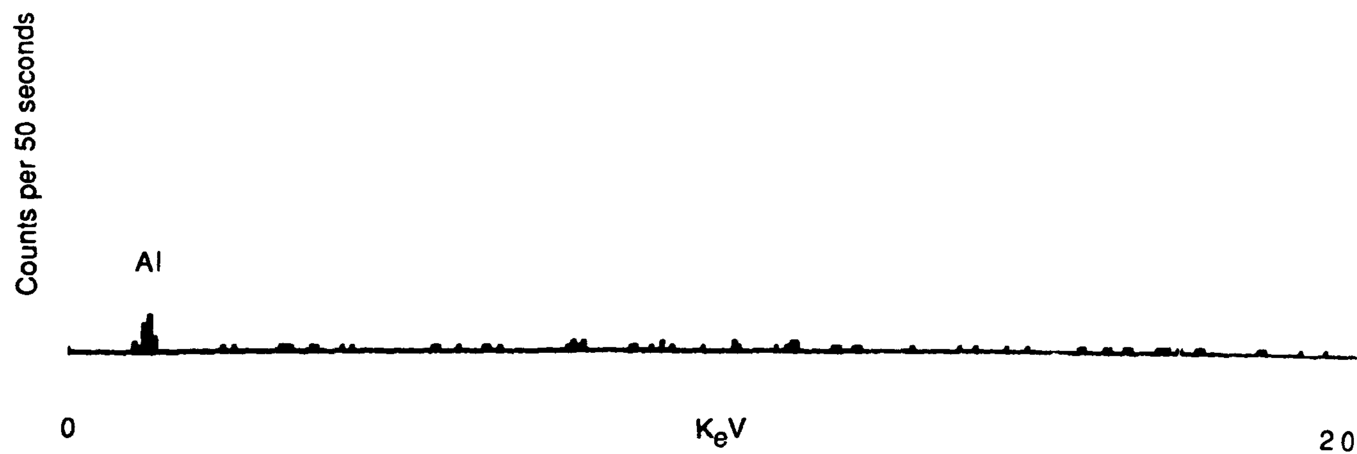


Fig. 3.13.c. **Copper supplementation. Week 5.** Eosinophil of rat small intestine. X-ray emission spectra of the electron dense eosinophilic granules (acted as the control or background). The aluminium (Al) peak was derived from the grid material.

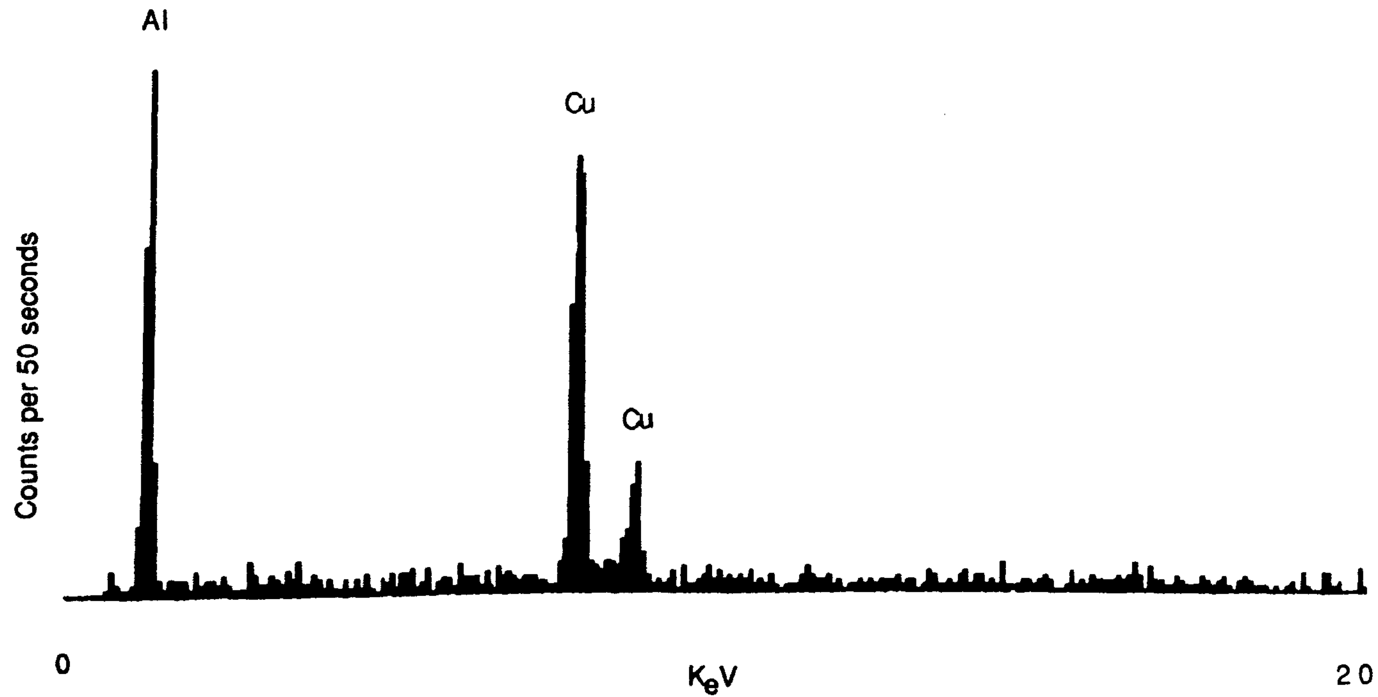


Fig. 3.13.d. **Copper supplementation. Week 16.** Paneth cell of rat small intestine. X-ray emission spectra of the apical cytoplasm of the homogenous oval electron dense granules. Copper (Cu) peaks marked. The aluminium (Al) peak was derived from the grid material.

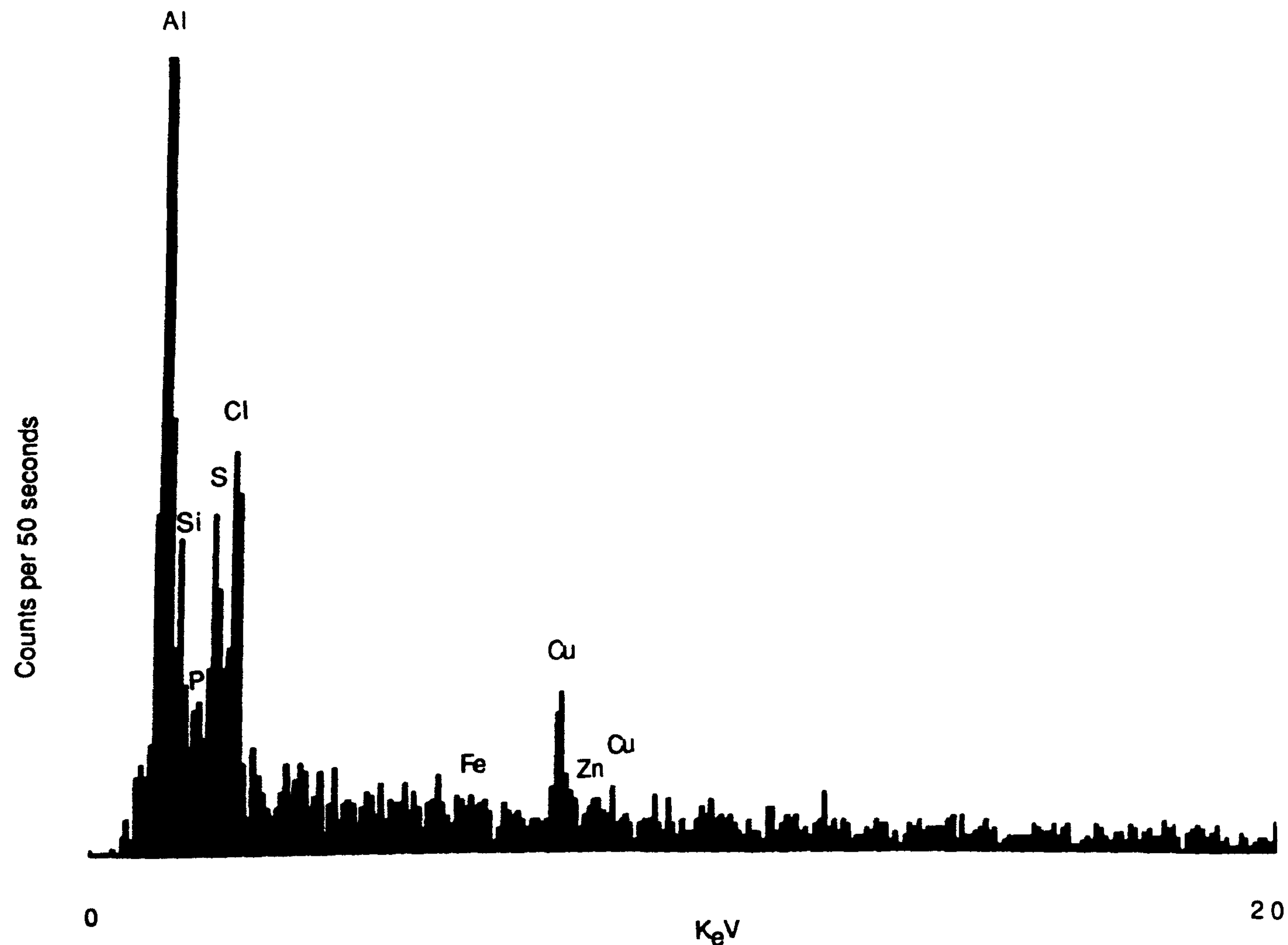


Fig. 3.13.e. **Copper supplementation. Week 16**. Paneth cell of rat small intestine. X-ray emission spectra of the apical cytoplasm of the homogenous oval electron dense granules. Copper (Cu), sulphur (S), chlorine (Cl), phosphate (P) and silica (Si), Iron (Fe) and zinc (Zn). The aluminium (Al) peak was derived from the grid material.

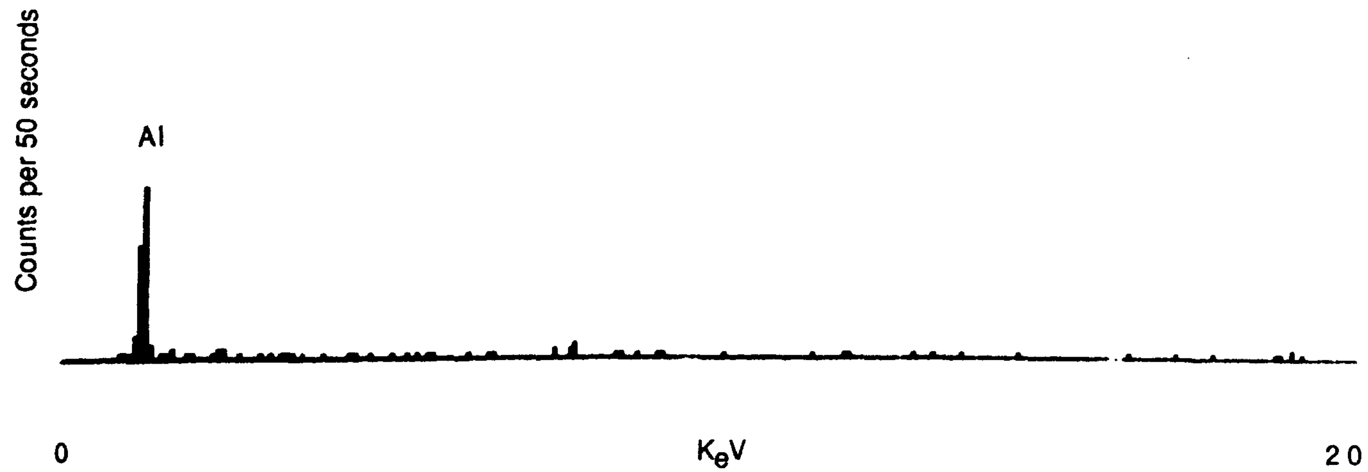
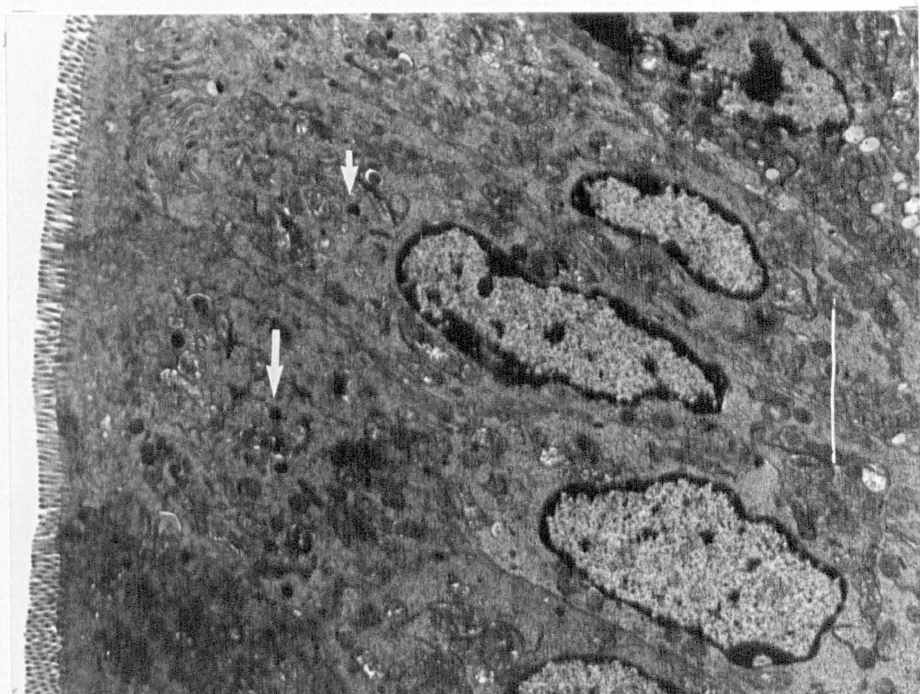
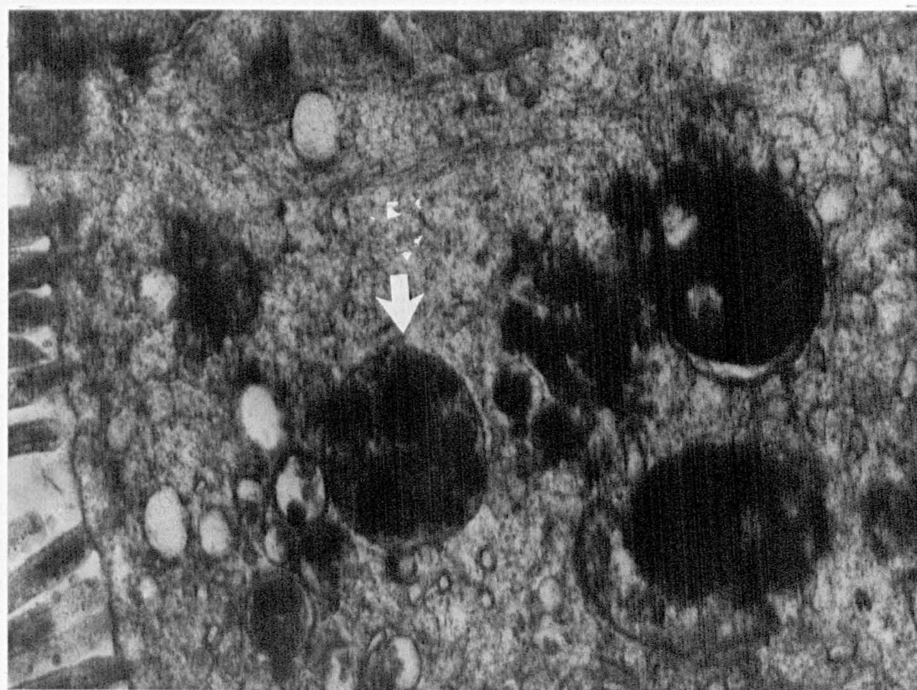


Fig. 3.13.f. **Copper supplementation. Week 16.** Paneth cell of rat small intestine. X-ray emission spectra of the apical cytoplasm of the homogenous oval electron dense granules. The aluminium (Al) peak was derived from the grid material.



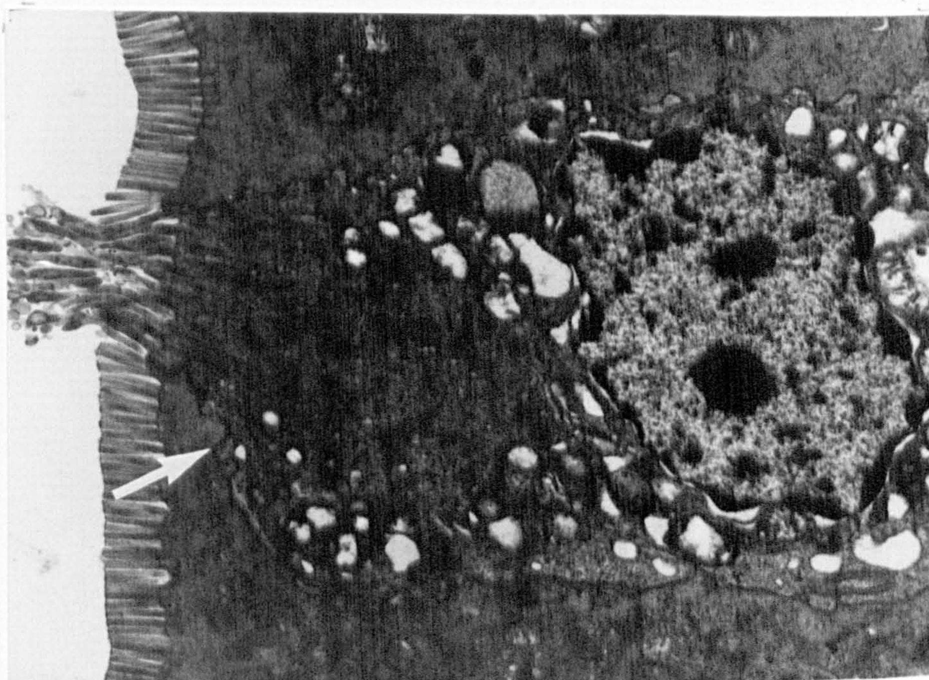
(a)



(b)

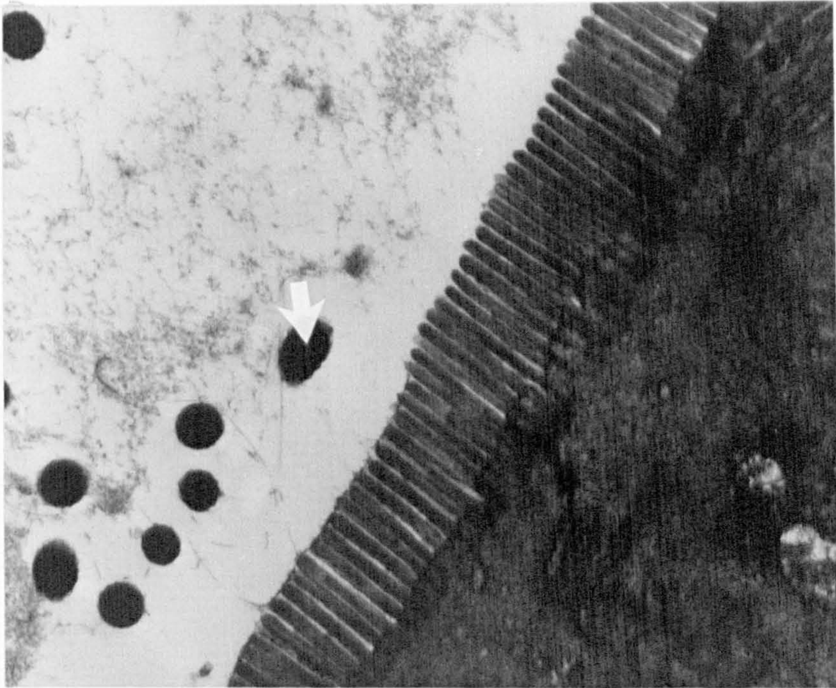
Fig. 3.14. **Copper supplementation. Week 3.** Caecum of rat. Enterocyte. Electron dense granules prominent at the apical cytoplasm (lysosomes) (arrow). (a) X 4500. (b) X 30000.

(c)

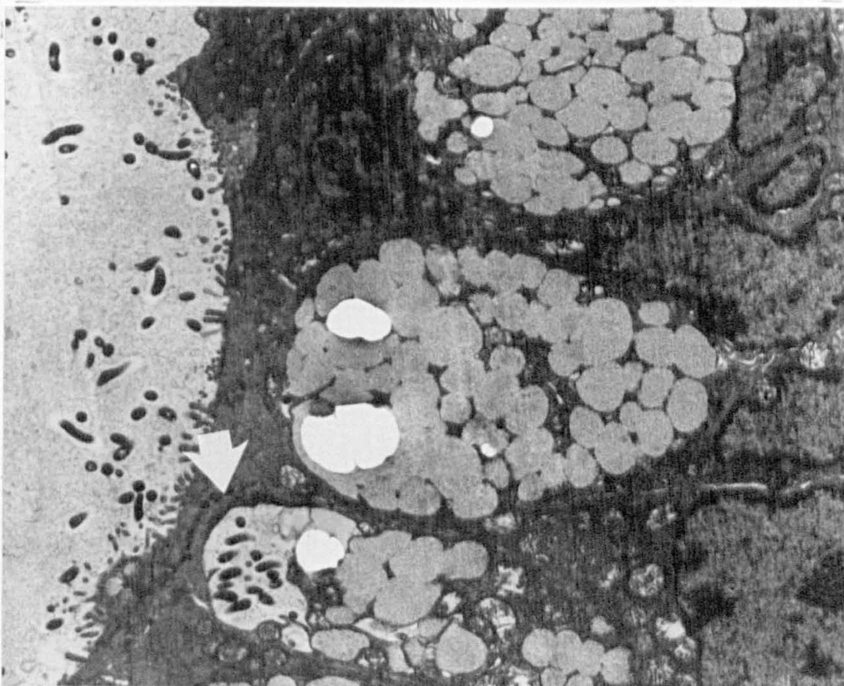


(d)

Fig. 3.14. **Cell extrusion (apoptosis)** (arrow). Caecal mucosa of rat. Copper supplementation. Week 1. (c) X 3750. (d) X 9000.



(e)



(f)

Fig. 3.14. **Bacterial colonies** (arrow). **Copper supplementation. Week 3.** Caecal mucosa of rat. (e) Upper region (X 15000) and (f) base of the mucosa. Invasion of the bacteria at the apical region of the epithelial cell (X 4 500).

3.4. DISCUSSION

This study further clarified the role of the gastrointestinal tract in copper tolerance in rats. Hyperplasia of gastric mucous secreting cells in copper supplemented animals was also associated with ultrastructural changes in the appearance of the mucous granule density of the surface mucous cells. Increased numbers of electron dense core mucous granules and the localisation of copper and other elemental residues including sulphur within the core in the copper supplemented groups further strengthened the possibly important role of mucus in promoting copper tolerance. Mucus-bound-copper may be complexed in an insoluble form and unavailable for further absorption.

Short term absorptive studies using copper isotopes in rats have indicated that copper is mainly absorbed from the stomach and upper small intestine (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984). Changes in the apical surface of the surface mucous cells associated with increased electron density at week 16 in copper supplemented groups may further contribute to the adaptive changes in copper tolerant rats which could prevent the uptake of luminal copper into the cell. It has been suggested that mucus may act as a medium in which macromolecules become insoluble and are prevented from contact with the cell membrane, whereas small molecules are soluble and free to diffuse (Edward, 1978). Thickened layers of mucosubstance could be a result of the excessive and rapid excretion of the mucus in response to high luminal copper contents (Appendix, 1.3).

The degeneration and death of the parietal cells could be due to the toxic effect of copper which caused a reduction of the parietal cell population (Chapter, 1). This cell has an important role in the secretion of hydrochloric acid and in the maintainance of the acidity of the stomach. The parietal cell mass has been reported to be well correlated to the acid secretory capacity (Ito, 1987). Furthermore, the presence of acid in the fetal stomach coincided with the differentiation of the cell (Ito, 1987). Thus, the depletion of the Parietal cell mass in the copper supplemented

groups may alter the acidity of the stomach and copper may be less available for further absorption.

Degenerative changes within the enterocyte microvilli of the villus upper third in copper supplemented animals are most probably due to the toxic effect of the metal. These changes could alter the uptake of copper from the lumen into the cytoplasm of the enterocyte. Recovery at week 16 may be a result of the complexing of luminal copper and its becoming less damaging, alternatively the rapid cellular turnover of the enterocyte populations (Chapter, 1) could immediately replace the damaged cells. Furthermore, the microvilli may be altered and incapable of absorbing copper having undergone a functional adaptation.

An increase in electron dense granules (lysosomes) in the cytoplasm of the enterocyte of copper supplemented rats during the first 5 weeks of copper supplementation corresponded with the metal contents in the organ (Chapters, 1, 2). Unfortunately, the X-ray elemental profile of the granule was not examined due to the difficulties in the identification of or ability to distinguish this granule from the other electron dense materials within the cytoplasm in the unstained sections for microanalysis. Intestinal lysosomes of copper supplemented rats were smaller than equivalant hepatic lysosome (Fuentealba, 1988) and far fewer lysosomes were observed within the enterocyte. These variations probably reflect differences in copper content in either organ in which hepatic copper content was about 100 times higher than intestinal copper (Chapter, 1 ; Appendix, 3.2).

The demonstration of electron dense granules at the intercellular space of the enterocytes possibly suggests that the transfer of copper from the enterocyte to the portal circulation may also follow this route, although likewise their copper content or otherwise could not be confirmed in the present study. Furthermore, copper may also be sequestrated within the intraepithelial leukocytes at this region and it may be extruded into the intestinal lumen following cellular extrusion or alternatively transfered into the portal circulation. This phenomenon may therefore suggest that intraepithelial leukocytes might play some role in copper homeostasis. The deletion

of intraepithelial leukocytes during copper supplementation (Chapter, 1) may contribute in the reduction of copper absorption.

Copper retention in the gastrointestinal tract may equally represent metal flux in the process of absorption, excretion or storage as suggested earlier (Chapter, 1). The detection of copper in the secretory granules of the Paneth cell of copper supplemented rats suggested that copper could be excreted through this route. This mechanism could play some significant role in the process of adaptation and copper tolerance.

The definitive role of the Paneth cell has not been established, although the nature of the conspicous granules in the cell which is very similar to that of other types of secretory cells such as pancreatic acinar cells suggested that the Paneth cell has an important secretory function (Sandow and Whitehead, 1979). The secretory granules of the Paneth cell have been reported to contain various substances such as lysozyme (Deckx et al., 1967 ; Peeters and Vantrappen, 1975), immunoglobin A (Satoh et al., 1986), and heavy metals (Elmes, 1976 ; Dinsdale, 1984). It appears that Paneth cells may play different roles in controlling both the intestinal bacterial populations and metal homeostasis at the absorptive sites.

In metal homeostasis despite the demonstration of the heavy metals in the secretory granules of the Paneth cell (Elmes, 1976 ; Sandow and Whitehead, 1979 ; Dinsdale, 1984), little attention has been focused in this area and if anything most of the studies seem to concentrate on the role of Paneth cells in the excretion of zinc. The demonstration of zinc in the Paneth cell after parenteral zinc administration, undetected in zinc deficient rats, suggests that the Paneth cell was an excretory pathway for zinc (Miller et al., 1961 ; Elmes, 1976). Furthermore, the depletion of plasma zinc was reported to coincide with the reduction of histochemical reactivity of the cell (Pascoe et al., 1971).

In the present study the demonstration of copper in the secretory granules of the Paneth cell of copper supplemented rats may possibly involve a similar excretory pathway to that of zinc. Furthermore, it has been reported that fecal copper

excretion from the parental injection of copper isotopes was not prevented by either ligation of the bile duct (Mahoney et al., 1955) or duodenostomy below the pancretic and biliary duct (Owen, 1964).

The morphological differentiation of Paneth cells in rat is not completed until about the second week after birth (Behnke and Moe, 1964). This seems to be correlated with the concentration of copper in both the intestinal tract (Johnson and Evans, 1980 ; Mason et al., 1981) and liver (Hurley et al., 1980) which were markedly elevated during the first 2 weeks of postnatal life, but were depleted to about the adults level by the third week. This adds support to the possible excretory role of the Paneth cell in the elimination of copper in addition to other possible changes which have been suggested previously including the maturation of the absorptive (Mistilis and Mearrick, 1969 ; Williams and Beck, 1969) and hepatic cells (Evans, 1973).

Paneth cells have been found in the small intestine of man, some other mammals, birds, amphibians and reptiles (Sandow and Whitehead, 1979). In domestic animals this cell is most numerous in the small intestine of horses, is less prominent in ruminants and is not found in dogs, cats and pigs (Barker and Van Dreumel, 1985). Among these animals , it appears that horses are less prone to copper poisoning than the other animals. Horses fed with copper supplemented diet up to 791 mg/kg for 6 months do not shows any sign of toxic effect (Smith et al., 1975). However, despite the presence of this cell in ruminants, sheep is still the most susceptible species to copper poisoning, although this may simply reveal that more than one mechanism is involved in copper homeostasis. The adaptive role of Paneth cell in the elimination of excess copper within different animal species needs further clarification.

The demonstration of sulphur from the secretory granules of the Paneth cell of copper supplemented rats in the present study coincided with intense immunoreactivity for metallothionein (MT) in this cell type (Chapter, 2), which could suggest that MT is induced by the cell in response to excess copper. However, immunocytochemical MT does not correspond in its localisation to granules being

more confined to a reticulate pattern superimposed on diffuse staining (Chapter, 2). This is similar to reports in copper loaded liver and kidney (Fuentealba et al., 1989b ; Evering et al., 1990) which has been ascribed to a likely loss of immunoreactivity in granule/lysosomal form (Evering et al., 1990).

The appearance of cytoplasmic lysosomes in the absorptive cells of the caecum paralleled to the copper content in this organ (Chapter, 1) and it may suggest that excess copper is being sequestrated within the lysosomes, although the localisation of the metal was not studied. Excess copper retention in the caecum may consist of this metal in the process of excretion rather than absorption, as it appears that copper is mainly absorbed from the proximal part of the gastrointestinal tract (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984). A continous cellular extrusion could promote copper excretion.

In conclusion this study has further clarified the changes and the possible role of the gastrointestinal tract in response to copper excess. Copper bound to gastric mucus may be unavailable for absorption, especially with the possible elevation of the pH of the organ and it may also reduce the potential toxic effect of the metal. Furthermore, the alterations in the absorptive surfaces of both gastric and intestinal mucosa may prevent the uptake of the metal into the cell. Rapid cellular turnover can facilitate the elimination of the metal and excess copper may also be excreted from the Paneth cell.

Addendum

The detection limit for X-ray microanalytical units is about 100 μg metal/g of tissue (Bearman and Isasi, 1972).

CHAPTER : 4

The Antagonistic Effect Between Copper and Zinc : Metallothionein Status and Metal Retention

4.1. INTRODUCTION

Copper and zinc are essential trace elements in both man and animals and disturbances in their bioavailability may predispose to deficiency or conversely toxicity. The existence of complex interactions between these metals has been recognised for many years. High dietary zinc intakes have been reported could induce copper deficiency in man (Prasad et al., 1978 ; Fischer et al., 1984) and animals (Underwood, 1977). Likewise, zinc has been shown to confer protection against copper toxicity in pigs (Ritchie et al., 1963 ; Suttle and Mills, 1966) and sheep (Mills, 1974 ; Bremner et al., 1976). Furthermore, disturbances in zinc metabolism have also been reported in association with excess dietary copper (Suttle and Mills, 1966 ; Evans et al., 1970 ; Van Campen, 1969 ; Mills, 1974).

The antagonistic effect between these metals may suggest that zinc, which is less toxic than copper (Bises et al., 1989) might be useful as an alternative therapy for copper associated diseases in both man and animals and indeed, zinc has been made use of in the treatment of familial copper storage disorder in Wilson's disease in man (Brewer et al., 1983 ; Hoogenraad and Van den Hamer, 1983 ; Caillie-Bertrand et al., 1985 ; Hill et al., 1987) and Bedlington terriers (Brewer, personal communication), although its usage is still far from universal.

The mechanisms that are involved in the antagonism between these metals are still poorly understood. However, it appears that this effect may occur mainly at the site of absorption, within the small intestine (Van Campen and Scaife, 1967 ; Van Campen, 1969 ; Oestreicher and Cousins, 1985). The absorption of copper was reported to occur mainly from the stomach and upper small intestine (Van Campen and Mitchell, 1965 ; Marceau et al, 1970 ; Van Barneveld and Van den Hamer,

1984), whereas zinc was absorbed mainly from the small intestine (Van Campen and Mitchell, 1965). It appears that the absorption of both copper (Crampton et al, 1965 ; Marceau et al., 1970) and zinc (Evans et al, 1975 ; Jackson et al., 1981) involves two separate mechanisms : the uptake of the metal from the intestinal lumen across the brush border into the cell and the transfer of the metal via the basolateral membrane into the portal circulation.

Isolation of a low molecular weight copper (Starcher, 1969 ; Evans et al., 1970) and zinc binding protein (Richards and Cousins, 1975, 1977) identified as metallothionein (MT) from the small intestine has given rise to speculation that MT may possibly be involved in the antagonism displayed by the metals. Zinc is a better inducer of the protein when compared to copper (Oestreicher and Cousins, 1985), however, MT has a higher affinity to bind copper than zinc (Bremner, 1980). Zinc induced intestinal MT may possibly sequester copper in the absorptive cells and make it unavailable for further absorption (Richards and Cousins 1977 ; Ogiso et al., 1979 ; Hall et al., 1979 ; Fischer et al., 1981, 1983). In contrast, it has been suggested that zinc could inhibit the absorption of copper by binding to and displacing copper from MT (Starcher, 1969 ; Evans et al., 1970).

The biological functions of MT in copper and zinc homeostasis are still poorly understood and it appears that the precise role of this protein at the absorptive site is still a matter of conjecture. In the previous study (Chapter, 2) it was demonstrated that the initial accumulation of copper in the gastrointestinal tract of copper supplemented rats was paralleled by a rise of MT. Subsequently (5-16 weeks) both copper and MT fell significantly; a similar sequence of events to that reported in the liver of copper tolerant rats (Evering, 1989). From which it was proposed that the protective adaptation and tolerance that occurs in chronic copper supplemented rats may possibly not directly be associated with MT (Chapter, 2). However, the protective interaction of zinc with regard to copper absorption may involve different mechanisms which may indeed invoke MT.

It is the aim of this study to assess the effect of oral zinc supplementation on both copper supplemented and unsupplemented rats over short and more prolonged periods with regard to metal retention in both liver and intestinal tract and intestinal MT status.

4.2. MATERIALS AND METHODS

4.2.1. Animals

One hundred and eight, 10 weeks old male Wistar rats of uniform weight (361 ± 36 (mean ± SD)) were randomly selected into 32 groups of 4 animals each. Each group of animals was allocated into a plastic cage with woodchips bedding. Four groups of rats were each fed respectively copper supplemented (1000 mg/kg), zinc supplemented (1000 mg/kg) and copper (1000 mg/kg) and zinc (1000 mg/kg) supplemented diets (12 groups in all). Controls were included and fed unsupplemented diet (3 groups) (Labsur Animal Diet, Lavender Mill, Manea Cambridgeshire) (Appendix, 1.0). Parenterally induced zinc MT was used as reference marker and four animals were killed at 20 hours post subcutaneous zinc injection (as 2.5% solution of zinc sulphate (Analar grade, BDH Chemical Ltd.) in 0.9% NaCl), at a dose rate of 100 mg/kg body weight. Furthermore, the intestinal MT status and metal retention in both the liver and small intestine after parenteral zinc injection were assessed in additional sixteen similar dietary groups including 4 controls.

Food and drinking water were always available and animals were observed regularly. An individual body weight and a group food consumption were recorded weekly (Appendix, 4.0.a, b ; 4.1.a, b). A group of animals from both with and without subcutaneous zinc injection was killed at intervals of 3 days, 3, 5 and 16 weeks. All animals were deprived of food overnight prior to killing. Euthanasia was performed by carbon dioxide inhalation and subsequent cervical dislocation.

A mid line incision was made into the abdominal cavity and the small intestine was immediately removed and placed on ice. The whole small intestinal tissue was taken for copper and zinc analysis, chromatographic separation and application of ELISA technique for MT (Chapter, 2). Formalin fixed tissue from the middle small intestine was also taken for immunocytochemical staining (Chapters, 1, 2). The right middle lobe of the liver was retained for copper and zinc analysis (Haywood, 1981).

4.2.2. Chromatographic separation

The details of the procedure are as described in Chapter 2. The small intestine of each individual rat was flushed with cold normal saline, cleaned and stored at -70°C in a plastic pot until required. Samples were then partially defrosted, scraped and diluted in 1 volume (w/v) or otherwise stated, of 10mM Tris-chloride buffer, pH 8.0, containing 0.02% sodium azide before they were homogenised and centrifuged. Metal and MT analysis were carried out on the soluble fractions on an individual basis. Chromatographic separation of pooled samples (4) was performed using Sephadex G-75 packed column and the copper, zinc and MT (on selected samples) contents of the eluant fractions estimated.

4.2.3. Copper and zinc analysis

The details of the procedure are as described in Chapter 1. Copper and zinc content in the eluant and soluble fractions of small intestine were directly analysed using Atomic Absorption Spectrophotometry (AAS). Metal concentrations are expressed as the mean and standard error of mean (mean ± SEM) in µg/g wet weight.

Triplicate samples from the right middle lobe of the liver (0.5 g) from each individual rat were taken and placed in plastic pots, oven dried at 70°C, weighed, digested in acid and diluted before they were analysed for copper and zinc contents (AAS). The recovery was included in each batch of acid digestion. Metal concentrations are express as the mean and standard error of mean (mean ± SEM) in µg/g dry weight.

4.2.4. Enzyme linked immunosorbent assay (ELISA) for MT

The details of the procedure are as described in Chapter 2. Assays were conducted on individual intestinal soluble fractions and on selected samples of the eluant fractions. MT concentrations are expressed as the mean and standard error of mean (mean ± SEM) in µg/g.

4.2.5. Immunocytochemistry for MT

Middle small intestine was fixed in freshly prepared 10% formalin and was embedded in paraffin wax as described in Chapter 1. Selected samples were stained for immunoreactive MT using the DNP-localisation system kit applied in conjunction with mouse IgG to monoclonal antibody to MT. This procedure was carried out at The University of Wales College of Medicine and the details of the procedure are as described in Chapter 2.

4.2.6. Stastical analysis

Stastical analysis was performed using Student's t-test in cricket software (version 1.1) for Macintosh (Rafferty et al., 1985).

4.3. RESULTS

4.3.1. Control diet

a. Small intestine

The concentration of copper (0.46 ± 0.01 µg/g) and zinc (9.55 ± 0.12 µg/g) remained unchanged (p>0.05) throughout the trial (Tables, 4.0, 4.1 ; Figs, 4.0a, 4.1a). Likewise MT showed no change (36.7 ± 3.4 µg/g) (Table, 4.2 ; Fig, 4.2a). Chromatographic studies failed to identify MT containing fraction (Figs, 4.8 - 4.11), although a MT peak was confirmed after 20 hours subcutaneous zinc injection (Fig, 4.5).

Copper and zinc concentrations (2.38 ± 0.62 µg/g and 33.64 ± 4.87 µg/g respectively) were elevated at 20 hours after parenteral zinc injection and remained high at day 3, but regressed to normal level thereafter (Tables, 4.0, 4.1). MT content which was elevated to about 25 fold normal during the first 3 days after parenteral zinc injection regressed to the control level by week 16 (Tables, 4.2 ; 4.2b).

Intense immunostaining for MT within the cytoplasm of the villus enterocytes and Paneth cells was demonstrated at 20 hours post parenteral zinc injection and remained unchanged by day 3 (Figs, 4.7a, b).

b. Liver

Copper and zinc concentrations (20.7 ± 0.6 µg/g and 133.9 ± 2.2 µg/g respectively) showed no significant changes (p>0.05) throughout the trial. The elevation of liver zinc content after subcutaneous zinc injection during the first 3 days fell to normal levels at week 3 and thereafter (Tables, 4.3, 4.4 ; Figs, 4.3a, b, c, d. ; Figs, 4.4a, b).

4.3.2. Copper supplemented diet

a. Small intestine

Copper rose to 1.88 ± 0.12 µg/g at week 3, but falling subsequently to 1.26 ± 0.02 µg/g at week 16 (Table, 4.0 ; Fig, 4.0a). Zinc however remained constant

throughout the trial (9.32 ± 0.33 μg/g) (Table, 4.1 ; Fig, 4.1a). MT profile paralleled copper with a peak at week 3 (110.3 ± 10.5 μg/g) and regressed to the normal levels at week 16 (37.1 ± 5.9 μg/g) (Table, 4.2 ; Fig, 4.2a). At no time could MT peak be detected chromatographically (Figs, 4.8 - 4.11).

MT content which was elevated to about 14 fold the control at day 3 after parenteral zinc, continously regressed thereafter, but was still about double of the control by week 16 (Table, 4.2 ; Fig, 4.2b), whilst zinc content which was elevated to about double the control at day 3 after parenteral zinc injection fell to the control level at week 3 and thereafter (Table, 4.1 ; Fig, 4.1b). Copper content was elevated to a maximum level at week 5 (2.91 ± 0.26 μg/g) and regressed by week 16 (1.35 ± 0.06 μg/g) (Table, 4.0 ; Fig, 4.0b).

b. Liver

Copper rose to maximum concentration at week 5 (2380.9 ± 172.8 μg/g) and had partially regressed by week 16 (1936.0 ± 179.3 μg/g), whereas zinc remained constant (132.5 ± 2.3 μg/g). A similar sequence of copper retention was demonstrated in rats with parenteral zinc injection. Zinc content rose to about double the control at day 3 after parenteral zinc injection, but fell to the normal level by week 3 and thereafter (Tables, 4.3, 4.4 ; Figs, 4.3a, b, c, d ; Figs, 4.4a, b).

4.3.3. Zinc supplemented diet

a. Small intestine

Copper remained constant (0.41 ± 0.01 μg/g) throughout the trial, but was low overall ($p<0.05$) when compared to the controls (0.46 ± 0.01 μg/g). Zinc concentration remained unchanged (9.19 ± 0.17 μg/g). MT showed modest increases at week 3 and 5, but regressed to the normal level at week 16 (Tables, 4.0, 4.1, 4.2 ; Figs, 4.0a, 4.1a, 4.2a). Chromatographic studies failed to identified MT peak (Figs, 4.8 - 4.11), except at day 3 after parenteral zinc injection (Fig, 4.6.a).

MT rose to about 20 fold the control at day 3 after parenteral zinc injection, but regressed continously to the normal level by week 16 (Table, 4.2 ; Fig, 4.2b), whilst zinc concentration which was elevated to about double the control at day 3, returned to the normal level at week 3 and thereafter. Copper content remained low ($p<0.05$) at week 3 and thereafter (0.35 ± 0.01 μg/g) when compared to the controls (0.46 ± 0.01 μg/g) (Tables, 4.0, 4.1 ; Fig, 4.0b ; Fig, 4.1b).

Immunostaining for MT at day 3 after parenteral zinc injection was localised within the cytoplasm of the villus enterocytes and the Paneth cells.

b. Liver

Copper had declined significantly ($p<0.05$) at week 16 (14.3 ± 0.1 μg/g) when compared to the controls (20.7 ± 0.6 μg/g), but zinc remained normal. Similar patterns of copper and zinc retention were demonstrated in animals receiving parenteral zinc injection, apart from the elevation of zinc concentration to about double of the control at day 3 (Tables, 4.3, 4.4 ; Figs, 4.3b, d ; Figs, 4.4a, b).

4.3.4. Copper and zinc supplemented diet

a. Small Intestine

Copper rose to maximum by week 5 (1.65 ± 0.12 μg/g), but reduced by week 16 (1.29 ± 0.25 μg/g). MT content paralleled to copper retention, whilst zinc remained unchanged (9.78 ± 0.21 μg/g) (Tables, 4.0, 4.1, 4.2 ; Figs, 4.0a, 4.1a, 4.2a). Chromatographic studies failed to isolate MT peak, except at day 3 after parenteral zinc injection (Figs, 4.6b, 4.8 - 4.11).

Copper content in the parenteral zinc injection groups rose to reach maximum at week 3 (2.40 ± 0.27 μg/g), but were reduced by week 16 (1.42 ± 0.19 μg/g), whilst zinc content which rose to about double of the control at day 3, returned to the normal level at week 3 and thereafter (Tables, 4.0, 4.1 ; Figs, 4.0b, 4.1b). MT retention which was elevated to about 18 fold the control at day 3, regressed to the control level by week 16 (Table, 4.2 ; Fig, 4.2b).

Immunostaining for MT at day 3 after parenteral zinc injection was localised within the cytoplasm of the villus enterocytes and the Paneth cells.

b. Liver

Copper rose more slowly to reach maximum at week 5 (2061.5 ± 84.9 μg/g) and fell more precipitately by week 16 (826.4 ± 354.1 μg/g). At all times copper concentration was less than the copper supplemented diet alone ($p<0.05$), although at week 5 it was statistically insignificant. A similar sequence of copper retention, rise and fall, was demonstrated in parenteral zinc injection groups. Zinc content remained unchanged throughout the trial, except at week 5 which was mildly elevated ($p<0.05$) in the both groups and at day 3 after parenteral zinc injection (Tables, 4.3, 4.4 ; Figs, 4.3a, c ; Figs, 4.4a, b).

Table 4.0. **Copper:** small intestine soluble fraction

Dietary supplementatation	Small intestine soluble fraction copper (µg/g wet weight) Day 3	Week 3	Week 5	Week 16
1. Control	0.45 ± 0.02	-	0.48 ± 0.01	0.46 ± 0.02
2. Copper	0.93 ± 0.02	1.88 ± 0.12	1.23 ± 0.04	1.26 ± 0.02
3. Zinc	0.42 ± 0.00	0.43 ± 0.01	0.40 ± 0.02	0.40 ± 0.00
4. Copper + Zinc	0.90 ± 0.10	1.39 ± 0.27	1.65 ± 0.12	1.29 ± 0.25
Zinc (subcutaneous) 20 hours prior to dietary supplementation				
1. Control	1.01 ± 0.03	0.47 ± 0.02	0.47 ± 0.01	0.46 ± 0.02
2. Copper	1.23 ± 0.08	2.36 ± 0.47	2.91 ± 0.26	1.35 ± 0.06
3. Zinc	0.48 ± 0.03	0.36 ± 0.02	0.33 ± 0.01	0.35 ± 0.01
4. Copper + Zinc	1.23 ± 0.09	2.40 ± 0.27	1.71 ± 0.20	1.42 ± 0.19

* Zinc (subcutaneous) 20 hours = 2.38 ± 0.62 µg/g wet weight.

Each value is the mean and standard error of mean of 4 rats.

Table 4.1. **Zinc:** small intestine soluble fraction

Dietary supplementatation	Small intestine soluble fraction zinc (µg/g wet weight) Day 3	Week 3	Week 5	Week 16
1. Control	9.46 ± 0.16	-	9.83 ± 0.31	9.35 ± 0.11
2. Copper	8.99 ± 0.40	10.23 ± 0.40	10.08 ± 0.59	7.98 ± 0.69
3. Zinc	9.00 ± 0.32	9.23 ± 0.35	9.26 ± 0.44	9.27 ±0.36
4. Copper + Zinc	9.14 ± 0.40	9.87 ± 0.40	10.08 ± 0.46	10.03 ± 0.37
Zinc (subcutaneous) 20 hours prior to dietary supplementation				
1. Control	30.69 ± 6.48	9.14 ± 0.32	9.49 ± 0.31	9.51 ± 0.26
2. Copper	19.84 ± 0.98	10.08 ± 0.62	9.56 ± 0.11	9.32 ± 0.29
3. Zinc	21.86 ± 1.84	9.43 ± 0.25	9.47 ± 0.34	9.43 ± 0.25
4. Copper + zinc	23.41 ± 2.98	9.91 ± 0.37	11.47 ± 0.60	9.77 ± 0.40

* Zinc (subcutaneous) 20 hours = 33.64 ± 4.87 µg/g wet weight.

Each value is the mean and standard error of mean of 4 rats.

Table 4.2. **Metallothionein:** small intestine soluble fraction

Dietary supplementation	Small intestine soluble fraction metallothionein (µg/g) Day 3	Week 3	Week 5	Week 16
1. Control	38.0 ± 8.1	-	35.5 ± 7.6	36.6 ± 1.8
2. Copper	45.9 ± 10.1	110.3 ± 10.5	89.3 ± 10.3	37.1 ± 5.9
3. Zinc	53.8 ± 9.5	80.5 ± 9.6	69.5 ± 5.2	34.6 ± 5.0
4. Copper + Zinc	63.3 ± 10.0	92.6 ± 5.7	111.4 ± 19.4	46.8 ± 12.0
	Zinc (subcutaneous) 20 hours prior to dietary supplementation			
1. Control	665.0 ± 94.2	74.4 ± 8.4	61.1 ± 9.7	48.2 ± 7.8
2. Copper	543.0 ± 63.1	124.9 ± 4.8	111.2 ± 16.1	70.1 ± 6.5
3. Zinc	820.2 ± 187.5	102.2 ± 11.1	76.3 ± 4.2	44.9 ± 5.6
4. Copper + Zinc	699.8 ± 85.7	147.9 ± 20.1	81.6 ± 9.6	58.8 ± 9.8

* Zinc (subcutaneous) 20 hours = 1021.5 ± 110.4 µg/g.

Each value is the mean and standard error of mean of 4 rats.

Table 4.3. **Liver copper**

Dietary supplementation	Liver copper (µg/g dry weight)			
	Day 3	Week 3	Week 5	Week 16
1. Control	21.9 ±1.5	-	19.7 ± 0.2	20.6 ± 0.7
2. Copper	88.1 ± 16.6	2103.8 ± 137.8	2380.9 ± 172.8	1936.0 ± 179.3
3. Zinc	17.2 ± 0.4	17.2 ± 1.1	19.2 ± 0.6	14.3 ± 0.1
4 Copper+Zinc	33.1 ± 2.5	892.7 ± 156.4	2061.5 ± 84.9	826.4 ± 354.1
Zinc (subcutaneous) 20 hours prior to dietary supplementation				
1. Control	19.1 ± 1.4	18.2 ± 0.2	19.8 ± 0.3	19.7 ± 1.3
2. Copper	78.9 ± 20.5	1562.0 ± 95.1	2308.1 ± 106.4	1807.0 ± 365.5
3. Zinc	18.8 ± 0.4	17.5 ± 0.6	19.7 ± 0.5	15.4 ± 0.4
4. Copper+Zinc	54.5 ± 7.0	1333.0 ± 222.0	1921.0 ± 190.2	1687.2 ± 203.0

* Zinc (subcutaneous) 20 hours = 20.2 ± 0.8 µg/g wet weight.
Each value is the mean and standard error of mean of 4 rats.

Table 4.4. **Liver zinc**

Dietary supplementation	Liver zinc (µg/g dry weight)			
	Day 3	Week 3	Week 5	Week 16
1. Control	139.0 ± 2.7	-	131.6 ± 4.5	131.1 ± 3.6
2. Copper	130.5 ± 5.5	131.2 ± 4.6	137.0 ± 3.5	131.1 ± 5.4
3. Zinc	128.5 ± 4.3	123.3 ± 7.1	125.2 ± 2.3	124.7 ± 3.0
4 Copper + Zinc	121.6 ± 3.0	126.6 ± 5.3	152.7 ± 6.3	134.3 ± 5.4
Zinc (subcutaneous) 20 hours prior to dietary supplementation				
1 Control	333.7 ± 51.2	125.2 ± 3.6	122.4 ± 1.0	127.3 ± 4.5
2. Copper	227.8 ± 8.3	137.9 ± 3.9	150.4 ± 7.7	134.9 ± 4.2
3. Zinc	258.5 ± 27.9	125.2 ± 3.6	140.8 ± 3.1	127.2 ± 4.2
4. Copper + Zinc	223.6 ± 18.5	138.0 ± 3.0	148.4 ± 3.2	140.5 ± 4.7

* Zinc (subcutaneuos) 20 hours = 375.3 ± 41.8 µg/g wet weight.
Each value is the mean and standard error of mean of 4 rats.

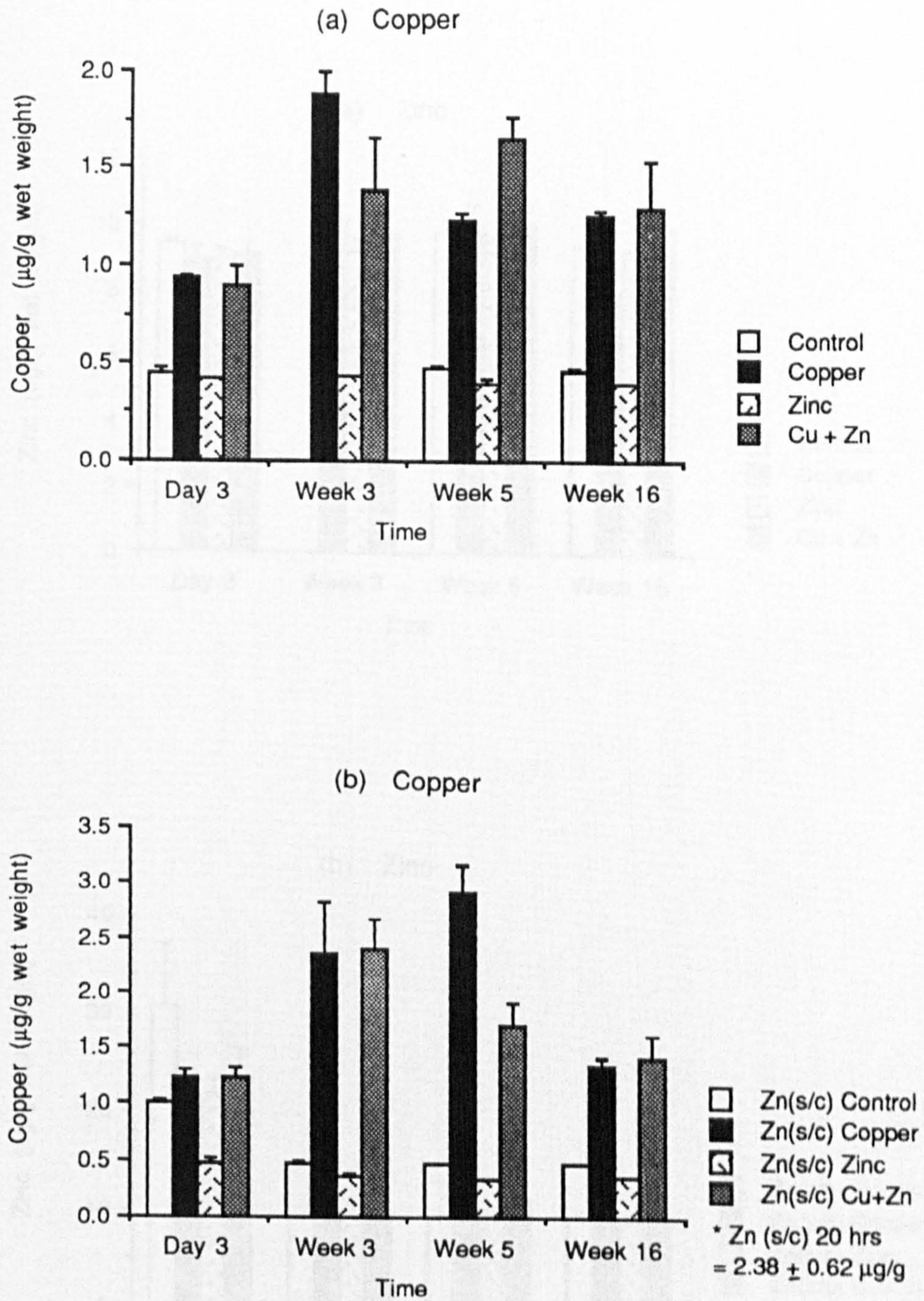


Fig. 4.0. **Small intestine soluble fraction copper.** (a) dietary supplemented and unsupplemented rats (b) plus subcutaneous zinc.

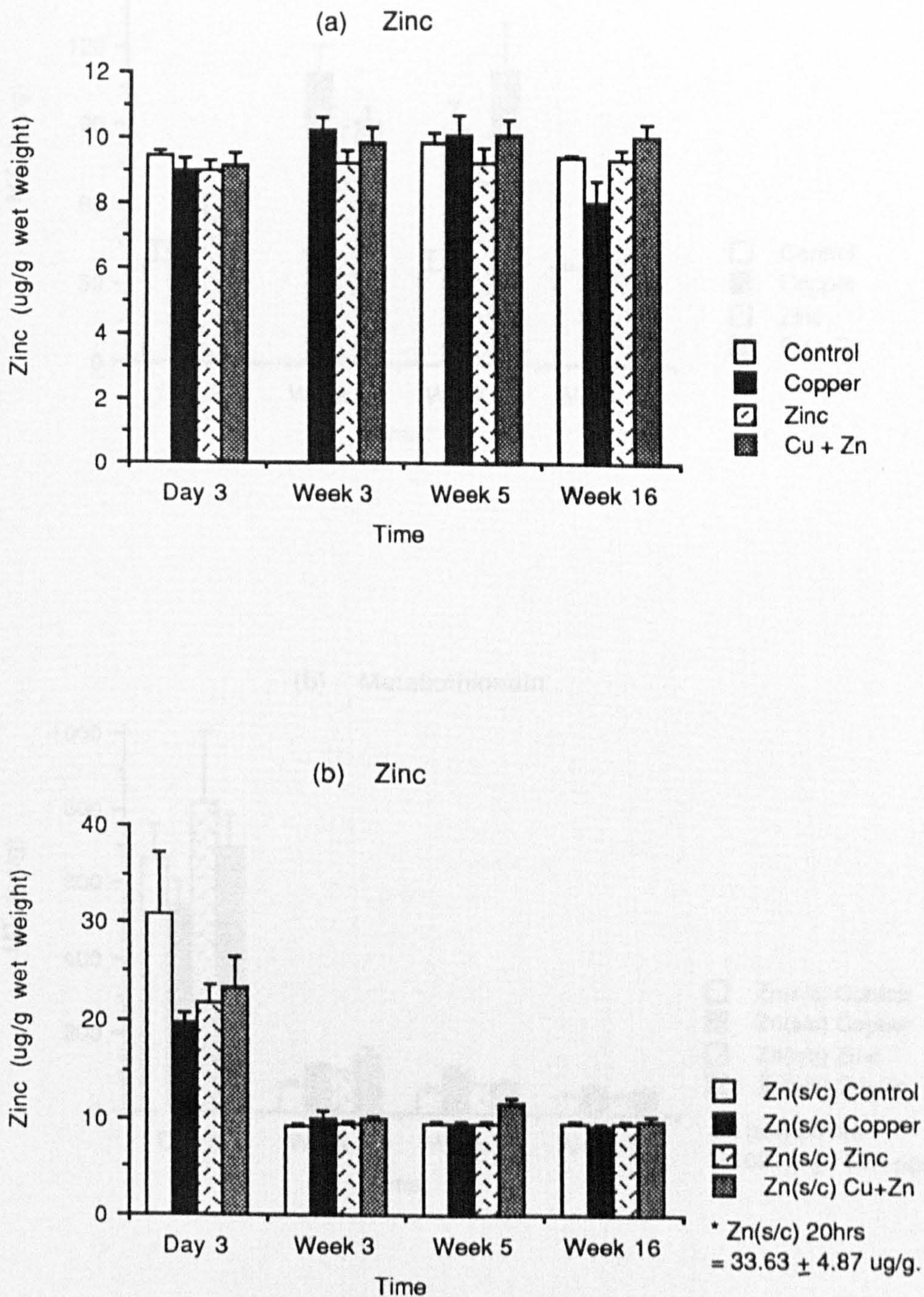


Fig. 4.1. **Small intestine soluble fraction zinc.** (a) dietary supplemented and unsupplemented rats (b) plus subcutaneous zinc.

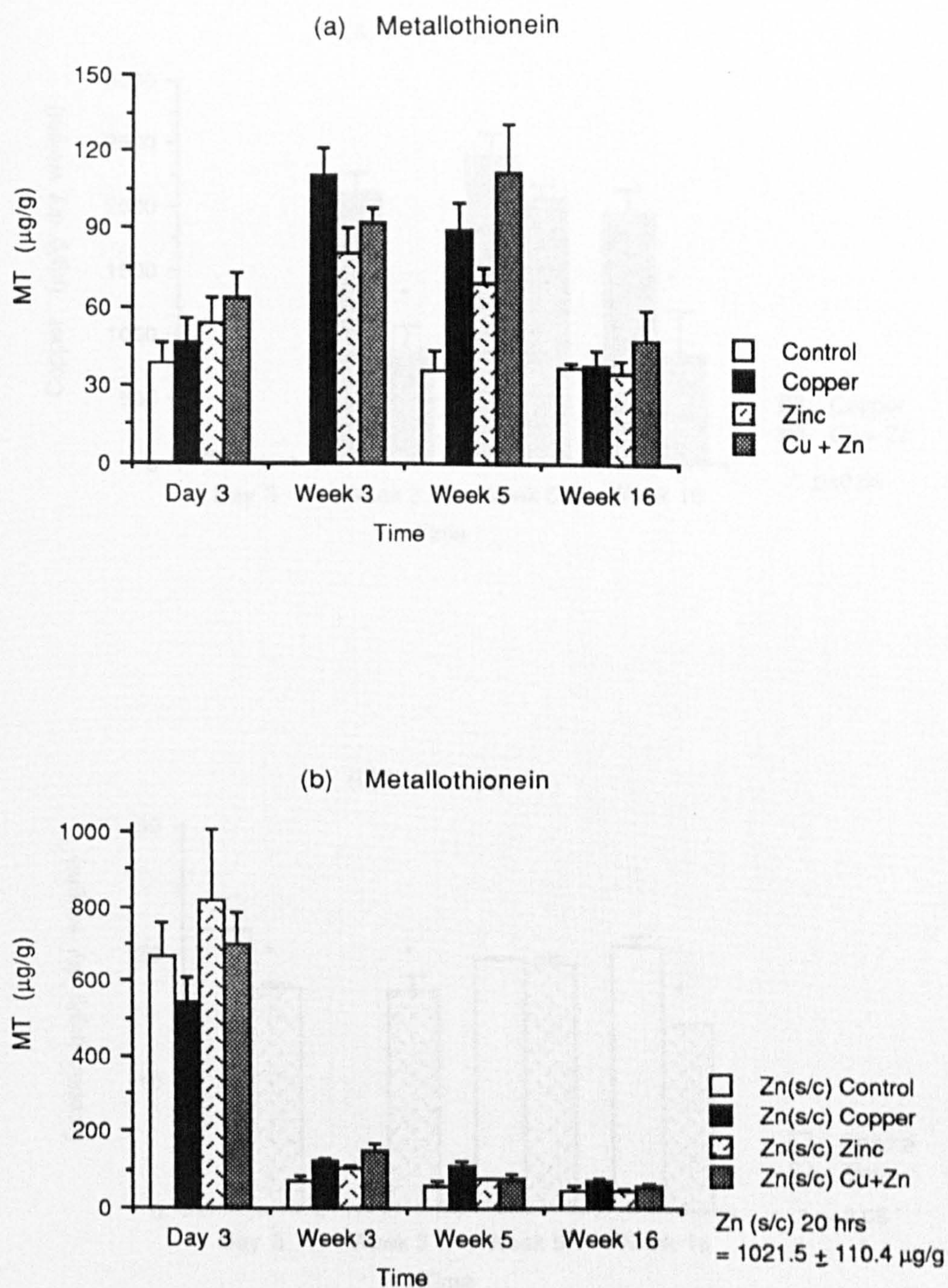
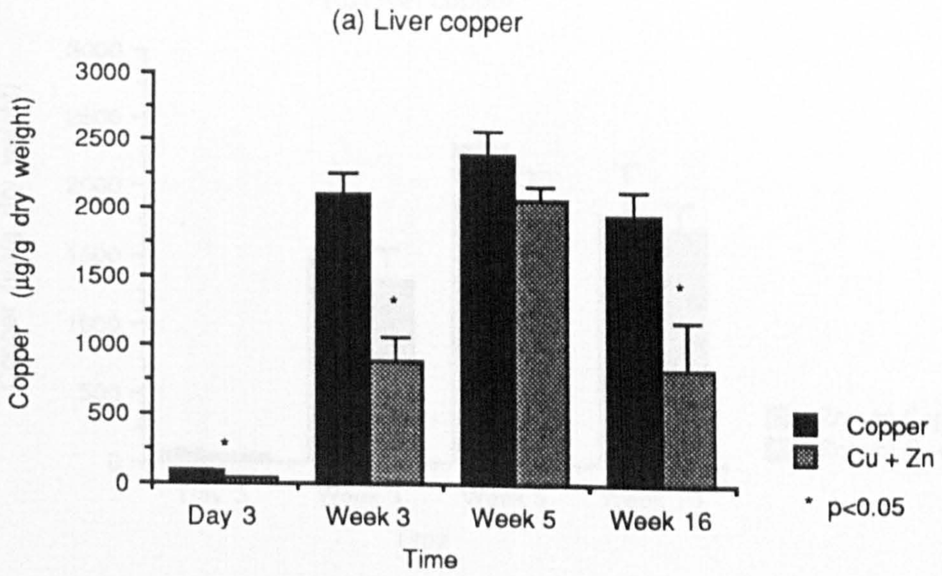


Fig. 4.2. **Small intestine soluble fraction metallothionein.** (a) dietary supplemented and unsupplemented rats (b) plus subcutaneous zinc.



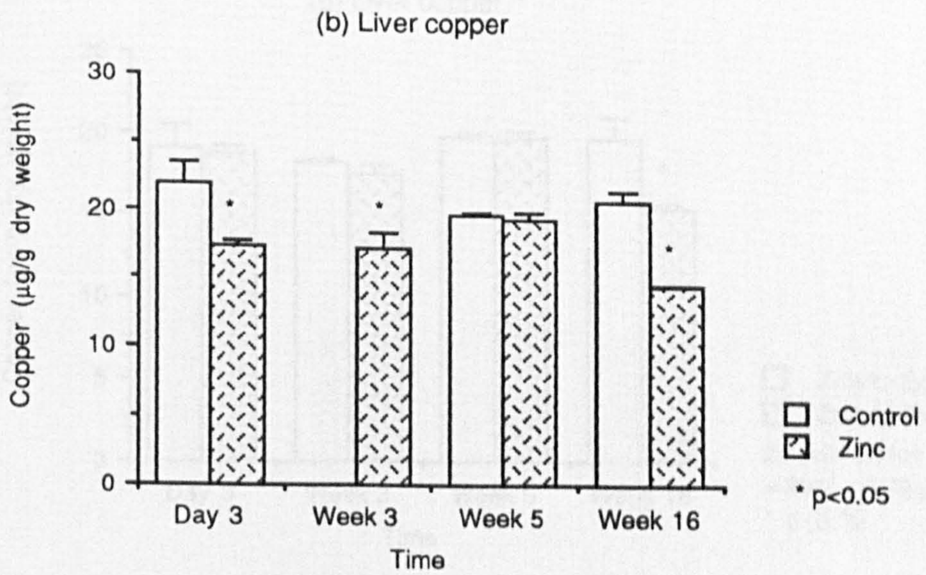
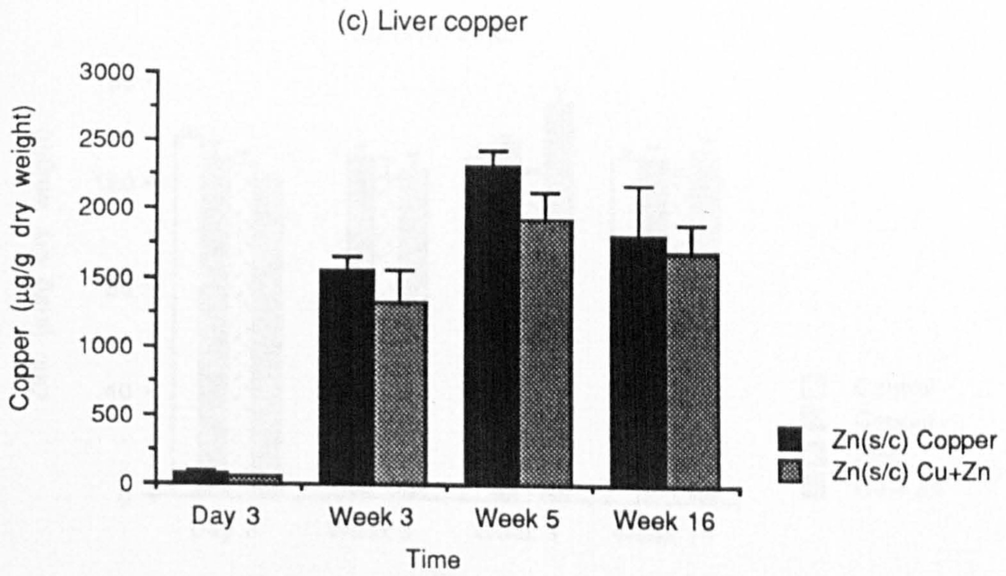


Fig. 4.3. **Liver copper.** Dietary supplemented and unsupplemented rats



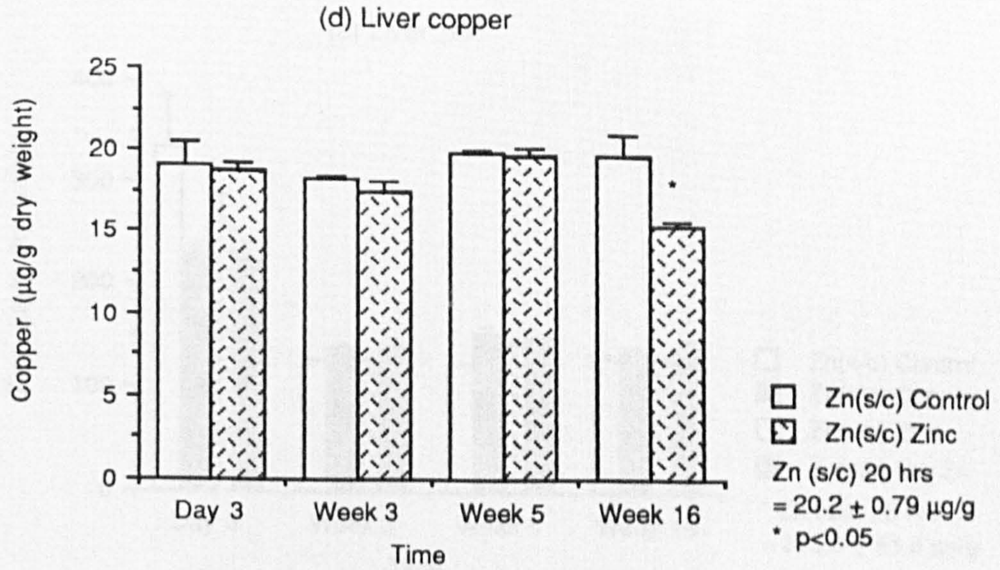


Fig. 4.3. **Liver copper.** Zinc (subcutaneous) prior to dietary supplemented and unsupplemented rats.

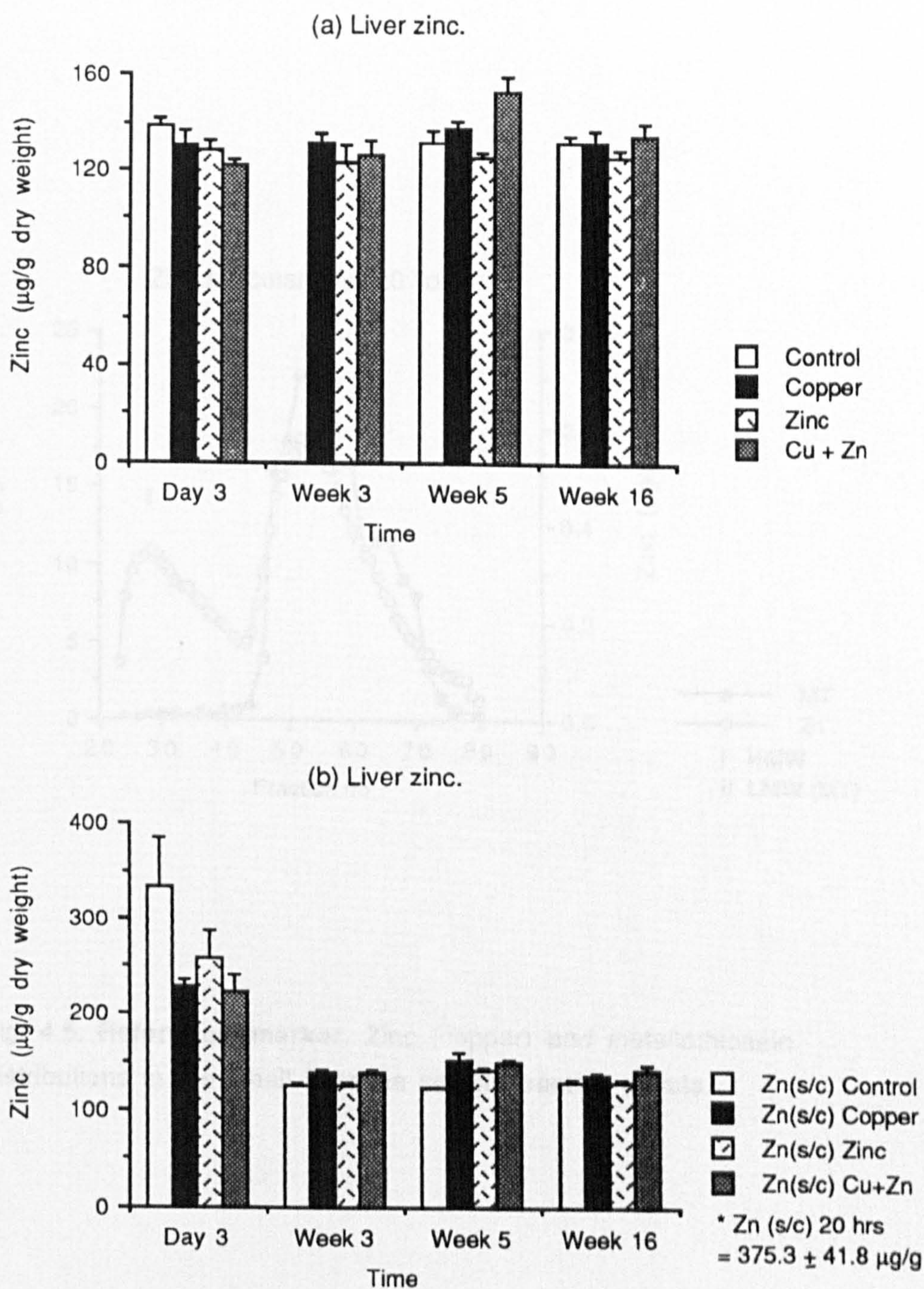


Fig. 4.4. **Liver zinc.** (a) dietary supplemented and unsupplemented rats (b) plus subcutaneous zinc.

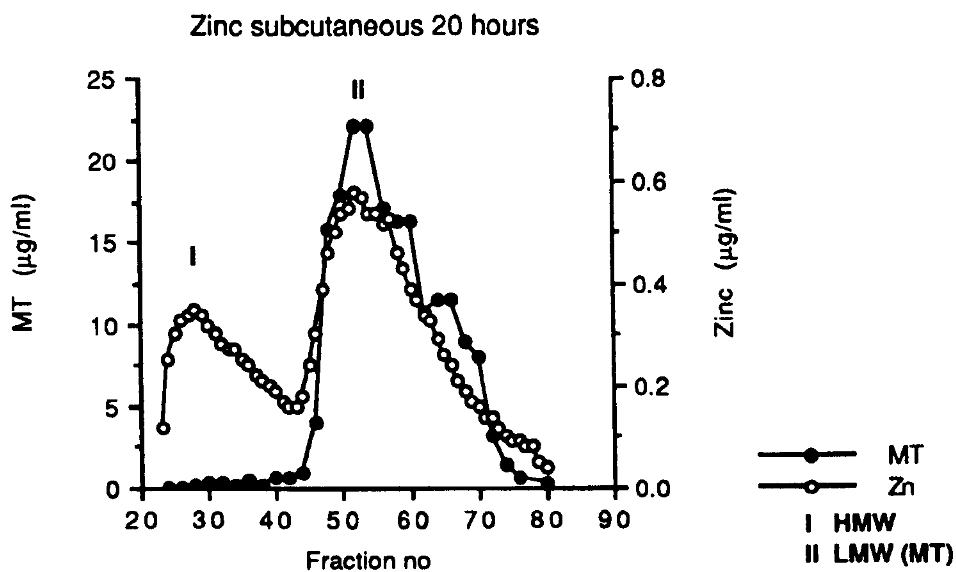


Fig. 4.5. **Reference marker.** Zinc (copper) and metallothionein distributions in the small intestine soluble fraction of rats.

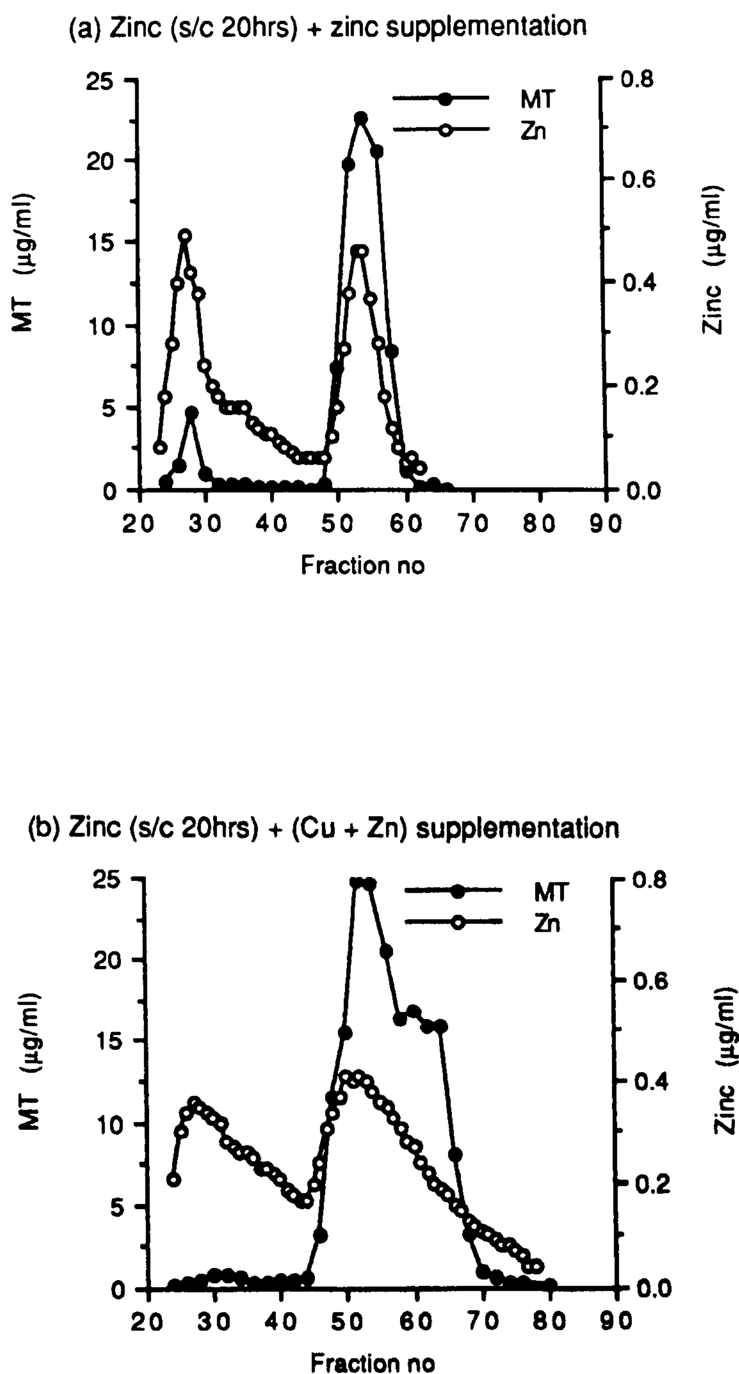
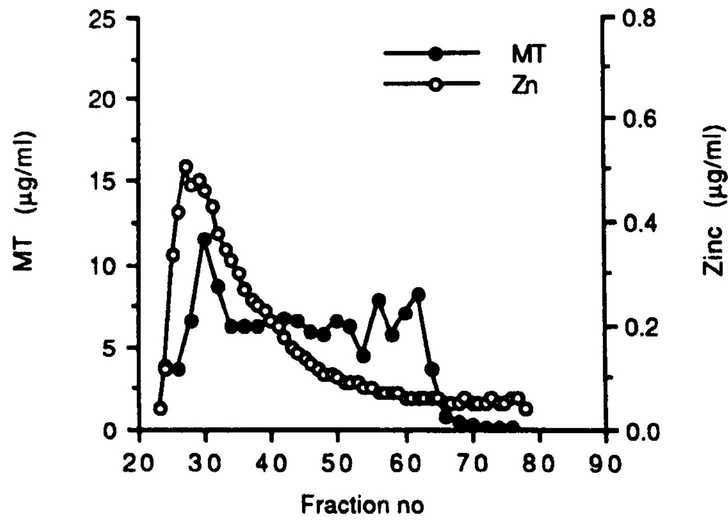


Fig. 4.6. **DAY 3.** Zinc (copper) and metallothionein distributions in the small intestine soluble fraction of rats.



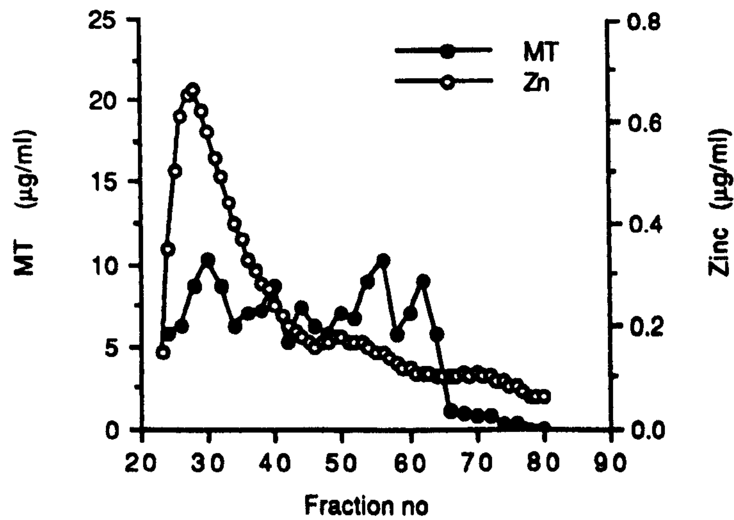
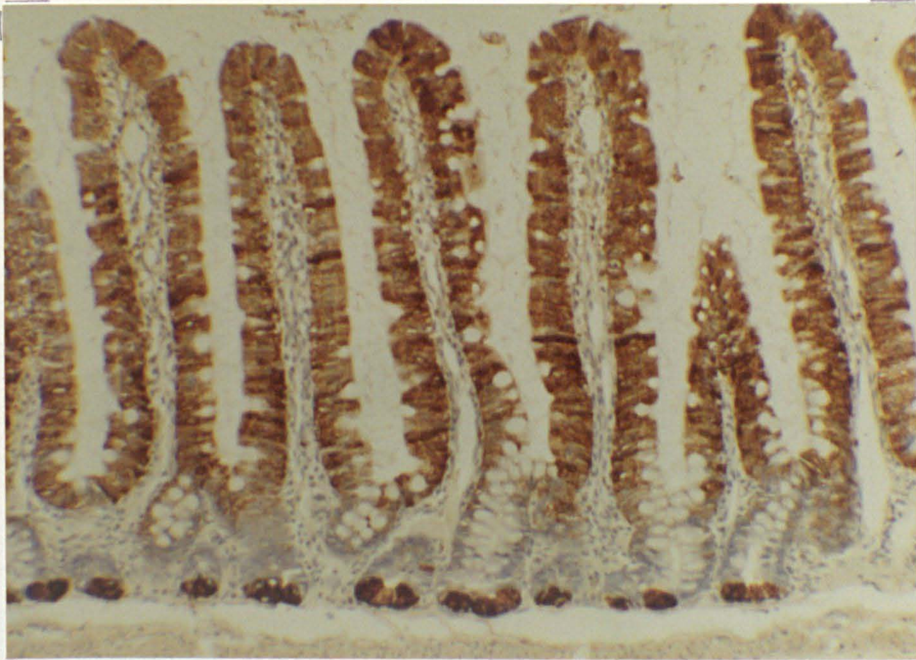
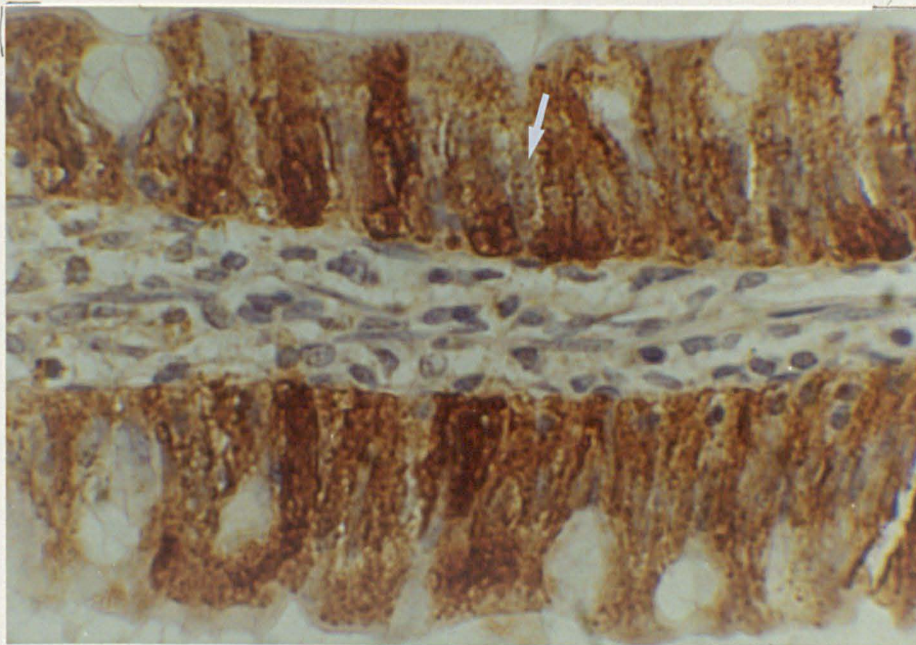


Fig. 4.6. **DAY 3. Zinc (copper) and metallothionein distributions in the small intestine soluble fraction of rats.**



(a)



(b)

Fig. 4.7. **Middle small intestine.** 20 hours after parenteral zinc injection. Intense immunoreactive staining for MT within the villus enterocytes and Paneth cells. Nuclei remained unstained (arrow). (a) X 100. (b) X 500. DNP-peroxidase.

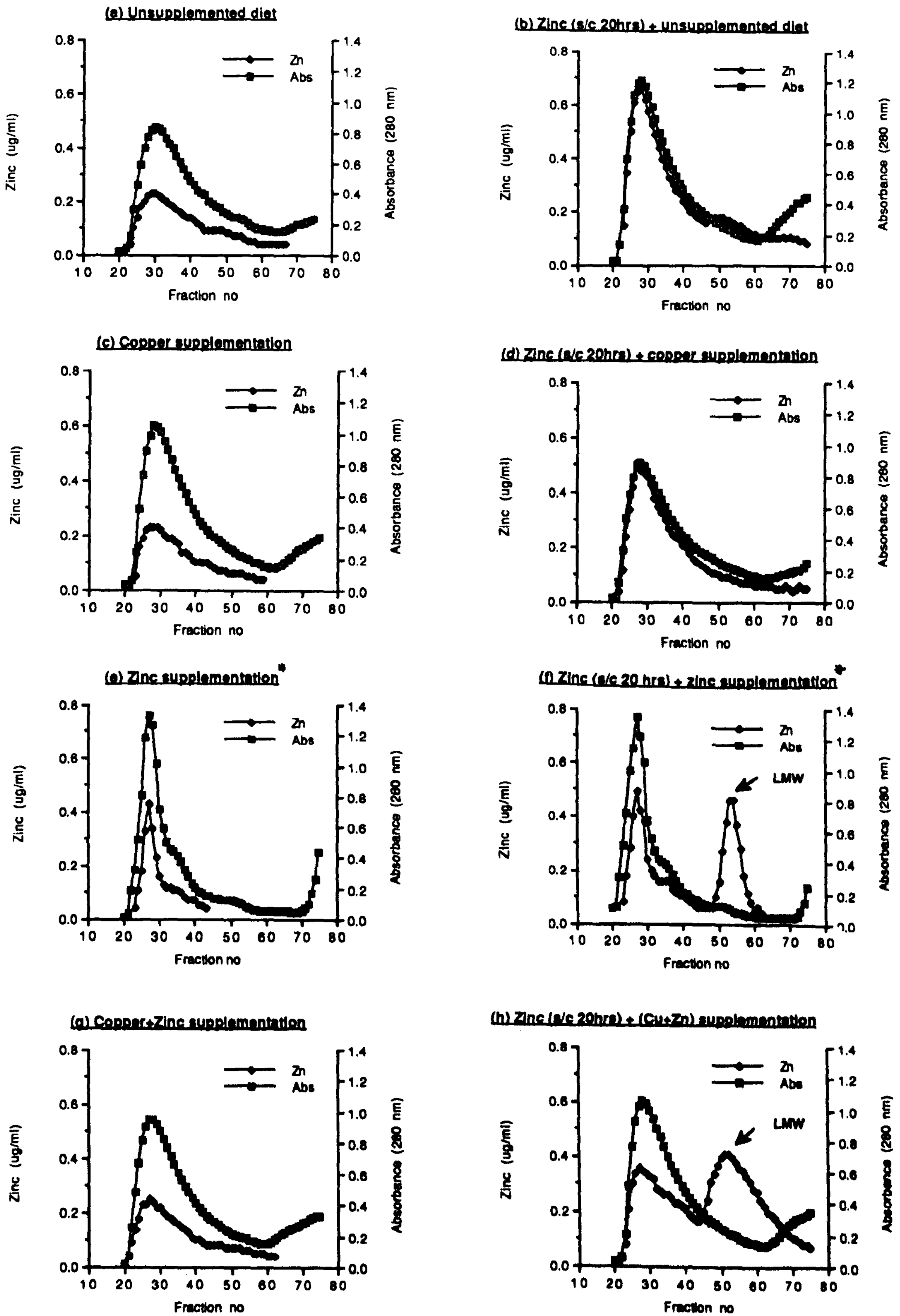


Fig. 4.8. **DAY 3**. Zinc (copper) distribution in the small intestine soluble fraction of rats. (*) Diluted in 2 volume (w/v).

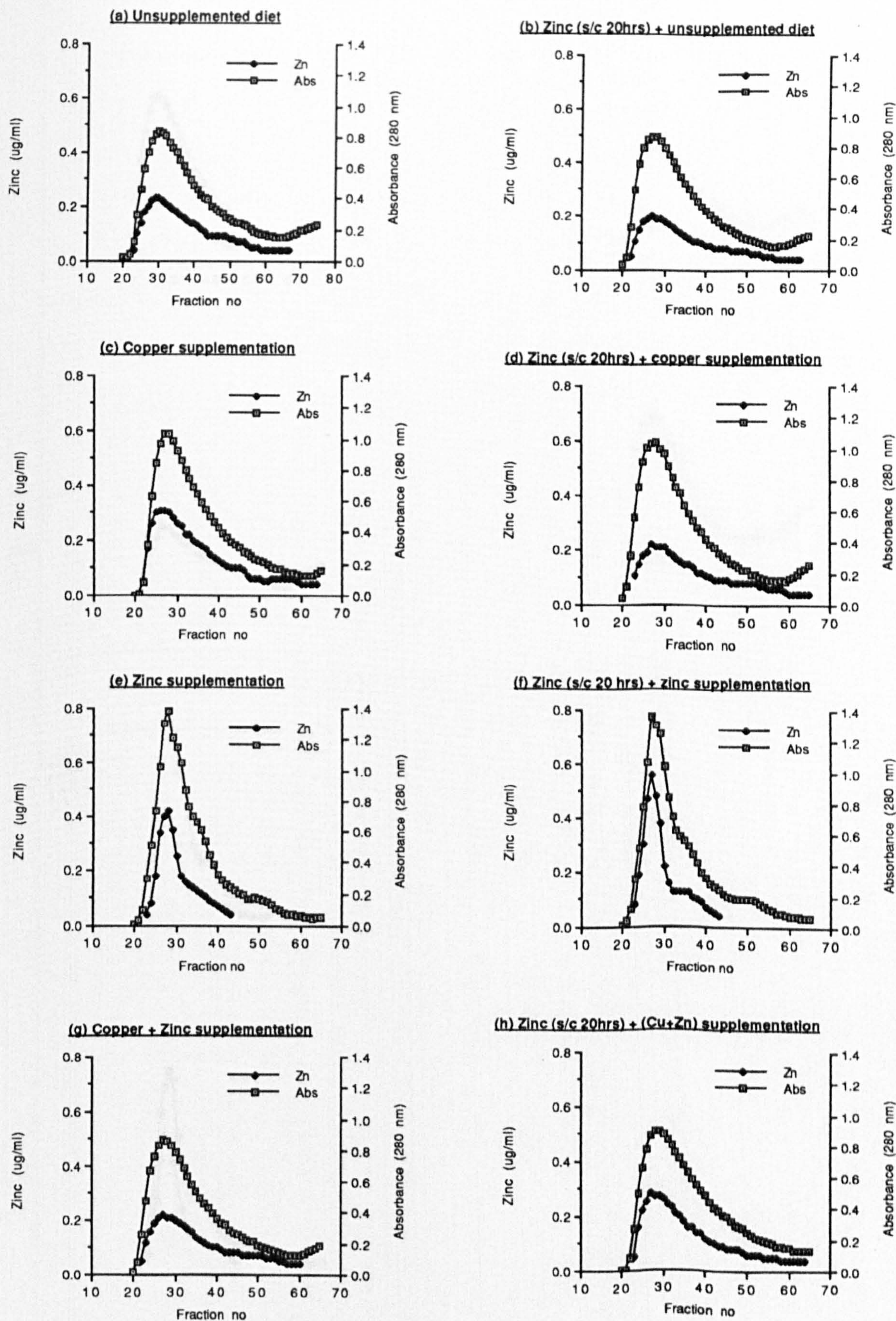


Fig. 4.9. **WEEK 3**. Zinc (copper) distribution in the small intestine soluble fraction of rats.

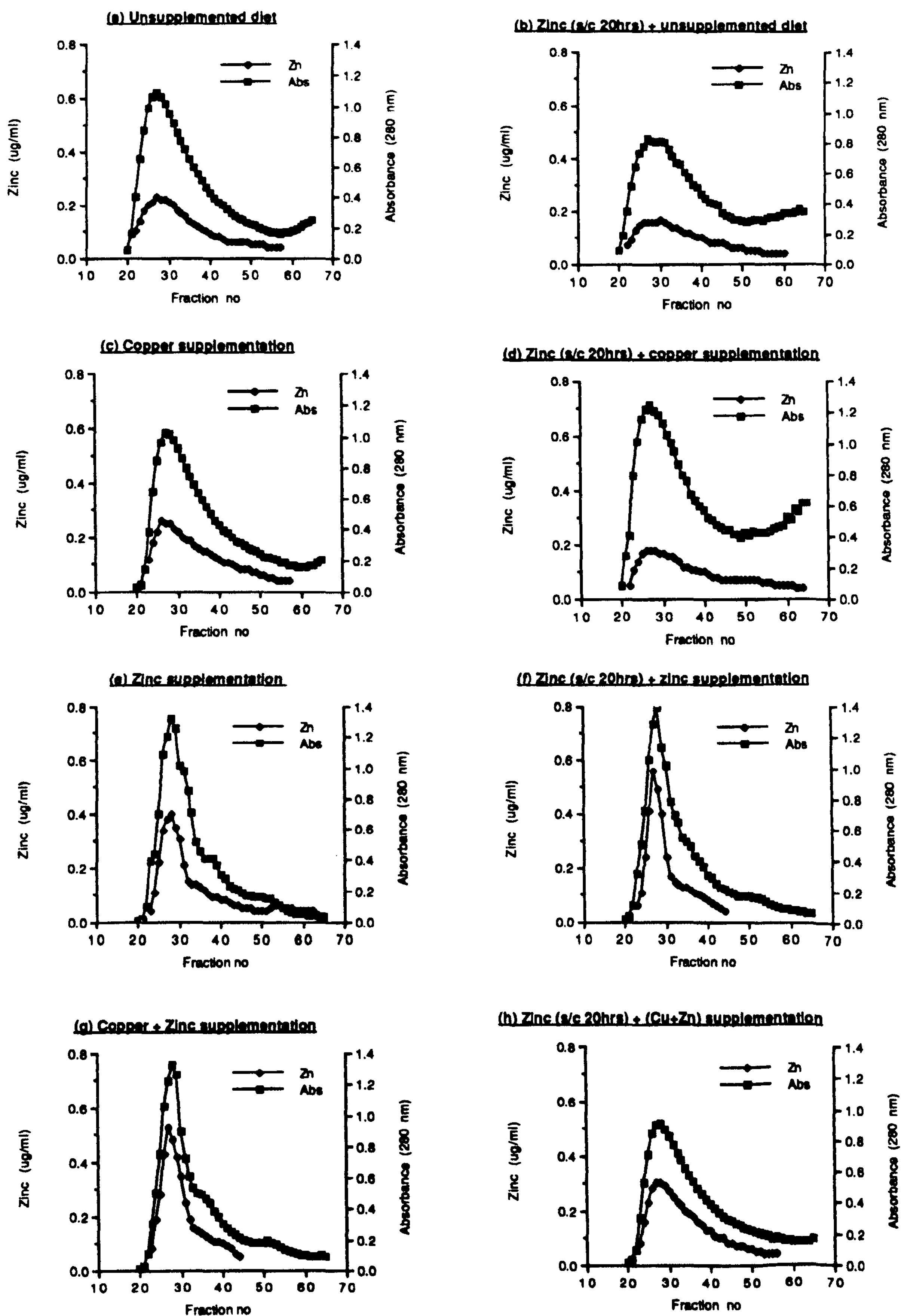


Fig. 4.10. **WEEK 5.** Zinc (copper) distribution in the small intestine soluble fraction of rats.

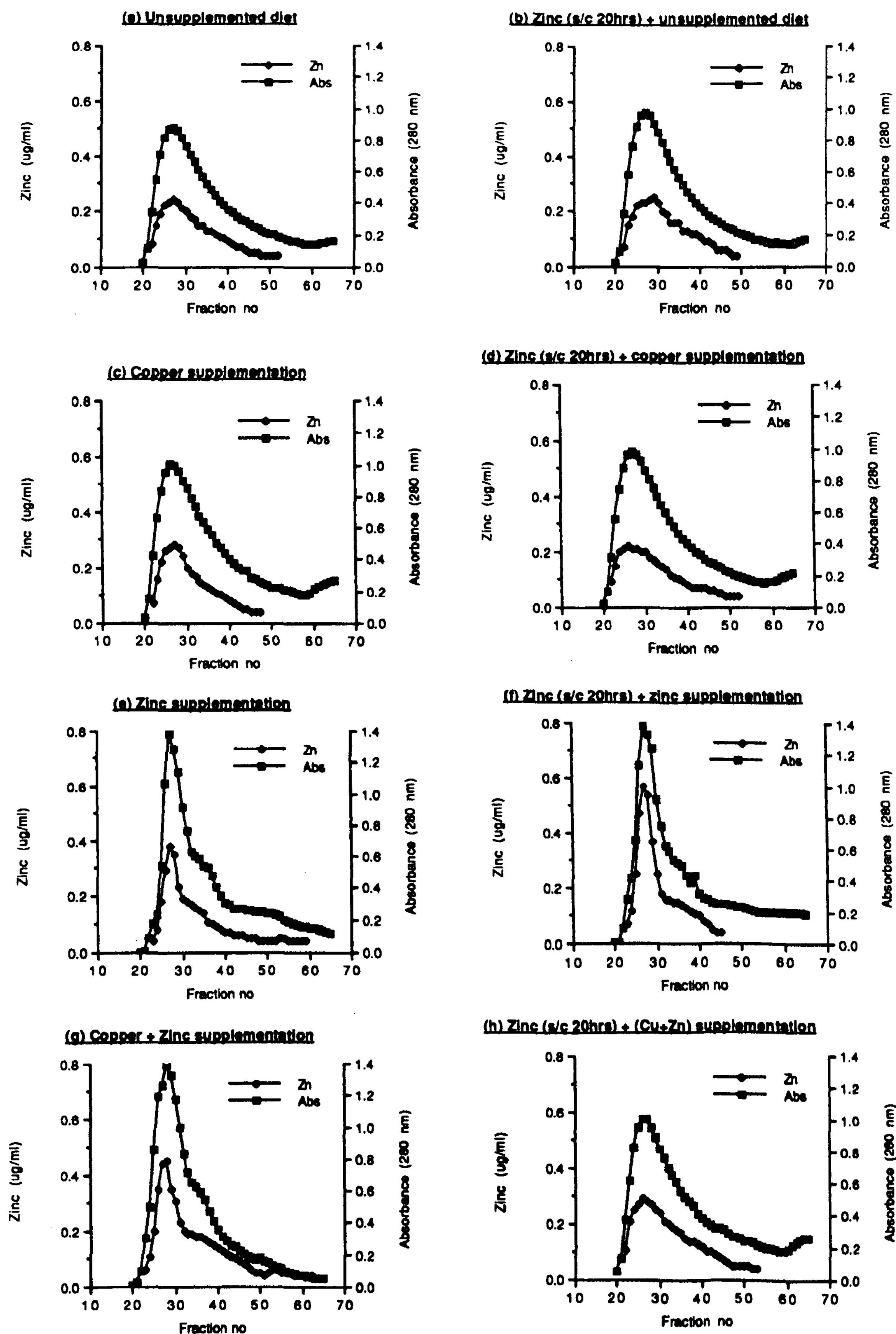


Fig. 4.11. **WEEK 16.** Zinc (copper) distribution in the small intestine soluble fraction of rats.

4.4. DISCUSSION

This study demonstrated unequivocally that zinc supplementation affects copper retention in both the liver and intestine in animals on copper supplemented and unsupplemented diets. In copper unsupplemented rats, additional zinc reduced intestinal and liver copper overall suggesting decreased copper absorption which was well established by 16 weeks. The effect of zinc on the copper supplemented animals was more complex and interpretation made more difficult by the natural process of adaptation which takes place in copper loaded states (Haywood, 1980, 1985).

The pattern of copper retention in the intestine of the copper supplemented groups alone follows that already established earlier (Chapters, 1, 2) whereby an initial rise in copper concentration is followed by a fall thereafter by week 16. An essentially similar copper profile occurs in the liver, an established pattern which has been reported many times previously (Haywood, 1980, 1985 ; Fuentealba, 1988 ; Evering, 1989) and demonstrates the development of copper tolerance which occurs in the normal copper loaded rat. In all, these changes would seem to reflect the transfer of copper to the liver via the intestinal tract and the functional modifications that occur with time reducing this transport (Chapters, 1, 3). The effect of zinc supplementation appears to further modify the process of natural adaptation whereby less copper becomes available for transport and hence liver copper accumulates more slowly and also is removed more quickly. The ratio of copper and zinc in the diet or at the luminal level may determine the antagonistic interaction of the metal and more studies require to be done on this aspect.

The unpredictable effect of zinc on hepatic copper accumulation or copper absorption has also been reported previously in Wilson's disease patients in response to zinc therapy (Callie-Bertrand et al., 1985 ; Hoogenraad and Van den Hamer, 1983), although it has also been reported that zinc supplementation can consistently inhibit hepatic copper accumulation (Hill et al., 1987). Furthermore, a zinc supplemented diet (420 μg/g) in sheep was found could inhibit hepatic copper accumulation (Bremner et al., 1976), but the inhibitory effect of zinc (543 μg/g)

was diminished with some elevation in copper content in the diets (Saylor and Leach, 1980). In laboratory animals high dietary zinc (900 μg/g) can inhibit copper absorption (Hall et al., 1979), but not at a lower concentration (180 μg/g) (Oestreicher and Cousins, 1985).

The inhibition of copper absorption in zinc supplemented animals may be associated with the elevation of excess copper retention within the intestinal mucosa bound to MT (Hall et. al., 1979 ; Fischer et al., 1983). Furthermore, it has been speculated that zinc induced intestinal MT could possibly sequester copper and make it unavailable for further absorption (Richards and Cousins, 1977 ; Ogiso et al.,1979 ; Hall et al., 1979 ; Fischer, 1981, 1983). However, the inability to increase MT in any marked or sustained manner after zinc supplementation in any of the dietary combinations is firm evidence that MT does not play a role in modifying copper uptake in this study. This failure appears even more marked after the demonstration that readily induced MT by parenteral zinc injection could not be maintained and further did not provide an enhanced barrier to copper accumulation in intestine or liver. This would appear to confirm the non importance of MT as a barrier to copper transport.

It must be construed moreover that interaction of copper and zinc takes place at the luminal level or within receptor sites on the brush border. Nevertheless, the ready inducibility of MT and its heavy concentration within the intestine after parenteral introduction of zinc requires explanation.

Metal retention within the intestinal tract may represent metal flux in the processes of absorption, excretion or storage. The elevation of intestinal zinc concentration and isolation of a zinc-MT fraction after parenteral zinc injection supports the previous reports (Richards and Cousins, 1975, 1977 ; Starcher et al., 1980 ; Jackson et al., 1986). Furthermore, parenteral zinc also has been reported to induce hepatic MT (Bremner et. al., 1973 ; Richard and Cousins, 1975 ; Lehman-McKeeman et al., 1988). MT is a low molecular weight protein (6000 - 7000 daltons) (Bremner, 1980), but it is isolated at about the same eluant volume as a

protein of 10000 daltons from chromatographic separation. This may possibly be due to the ellipsoidal configuration of the protein (Richards and Cousins, 1977). MT contains no aromatic acids and hence is poorly absorbed at 280 nm (Richards and Cousins, 1977).

The persistence of a zinc-MT peak at day 3 after zinc injection in zinc supplemented animals could be associated with the rate of MT degradation and the distribution of zinc within the fraction I (HMW) and fraction II (MT) of the protein constituents. High dietary zinc could possibly inhibit the rate of MT degradation and the transfer of the metal from peak II to peak I. The half life of hepatic and renal zinc MT has also been reported to be delayed in animals fed a zinc supplemented than unsupplemented (Whanger and Ridlington, 1982) or deficient zinc diets (Bremner et al., 1978). It has been suggested that the maintainence of the native structure of MT is dependent on the presence of bound metal and zinc may be an important determinant on the rate of MT degradation since this metal stablised the structure properties of the protein (Chen and Failla, 1989).

The isolation of a zinc-MT peak in zinc plus copper supplemented rats at day 3 after parenteral zinc was associated with an increase in copper retention, although, on the zinc supplemented diet alone the copper content remained unchanged. Furthermore, the MT peak failed to be demonstrated in both copper supplemented and unsupplemented rats, despite elevation in copper content. Thus, this may suggest that intestinal copper concentration can be influenced by factors other than MT. It appears that the intestinal copper uptake from the lumen into the absorptive cell can be inhibited when the luminal concentration of zinc is very much higher than copper and this may inhibit the absorption of copper.

Absorption studies using a vascular perfused rat intestine system demonstrated that high luminal zinc concentrations in the perfusate decreased the copper concentration in the mucosa cell cytosol and the amount transfered to the portal effluent (Oestreicher and Cousins, 1985). Furthermore, it has been demonstrated that parenteral zinc injection has no influence on ^{64}Cu absorption from the ligated

duodenum, but the absorption of the isotopes was depressed when both zinc and copper were administrated intraduodenally (Van Campen and Scaife, 1967).

The localization of immunoreactive staining for MT within the cytoplasm of the villus enterocytes and Paneth cells after parenteral zinc injection suggests that MT may play an important role as a transport protein in the elimination of excess zinc from the body. The role of Paneth cells in the excretion of zinc has been suggested previously (Miller et al., 1961 ; Elmes, 1976 ; Sandow and Whitehead, 1979 ; Dinsdale, 1984). Zinc bound to MT within the enterocytes could be excreted into the intestinal lumen following cellular extrusion.

It appears that rats can adapt to excess zinc. Furthermore, apart from being less toxic than copper (Bises et al., 1989), intestinal and hepatic zinc retention in zinc supplemented rats remained unchanged throughout the trials, suggesting that the absorption of the metal is inhibited or/and there is increased excretion. This may partly explain the lower incidence of zinc toxicity in animals when compared to copper (Underwood, 1977).

In conclusion this study has added support to the work of others that high dietary zinc can depress hepatic copper retention as a result of reduced transfer of copper across the gastrointestinal barrier. This interaction takes place at the luminal level and may involve unknown complex mechanisms, but is not mediated by MT. It appears that MT may rather act as a temporary transport protein as much for export as for import and does not serve to inhibit copper absorption.

CHAPTER : 5

The Absorption of Copper (^{64}Cu) in Copper and Zinc Supplemented Rats

5.1. INTRODUCTION

Copper is absorbed from the gastrointestinal tract (GIT), accumulated mainly in the liver and excess excreted through the bile (Evans, 1973 ; Cousins, 1985). The absorption of the metal seems to involve two separate mechanisms : the uptake of copper from the lumen into the absorptive cell and the transfer of the metal from the cell to the portal circulation (Crampton et al., 1965) and has been reported to occur mainly in the stomach and the upper small intestine (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984). Studies with both stable and radioactive copper have shown the metal was poorly absorbed (Underwood, 1977). The absorption of the metal was further reduced in response to high ^{64}Cu dosage (Mistilis and Farrer, 1968 ; Marceau et al., 1970) and in dietary copper supplementation (Bremner et al., 1979 ; Johnson and Lee, 1988). The previous studies (Chapters, 1-4) also suggest that the absorption of the metal in copper tolerant rats (Haywood, 1980, 1985) may be inhibited.

The reasons for this reduced absorption are little understood, although it has been speculated that copper may bind to metallothionein (MT), a low molecular weight copper binding protein within the enterocytes making the metal unavailable for absorption (Evans and Johnson, 1978). However, the previous studies in copper supplemented rats have shown that elevation of intestinal MT did not correspond to the hepatic copper accumulation suggesting that the protein is not directly associated with the absorption of the metal (Chapters, 2, 4).

It seems more likely that the gastrointestinal tract adapts by various means to high dietary copper in order to limit absorption (Chapters, 1, 3). Thus, copper may bind to the mucus and become less available for absorption, particularly with the possible elevation in the pH of the stomach. Alternatively, a defect in the transport of

copper from the absorptive cell to the portal circulation as occurs in Menkes' disease patients may become operable (Danks et al., 1973 ; Bremner, 1987a). However, endogenous copper may also be excreted from the gastrointestinal tract (Mahoney et al., 1955 ; Owen, 1964) and there is evidence to suggest that copper can be excreted from the Paneth cells in the intestinal tract (Chapter, 3). Furthermore, excess copper within the enterocytes can be eliminated through cellular extrusion (Chapters, 1, 3). This may enhance excretion and effectively reduce hepatic build up of the metal.

Likewise, zinc has also been shown to interfere with hepatic build up of copper; an effect that occurs at the luminal level and is unassociated with MT (Chapter, 4).

It is the aim of this study to assess the absorption of copper (^{64}Cu) from the gastrointestinal tract, in both copper and zinc supplemented rats and thereby confirm or otherwise that the reduced hepatic build up of copper is due to inhibited absorption of the metal.

5.2. MATERIALS AND METHODS

5.2.1. Animals

Ten-week-old male Wistar rats of uniform weight (344 ± 19 (mean ± SD)) were randomly selected and allocated in groups of 4 into plastic cages with woodchips bedding. They were fed a pelleted copper supplemented (1000 mg/kg) or zinc supplemented (1000 mg/kg) diet. Controls were included and fed the unsupplemented diet (Labsur Animal Diet, Lavender Mill, Manea Cambridgeshire) (Appendix, 1.0). Food and tap water were freely available and animals were regularly observed. The individual body weight and dietary food intake per group were recorded weekly (Appendix, 5.0, 5.1). Each group of animals was euthanised at intervals of 1, 3, 5 and 16 weeks. They were deprived overnight of food prior to killing.

5.2.2. Sampling procedure

Radioactive copper (^{64}Cu) with initial specific activity of 1.5 to 2.5 milicuries (mc) per 4.98 mg copper as anhydrous copper sulfate (Aldrich Chemical Co. Ltd.) was provided by the Universities Research Reactor, Risley Warrington Cheshire. It was diluted to a copper concentration of 100 µg per ml using distilled water. One ml of the radioactive solution (100 µg Cu) was administered intragastrically using a 16G blunt-ended needle cannula into each animal under mild diethyl ether anaesthesia (Waynforth, 1980). Four hours later (Appendix, 5.2 ; Hall et al., 1979 ; Van Barneveld and Van den Hamer, 1984) animals were put under general anaesthesia using ether and blood was collected from the posterior vena cava and kept in lithium heparin. Animals were subsequently killed by cervical dislocation and the liver and kidneys were retained. The gastrointestinal tracts were subdivided into the stomach, small intestine, caecum and colon. The wet weights of the liver and kidneys were recorded (Appendix, 5.3).

Radioactivity of the gastrointestinal subdivisions, liver, kidneys and triplicate (duplicate) 2.0 ml blood samples were immediately analysed in an automatic gamma counter (Riastar 5400, Packard Instrument Company). The radioactivity of the dose

solution was included in each analysis and was used as a reference in the appropriate corrections for the decay of the isotopes (Appendix, 5.5).

Results are expressed as the percentage of dose administered per minute (mean $\pm$ SEM). The proportion of absorbed ^{64}Cu distributed in the liver, kidneys and blood was also calculated (Appendix, 5.6). Values for blood were arrived by multipling the counts per minute per ml of blood by 7% of the body weight (Van Campen and Mitchell, 1965).

5.2.3. Stastical analysis

Stastical analysis was performed using Student's t-test in cricket software (version 1.1) for Macintosh (Rafferty et al., 1985).

5.3. RESULTS

5.3.1. ^{64} Cu absorption Week 1

a. Gastrointestinal tract

Total retention of ^{64}Cu in the copper supplemented rats (70.1 ± 4.0 %) was increased ($p<0.05$) when compared to the controls (48.3 ± 3.1 %). ^{64}Cu was disproportionately retained in the stomach at the expense of the small intestine and caecum (Table, 5.0 ; Fig, 5.0a).

Total ^{64}Cu retention in zinc supplemented groups (54.0 ± 4.4 %) did not differ significantly ($p>0.05$) from the controls (48.3 ± 3.1 %). The differential distribution of ^{64}Cu throughout the gastrointestinal did not differ from controls, except for the colon in which was increased ($p<0.05$) (Table, 5.0 ; Fig, 5.0a).

b. Blood, liver and kidneys

^{64}Cu concentrations in the liver (2.1 ± 0.1 %) and kidneys (0.9 ± 0.1 %) of the copper supplemented rats were decreased ($p<0.05$) when compared to the controls (2.7 ± 0.1 % and 1.1 ± 0.1 % respectively). The blood ^{64}Cu remained unchanged (Table, 5.1 ; Fig, 5.0b). However, the proportions of absorbed ^{64}Cu in the blood, liver and kidneys were significantly elevated (Fig, 5.0c).

^{64}Cu content in the blood, liver and kidneys of zinc supplemented rats remained unchanged when compared to the controls (Table, 5.1,. ; Figs, 5.0b, c).

5.3.2. ^{64} Cu absorption Week 3

a. Gastrointestinal tract

^{64}Cu retention was higher ($p<0.05$) in the copper supplemented groups (37.6 ± 2.4 %) than the controls (25.0 ± 2.5 %). The retention of the isotopes was increased ($p<0.05$) in the stomach and although elevated in the small intestine and caecum, this was not significant ($p>0.05$). However, ^{64}Cu was decreased ($p<0.05$) in the colon (Table, 5.0 ; Fig, 5.1a).

Total ^{64}Cu retention was now increased ($p<0.05$) in zinc supplemented rats (40.1 + 4.5 %) when compared to the controls (25.0 + 2.5 %). In contrast to copper supplemented rats, ^{64}Cu in zinc supplemented rats passed rapidly through the upper gastrointestinal tract to become concentrated in the caecum (Table, 5.0 ; Fig, 5.1a).

b. Blood, liver and kidneys

^{64}Cu content in the blood (1.5 + 0.1 %) and liver (2.5 + 0.6 %) of the copper supplemented rats remained unchanged ($p>0.05$) when compared to the controls (1.5 + 0.1 % and 1.50 + 0.1 % respectively), but significantly decreased ($p<0.05$) in the kidneys. However, the proportion of absorbed ^{64}Cu was increased ($p<0.05$) in the liver and blood and remained unchanged in the kidneys (Table, 5.1 ; Figs, 5.1b, c).

^{64}Cu content in the blood, liver and kidneys of zinc supplemented rats remained unchanged ($p>0.05$) when compared to the controls, although the proportion of absorbed ^{64}Cu in the blood was significantly elevated ($p<0.05$) (Table, 5.1 ; Figs, 5.1b, c).

5.3.3. ^{64}Cu absorption Week 5

a. Gastrointestinal tract

^{64}Cu retention was elevated ($p<0.05$) in copper supplemented rats (38.8 +3.4 %) when compared to the controls (25.4 + 2.0 %). ^{64}Cu was significantly increased ($p<0.05$) in the stomach and colon, but not the small intestine and caecum (Table, 5.0 ; Fig, 5.2a).

Total ^{64}Cu retention in zinc supplemented rats (39.9 + 5.3 %) was increased when compared to the controls (25.4 + 2.0 %). ^{64}Cu was markedly elevated ($p<0.05$) in the colon (Table, 5.0 ; Fig, 5.2a).

b. Blood, liver and kidneys

^{64}Cu content in the kidneys of copper supplemented rats (0.4 + 0.1 %) was decreased ($p<0.05$) when compared to the controls (0.6 + 0.1 %), but the blood and liver contents remained unchanged (Table, 5.1 ; Fig, 5.2b). The proportion of absorbed

^{64}Cu was elevated in the liver, but remained constant in the blood and kidneys (Fig, 5.2c).

^{64}Cu concentration in the blood, liver and kidneys of zinc supplemented rats remained unchanged ($p>0.05$) when compared to the controls, however, the proportion of absorbed ^{64}Cu in the liver and blood were increased ($p<0.05$) (Figs, 5.2b, c).

5.3.4. ^{64}Cu absorption Week 16

a. Gastrointestinal tract

^{64}Cu retention in the copper supplemented groups (47.9 ± 1.7 %) was elevated when compared to the controls (27.3 ± 3.2 %). The retention of the isotopes was significantly elevated ($p<0.05$) in the stomach, but not the small intestine, caecum and colon (Table, 5.0 ; Fig, 5.3a).

Total ^{64}Cu retention was higher ($p<0.05$) in the zinc supplemented rats (43.6 ± 1.7%) than the controls (27.3 ± 3.2 %). ^{64}Cu retention remained unchanged in the stomach, but was markedly elevated ($p<0.05$) in the colon (Table, 5.0 ; Fig, 5.3a).

b. Blood, liver and kidneys.

^{64}Cu concentrations in both the blood (2.1 ± 0.1 %) and kidneys (0.5 ± 0.1 %) were decreased ($p<0.05$) in copper supplemented rats at the last sampling point when compared to the controls (2.8 ± 0.2 % and 0.8 ± 0.0 % respectively), but remained unchanged in the liver (Table, 5.1 ; Fig, 5.3b). However, the proportion of absorbed ^{64}Cu in both the blood and kidneys remained unchanged, but was elevated ($p<0.05$) in the liver (Fig, 5.3c).

^{64}Cu concentration in the blood, liver and kidneys remained unchanged in zinc supplemented groups when compared to the controls (Table, 5.0 ; Fig, 5.3b), although the proportion of absorbed ^{64}Cu in the liver was significantly increased ($p<0.05$) (Fig, 5.3c).

Table 5.0

Distribution of ^{64}Cu in the gastrointestinal tissues and contents of control, copper and zinc supplemented rats after 4 hours intragastric administration of the isotopes

Time (wks)	Dietary supplementation	^{64}Cu retained, counts per minute (% of dose) Stomach	Small intestine	Caecum	Colon	Total retention
1	Control	4.1 ± 1.0	20.1 ± 3.5	23.2 ± 2.6	0.9 ± 0.2	48.3 ± 3.1
	Copper	41.6 ± 5.8*	11.4 ± 2.5	15.3 ± 6.1	1.8 ± 0.6	70.1 ± 4.0*
	Zinc	3.6 ± 0.9	24.3 ± 2.2	22.5 ± 5.4	3.6 ± 0.7*	54.0 ± 4.4
3	Control	3.2 ± 1.4	8.2 ± 1.1	10.7 ± 2.8	2.9 ± 1.2	25.0 ± 2.5
	Copper	10.3 ± 0.8*	12.2 ± 2.4	14.9 ± 0.8	0.2 ± 0.0*	37.6 ± 2.4*
	Zinc	0.8 ± 0.1	10.5 ± 3.7	28.6 ± 2.2*	0.2 ± 0.0*	40.1 ± 4.5*
5	Control	2.0 ± 0.8	6.8 ± 1.9	16.3 ± 2.8	0.3 ± 0.0	25.4 ± 2.0
	Copper	6.1 ± 1.0*	9.1 ± 2.9	20.1 ± 3.1	3.5 ± 0.4*	38.8 ± 3.4*
	Zinc	1.6 ± 0.3	8.7 ± 2.5	25.0 ± 6.4	4.7 ± 0.5*	39.9 ± 5.3*
16	Control	1.4 ± 0.6	3.7 ± 0.7	15.4 ± 1.0	6.9 ± 1.9	27.3 ± 3.2
	Copper	8.5 ± 2.8*	12.0 ± 4.1	19.7 ± 4.4	7.7 ± 2.7	47.9 ± 1.7*
	Zinc	1.4 ± 0.4	8.9 ± 2.1	18.2 ± 3.9	15.1 ± 2.0*	43.6 ± 1.7*

Each value is the mean and standard error of mean of 4 rats. (*) $p<0.05$.

Table 5.1

Distribution of ^{64}Cu in the blood, liver and kidney of control, copper and zinc supplemented rats after 4 hours intragastric administration of the isotopes

Time (wks)	Dietary supplementation	^{64}Cu absorbed, counts per minute (% of dose) Blood	Liver	Kidneys
	Control	6.5 ± 0.2	2.7 ± 0.1	1.1 ± 0.1
1	Copper	6.4 ± 0.4	2.1 ± 0.1*	0.9 ± 0.1*
	Zinc	6.4 ± 0.4	2.8 ± 0.2	1.2 ± 0.1
	Control	1.5 ± 0.1	1.5 ± 0.1	0.6 ± 0.1
3	Copper	1.5 ± 0.1	2.5 ± 0.6	0.4 ± 0.1*
	Zinc	1.6 ± 0.1	1.6 ± 0.2	0.6 ± 0.1
	Control	2.0 ± 0.2	1.1 ± 0.1	0.6 ± 0.1
5	Copper	1.6 ± 0.1	1.3 ± 0.1	0.4 ± 0.1*
	Zinc	2.2 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
	Control	2.8 ± 0.2	1.6 ± 0.1	0.8 ± 0.0
16	Copper	2.1 ± 0.1*	2.1 ± 0.3	0.5 ± 0.1*
	Zinc	2.6 ± 0.2	1.9 ± 0.1	0.8 ± 0.2

Each value is the mean and standard error of mean of 4 rats. (*) $p<0.05$

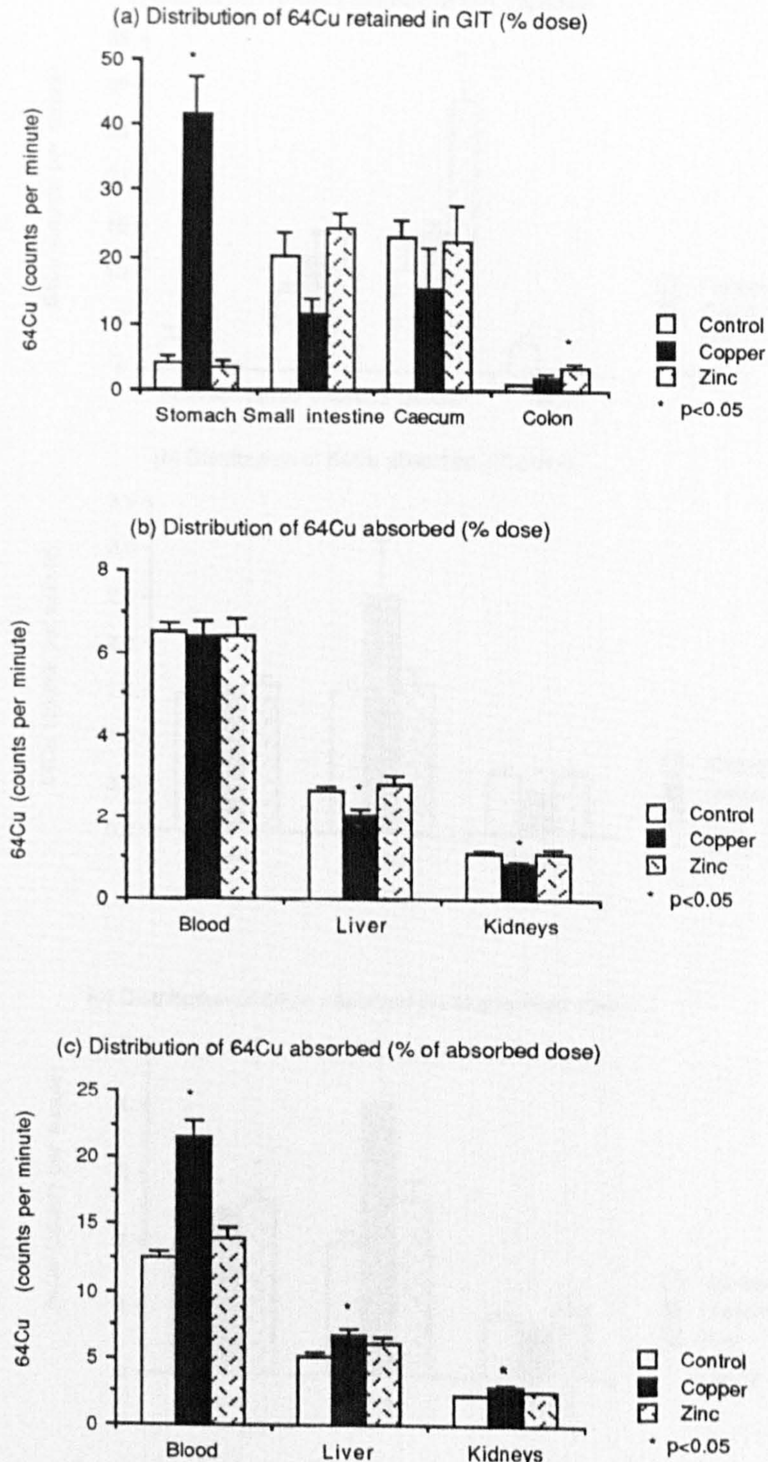


Fig. 5.0. **Week 1**. Distribution of ^{64}Cu in the GIT subdivisions, blood, liver and kidneys after 4.0 hours intragastric administration of the isotopes.

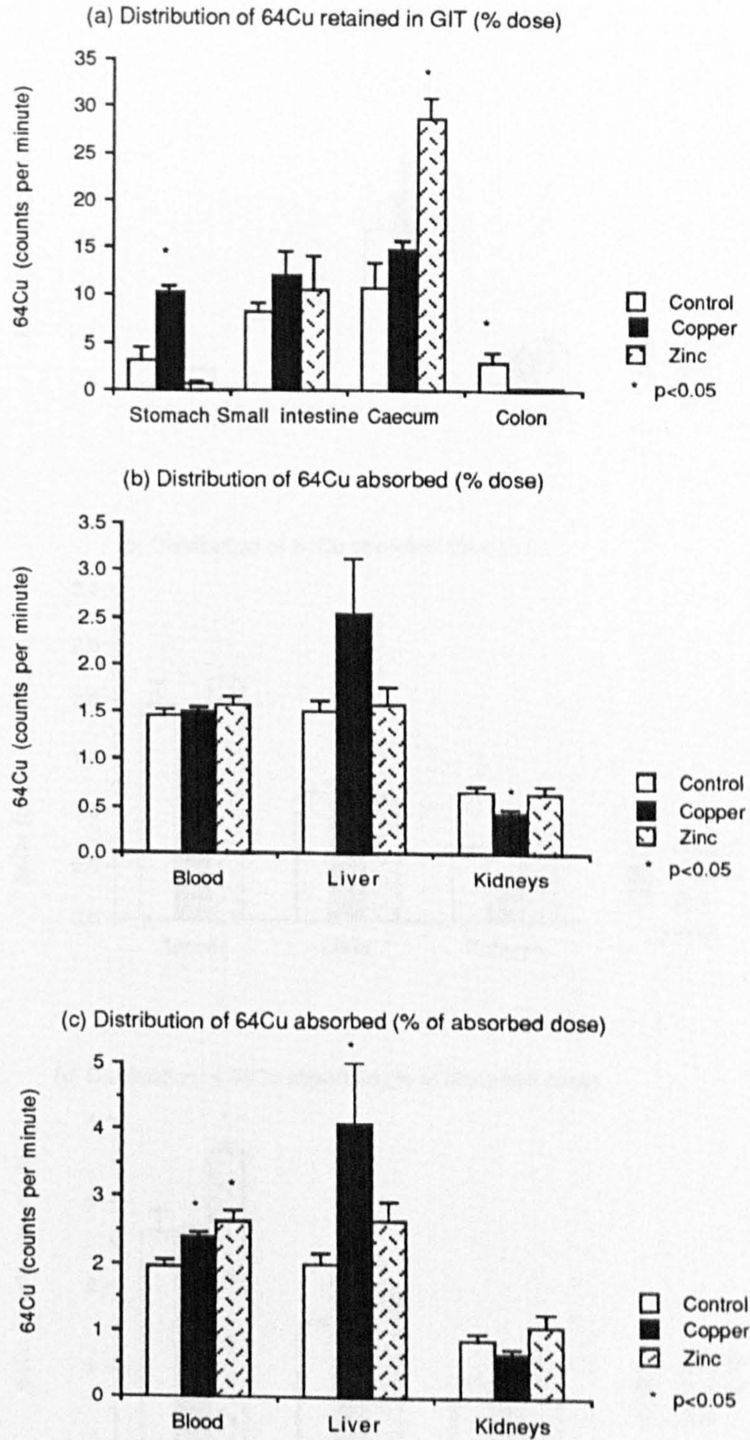


Fig. 5.1. **Week 3.** Distribution of ^{64}Cu in the GIT subdivisions, blood, liver and kidneys after 4.0 hours intragastric administration of the isotopes.

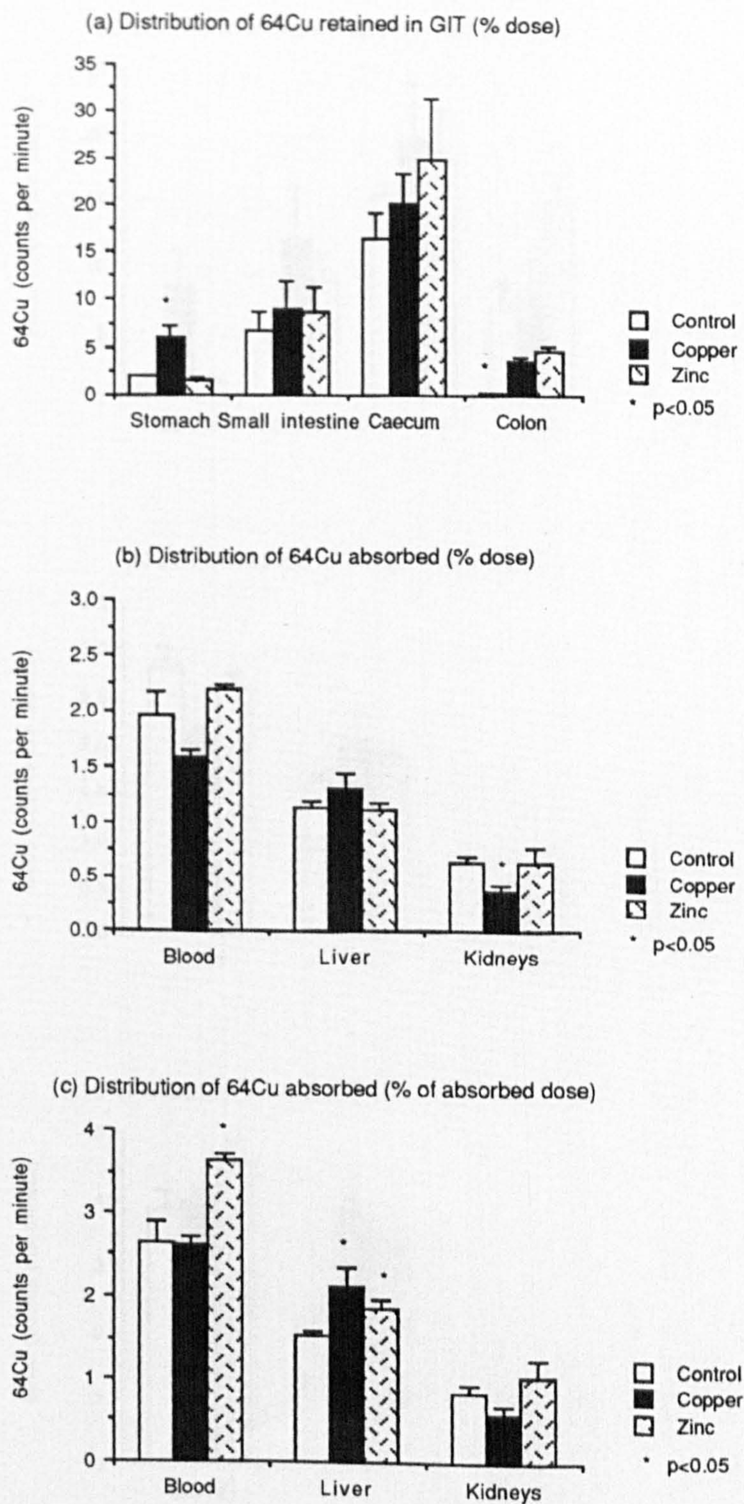


Fig. 5.2. **Week 5**. Distribution of ^{64}Cu in the GIT subdivisions, blood, liver and kidneys after 4.0 hours intragastric administration of the isotopes.

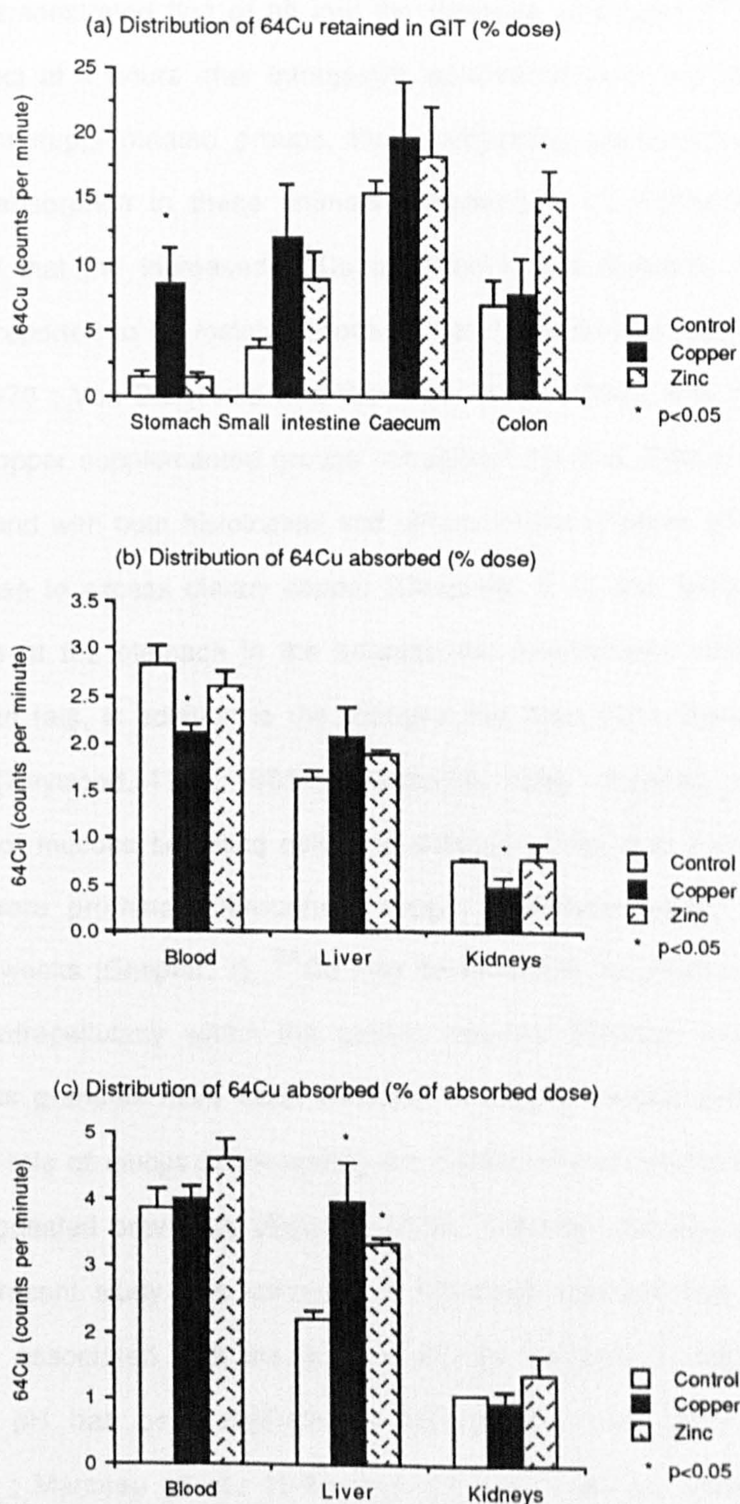


Fig. 5.3. **Week 16.** Distribution of ^{64}Cu in the GIT subdivisions, blood, liver and kidneys after 4.0 hours intragastric administration of the isotopes.

5.4. DISCUSSION

This study demonstrated first of all that the retention of copper (^{64}Cu) in the gastrointestinal tract at 4 hours after intragastric administration of the isotope was elevated in copper supplemented groups, thus, supporting previous evidence of inhibited copper absorption in these animals (Chapters, 1-4). Furthermore, it is interesting to find that the increased ^{64}Cu retention in the stomach, at the site copper has been reported to be mainly absorbed (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984), was consistently observed in the copper supplemented groups throughout the trial. This phenomenon seems to correspond with both histological and ultrastructural changes of the gastric mucosa in response to excess dietary copper (Chapters, 1, 3) and further supports the important role of the stomach in the adaptational mechanisms contributing to copper tolerance in rats, in addition to the changes that have been observed in the liver and kidney (Haywood, 1980, 1985 ; Fuentealba, 1988 ; Evering, 1989).

Hyperplasia of mucous secreting cells and deletion of the acid producing cells (parietal cells) were prominent throughout copper supplementation, particularly during the first 5 weeks (Chapter, 1). ^{64}Cu may be bound to the mucus both within the lumen and intracellularly within the gastric mucosa. Electron dense copper containing mucous granules have been detected in copper supplemented animals (Chapter, 3). The role of mucus in preventing the uptake of macromolecules into the cell has been suggested previously (Edward, 1978). Although, the biological nature of ^{64}Cu in the present study was unknown, it has been reported that at high pH copper is mainly associated with the macromolecular complexes (Gollon, 1975). Conversely, low pH has been reported to facilitate the absorption of copper (Tompsett, 1940 ; Marceau et al., 1970) and the acid secretory capacity of the stomach seems to be well correlated with the parietal cell mass in the gastric mucosa (Ito, 1987). Thus, it seems likely that a further inhibition of copper absorption occurs with the less acid conditions produced by parietal cell loss.

The retention of ^{64}Cu in the small intestine remained unchanged overall throughout the trial, although with minor fluctuations which seemed to be influenced by the retention of isotope in the stomach. As well as unabsorbed copper, there is likely to be excreted copper from the bile, enterocytes and Paneth cells (Mahoney et al., 1955 ; Owen, 1964 ; Chapter, 3), although the short time scale of this experiment is unlikely to have given rise to a major effect of ^{64}Cu excretion (Hall et al., 1979). It had been speculated that copper may bind to MT within the enterocytes and be not available for absorption (Evans and Johnson, 1978). Conversely, reduced ^{64}Cu absorption in copper supplemented rats has been reported to be unassociated with intestinal MT (Bremner et al., 1979). The nature of ^{64}Cu in the present study was unknown, but the previous evidence (Chapters, 2, 4) suggests that the elevation of intestinal MT is not directly associated to the absorption of the metal.

Copper is transfered from the absorptive cell to the portal circulation, bound to albumin and transported to the liver where it mainly accumulates (Dowdy, 1969 ; Cousins, 1985). It might have been expected that if copper absorption was reduced, blood and liver ^{64}Cu would decline also. This was not generally the case in the present investigation. Blood ^{64}Cu uptake remained unchanged until week 16, whilst liver ^{64}Cu uptake remained constant from week 3 thereafter, indeed as a proportion of absorbed dose it was increased.

Circulating ^{64}Cu can represent copper incorporated into ceruloplasmin, bound to albumin, MT and other proteins (Cousins, 1985 ; Gordon et al., 1987). Any short fall of blood ^{64}Cu might be made up by recycled copper that is blood copper of hepatic derivation. The decline in blood ^{64}Cu at week 16 paralleled the reduction of gastrointestinal copper concentration, which regressed to about the control level (Chapter, 1). Thus, this would seem to indicate functional changes in the absorptive capacity of the gastrointestinal tract whereby copper uptake into the absorptive cell is further blocked and may be associated with the physical alteration of the gastric luminal mucous membrane (Chapter, 3); alternatively receptor sites may be less

available in functionally adapted brush borders of enterocytes which have been shown to have undergone recovery (Chapter, 3).

With regard to the puzzling findings that liver uptake of ^{64}Cu was generally increased, this receives support from earlier work in both copper supplemented (Owen and Hazelrig, 1968) and unsupplemented animals (Owen, 1965 ; Terao and Owen, 1973). It has been proposed that at least three distinct pools of copper exist in the liver: the preparation of copper for excretion in the bile, temporary storage of copper and the incorporation of copper into ceruloplasmin (Hazelrig et al., 1966). Furthermore, hepatic radioisotope copper was found to be associated with a low molecular weight protein resembling metallothionein (MT) (Evans et al., 1970 ; Terao and Owen, 1973). MT can be a major copper binding protein in the liver and may play an important role in copper metabolism particularly in copper supplemented animals (Bremner, 1987b). Increased hepatic MT production has been reported to occur in copper supplemented rats (Evering, 1989).

The elevation of hepatic ^{64}Cu accumulation in the present study may thus reflect a preferential increase of ^{64}Cu bound to MT and as such is not a true indicator of total ^{64}Cu absorption from the gastrointestinal tract. The binding of hepatic copper to MT is a relatively short lived pool that is readily excreted in the bile (Bremner, 1987b). Thus, the increase of ^{64}Cu accumulated in the liver may be associated with an increase in ^{64}Cu excretion from the bile. Direct relationship between liver and biliary copper concentrations have been reported in copper supplemented rats (Evering, 1989) and pigs (Skalicky et al., 1978).

Preferential hepatic ^{64}Cu accumulation may also serve to reduce the concentration of the isotope in the kidney which was consistently observed throughout the trial. Decreased urinary ^{64}Cu concentration (% of dose) has been reported in copper supplemented rats after intravenous injection of the isotope and this was associated with the elevation of hepatic ^{64}Cu accumulation (Owen and Hazelrig, 1968).

The second part of the study which was concerned with the effect of zinc supplementation on copper absorption showed that zinc supplemented rats also have a greater total retention of ^{64}Cu (week 3 - week 16) than the controls. However, in contrast to the copper supplemented group there was no retention of isotope in the stomach, but rather concentration appeared to be at the distal part of the tract implying rapid passage along the gastrointestinal tract and suggesting different mechanisms involved. This supports the earlier contention that the interaction between copper and zinc takes place at the luminal level (Van Campen and Scaife, 1967 ; Oestreicher and Cousins, 1985), probably by blocking of receptor mechanisms and is not associated with intestinal MT status (Chapter, 4).

The failure to identify lowered blood, hepatic and kidney ^{64}Cu concentrations can not be explained and indeed the proportion of absorbed ^{64}Cu in the blood (week 3 and 5) and liver (week 5 and 16) were increased. ^{64}Cu may bind to hepatic MT and may be excreted in the bile. The elevation of hepatic MT in zinc supplemented rats have been reported previously (Chen et al., 1977).

In conclusion this study suggests that the absorption of ^{64}Cu is inhibited in both copper and zinc supplemented rats. The interaction takes place at the luminal level, but it seems to occur at different locations and involve different mechanisms. Copper supplemented rats invoke an additional functional adaptation, particularly at the proximal part of the gastrointestinal tract, whilst in zinc supplemented rats the passage of ^{64}Cu along the gastrointestinal tract was more rapid and may be associated with the competition of metal uptake at the brush border receptor sites. Furthermore, redistribution of copper seems to occur internally whereby copper preferentially accumulates in the liver and may facilitate its removal.

Addendum

i. Absorption is a process by which copper is transferred across the absorptive cell into the portal circulation.

ii. Changes in total gastrointestinal tissue copper concentration in the copper supplemented rats could cause isotope dilution which might affect the retention of ^{64}Cu.

CONCLUSION

This study demonstrated that the gastrointestinal tract of rats can adapt to chronic high copper exposure with repair of damage and exhibited the important role of this organ in the adaptational mechanisms contributing to copper tolerance, in addition to changes that have been observed in both the liver and kidney (Haywood, 1980, 1985 ; Fuentealba, 1988 ; Evering, 1989). Furthermore, copper (^{64}Cu) absorption is reduced. Gastrointestinal mural copper concentration rose initially, but was reduced after prolonged exposure to the metal, whilst zinc remained unchanged.

Morphological changes were most remarkable in the stomach, at the site where most copper is reputably absorbed (Van Campen and Mitchell, 1965 ; Marceau et al., 1970 ; Van Barneveld and Van den Hamer, 1984). Changes were related in magnitude to overall copper content within this organ. Cytotoxic damage (single cell necrosis) occurred initially, but subsequently subsided and was replaced by a wave of regenerative activity which inturn declined by week 16. The profile of cytopathological changes are similar to those recorded in the copper loaded rat liver whereby adaptation and recovery succeeds the initial cytotoxic effect (Haywood, 1980, 1985).

Hyperplasia of gastric mucous secreting cells and depletion of acid producing cells (parietal cells) which were both prominent in the trial groups, particularly during the first 5 weeks, might alter the availability of copper for absorption. Copper may bind to mucus both within the lumen and intracellularly within the gastric mucosa, presumeably making it less toxic and less available for absorption. Parietal cell depletion could be associated with an increase of gastric pH and this may also inhibit copper absorption. Furthermore, the uptake of copper may be inhibited by the adaptional changes exhibited at the apical surface of the gastric mucous secreting cell which was covered with a thickened electron dense membrane by week 16.

Despite broadly similar fluctuations of copper content histological changes in the small intestine, caecum and colon corresponded less obviously with changing copper than did the stomach, although villus atrophy was recorded in the distal small intestine during the first 5 weeks of copper supplementation with recovery in the later weeks. However, at the ultrastructural level degenerative changes within the enterocyte microvilli of the villus upper third were prominent initially, with subsequent recovery. This may be very important and signal a functional adaptation at the luminal absorptive site whereby copper uptake is inhibited.

Gastrointestinal mural copper may equally represent metal flux in the processes of excretion as much as absorption. Copper can be eliminated following cellular extrusion. Rapid cellular turnover was a feature of the copper supplemented groups suggesting enhanced elimination of the metal by this activity. Furthermore, localisation of copper containing Paneth cell granules suggests that excess copper in the body may be excreted by this route and that elimination of toxic metals may be an important functional role of these cells.

The pattern of response of the gastrointestinal MT and copper in copper supplemented rats, elevated initially and declining with time, is similar to that occurring in both the intestinal tract and liver of newborn rats (Johnson and Evans, 1980 ; Hurley et al., 1980 ; Mason et al., 1981) and also in the liver during the development of copper tolerance (Evering, 1989). MT corresponded to copper concentration rather than zinc suggesting that intestinal MT is induced by copper and supporting previous reports on the ability of copper to induce MT (Evans, 1979 ; Hall et al., 1979 ; Blalock et al., 1988 ; Scarino et al., 1988). Elevated MT was identified in the soluble fraction and in the tissue sections, in which it appeared superior to histochemical methods for the identification of copper confirming earlier reports (Elmes et al., 1989 ; Evering et al., 1990).

MT immunostaining within villus lower third probably indicates an increase in copper retention within this region which may be associated with the functional maturity of the enterocyte (Leblond and Stevens, 1948 ; Toner, 1968). Excess

copper might be retained within nonfully functional enterocytes as occurs in neonatal animals (Johnson and Evans, 1980 ; Hurley et al., 1980 ; Mason et al., 1981) and may act as a temporary barrier to absorption. However, the failure to maintain this barrier indicates that copper tolerance in rats relies on mechanisms other than a MT mucosal block to inhibit absorption. Furthermore, copper bound to MT appears to represent a temporary binding of metal in transport across the cell since intense immunostaining was also localised within the cytoplasm of the Paneth cells.

Animals on the zinc supplemented diet exhibited a retarded accumulation of liver copper, followed by its more rapid depletion. Radioactive isotope tracer (^{64}Cu) confirmed a delay in copper absorption. However, intestinal MT elevation was of a very minor nature and could not be maintained. Furthermore, chromatographic studies failed to identify significantly enhanced MT revealing that the depressant effect of high oral zinc on copper absorption and hepatic copper accumulation takes place at the luminal level and is not mediated by MT. The isolation of zinc induced MT after parenteral zinc injection with both chromatographic separation and immunoreactive techniques and the localisation of MT staining within the cytoplasm of the villus enterocytes and Paneth cells suggests that MT may rather act as a zinc transport protein within the intestinal mucosa in the process of excretion than a barrier to copper absorption. A regulatory control of zinc absorption is also indicated in oral zinc supplementation by the absence of excess zinc in both the intestine and liver. This process seems to take place at the luminal level or within the brush border.

Finally, the radioactive tracer studies suggest that the inhibition of copper (^{64}Cu) absorption in both copper and zinc supplemented animals occurs at different locations and may involve different mechanisms. Copper tolerant rats invoke an additional functional adaptation, particularly at the proximal part of the gastrointestinal tract, whilst in zinc supplementation the passage of ^{64}Cu along the gastrointestinal tract was more rapid and may be associated with the competition of metal uptake at the brush border receptor sites.

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Addendum

BEARMAN D. R. & ISASI J. A., (1972). Electron beam microanalysis. Am. Soc. Test. Spec. Tech. Pub., 506: 1-80.

Appendix 1.0

Calculated nutrient levels in pelleted CRM diet

PROXIMATE ANALYSIS (%)		
Ash		5.40
Crude Oil		2.50
Crude Protein		17.90
Crude Fibre		3.60
Calcium	(as Ca)	0.82
Phosphorus	(as P)	0.73
Sodium	(Na)	0.32
Potassium	(K)	0.72
Magnesium	(Mg)	0.16
Chloride	(Cl)	0.47
Carbohydrate		57.00
Starch		45.00
Digestable energy, MJ/kg		13.30

VITAMINS (per kg)	
Vitamin A (Retinol) iu	15000.000
Vitamin D3 (Cholecalciferol) iu	2400.000
Vitamin E (- Tocopherol) iu	80.000
Vitamin K3 added mg	150.000
Vitamin B1 (Thiamin) mg	12.000
Vitamin B2 (Riboflavin) mg	13.000
Vitamin B6 (Pyridoxin) mg	17.000
Niacin (Nicotinic Acid) mg	38.000
Pantothenic Acid mg	20.000
Folic Acid mg	3.100
Vitamin B12 (Cyanocobalamin) mg	0.079
Choline mg	1200.000
Biotin (available) mg	0.300

TRACE ELEMENTS (mg/kg)		
Manganese	(Mn)	63.00
COPPER	**(Cu)**	**14.00**
Cobalt	(Co)	0.40
Iron	(Fe)	106.00
Iodine (added)	(I)	0.28
ZINC	**(Zn)**	**61.00**
Selenium	(Se)	0.17
Chromium (added)		0.49

AMINO ACIDS (g/kg)	
Aspartic Acid	14.5
Glutamic Acid	33.8
Alanine	9.3
Threonine	6.6
Proline	13.0
Serine	9.5
Glycine	9.4
Valine	9.0
Cystine	2.8
Methionine	3.1
Isoleucine	7.8
Leucine	14.3
Tyrosine	6.6
Phenylalanine	8.5
Lysine	10.0
Histidine	4.4
Arginine	11.1
Tryptophan	2.1

Copper supplemented diet = CRM diet + 1000 mg/kg of copper as copper sulphate

Zinc supplemented diet = CRM diet + 1000 mg/kg of zinc as zinc sulphate

Copper and zinc supplemented diet = CRM diet + 1000 mg/kg of copper as copper sulphate + 1000 mg/kg of zinc as zinc sulphate

Appendix 1.1

Body weights of copper supplemented and control rats

Time (weeks)	Body weight, g (mean ± SEM) Copper supplementation	Control
0	320 ± 5 (40)	332 ± 7 (12)
1	342 ± 5 (40)	361 ± 6 (12)
2	361 ± 5 (36)	383 ± 8 (8)
3	382 ± 6 (32)	402 ± 9 (8)
4	397 ± 6 (28)	420 ± 8 (8)
5	411 ± 6 (24)	432 ± 7 (8)
6	428 ± 6 (20)	447 ± 8 (8)
7	443 ± 7 (16)	464 ± 8 (8)
8	450 ± 7 (16)	474 ± 8 (8)
9	462 ± 9 (12)	475 ± 9 (4)
10	471 ± 9 (12)	482 ± 8 (4)
11	477 ± 12 (8)	491 ± 7 (4)
12	484 ± 12 (8)	499 ± 8 (4)
13	478 ± 13 (4)	505 ± 7 (4)
14	483 ± 13 (4)	513 ± 8 (4)
15	487 ± 14 (4)	521 ± 11 (4)
16	492 ± 14 (4)	

Figure in brackets is the number of rats

Appendix 1.2

Food consumption per group of copper supplemented and control rats

Time (weeks)	Food consumption, g (mean ± SEM) Copper supplementation	Control
1	697 ± 24 (10)	703 ± 44 (3)
2	798 ± 23 (9)	701 ± 2 (2)
3	726 ± 32 (8)	708 ± 7 (2)
4	780 ± 34 (7)	722 ± 1 (2)
5	789 ± 27 (6)	714 ± 2 (2)
6	809 ± 13 (5)	691 ± 14 (2)
7	820 ± 38 (4)	735 ± 15 (2)
8	828 ± 37 (4)	745 ± 18 (2)
9	845 ± 43 (3)	711 (1)
10	841 ± 44 (3)	676 (1)
11	912 ± 60 (2)	701 (1)
12	941 ± 65 (2)	738 (1)
13	898 (1)	759 (1)
14	875 (1)	745 (1)
15	876 (1)	768 (1)
16	879 (1)	

Figure in brackets is the number of groups of 4 rats each

Appendix 1.3

Copper concentration of the gastrointestinal contents and feces of copper supplemented and control rats

Time (weeks)	Copper concentration (μg/g dry weight)				
	Stomach	Intestine	Caecum	Colon	Feces
	Copper supplementation				
1	3199.9	2236.3	3427.3	4378.2	4377.0
2	1914.7	1776.4	2219.3	3447.5	3506.7
3	3011.1	2469.6	3154.5	4293.6	3988.6
4	2699.1	1651.6	3007.9	4300.1	4683.1
5	2919.1	2630.4	2491.9	2939.5	3885.3
6	3358.6	2030.4	2992.9	3603.3	4398.8
8	3031.7	2830.1	3340.3	3669.1	4191.8
10	3397.0	3051.1	2479.3	3688.1	3984.1
12	1845.5	1432.1	2179.1	3282.7	4315.0
16	2975.0	3360.4	2701.8	4251.8	4085.5
Mean	2835.0*	2346.8*	2799.4*	3785.4*	4141.6*
± SEM	172.0	200.3	142.3	157.4	103.7
	Control				
1	32.3	33.8	54.9	58.4	49.9
8	30.8	27.5	42.7	47.1	63.2
15	32.7	38.0	43.1	54.3	51.3
Mean	31.9	33.1	46.9	53.3	54.8
± SEM	0.6	3.1	4.0	3.3	4.2

Each value is the mean of pooled samples of 4 rats in the group. (*) $p<0.05$.
Estimated recovery = 101 ± 6% (mean ± SD).

Appendix 1.4

Zinc concentration of the gastrointestinal contents and feces of copper supplemented and control rats

Time (weeks)	Zinc concentration (μg/g dry weight)				
	Stomach	Intestine	Caecum	Colon	Feces
	Copper supplementation				
1	112.0	175.0	322.3	350.4	356.0
2	41.6	206.8	213.2	323.8	300.4
3	39.2	307.3	306.6	398.2	336.7
4	62.6	122.0	326.8	460.8	399.5
5	67.6	250.7	340.7	400.1	406.3
6	142.2	140.8	336.3	365.0	339.4
8	137.8	374.2	552.2	519.6	365.2
10	46.1	356.8	270.8	348.1	353.5
12	81.7	204.7	211.5	283.4	345.5
16	203.7	293.5	280.4	424.6	377.9
Mean	93.5	243.2	316.1	387.4	358.0
± SEM	17.2	27.8	30.2	21.8	9.9
	Control				
1	69.7	415.6	433.2	426.2	439.5
8	62.4	246.1	363.3	406.9	366.9
15	58.1	301.5	257.6	321.2	322.2
Mean	63.4	321.1	351.8	384.8	376.2
± SEM	3.4	49.9	51.0	32.4	34.2

Each value is the mean of pooled samples of 4 rats in the group.
Estimated recovery = 102 ± 3% (mean ± SD).

Appendix 1.5

Experimental data for copper and zinc concentrations (ug/g dry weight) of the stomach tissues and stomach contents of copper supplemented and control rats

Time (weeks)	Stomach tissues Copper	Stomach tissues Zinc	Stomach contents Copper	Stomach contents Zinc
	Copper supplementation			
1	22.97	116.08	3485.99	119.81
	21.49	106.61	3112.21	121.01
	21.19	111.23	3001.56	95.02
2	26.25	100.15	1841.61	40.19
	31.28	107.42	2088.91	41.96
	22.11	116.66	1813.59	42.65
3	27.43	103.45	3024.60	26.93
	30.63	119.45	2928.57	53.57
	26.51	111.85	3080.05	36.95
4	23.14	124.24	3058.25	67.96
	23.81	122.86	2340.00	57.14
	34.24	130.74		
5	28.27	109.54	2992.70	62.04
	29.98	115.63	2844.50	61.00
	25.97	125.23	2920.23	79.77
6	15.12	99.80	3107.58	105.13
	16.39	103.83	3456.67	163.93
	15.96	106.38	3511.57	157.41
8	11.20	94.10	3010.63	127.54
	17.63	113.35	3052.81	148.05
	13.32	101.24		
10	14.02	108.49	3413.43	51.09
	15.64	104.99	3512.10	47.49
	19.68	111.28	3265.49	39.82
12	13.69	94.46	1824.61	98.17
	14.84	108.39	1829.52	72.08
	15.98	103.58	1882.24	74.85
16	9.06	79.31	3058.25	184.47
	9.72	89.95	2891.80	222.95
	11.30	85.84		
	Control			
1	10.26	112.88	34.36	68.73
	9.18	107.44	32.49	70.40
	11.29	121.35	29.96	69.90
8	10.17	99.82	30.04	64.38
	9.17	100.62	23.32	34.99
	8.67	114.38	39.02	87.80
15	8.98	100.41	18.89	61.32
	9.71	104.37	46.41	54.85
	9.49	99.68		

Appendix 1.6

Experimental data for copper and zinc concentrations (ug/g dry weight) of the subdivided part of small intestinal tissues and intestinal contents of copper supplemented and control rats

Time	Copper			Zinc			Copper	Zinc
(weeks)	Prox.	Mid.	Distal	Prox.	Mid.	Distal	Intestinal contents	
				Copper supplementation				
1	13.32	12.50	14.29	111.03	135.94	197.14	1922.69	144.49
	13.14	12.69	12.54	116.40	135.40	213.21	2506.11	198.04
	10.68	11.74	14.86	116.83	135.03	201.92	2280.17	182.58
2	15.64	16.39	15.35	113.92	142.08	172.05	1621.02	179.36
	18.27	17.29	15.99	107.58	146.97	189.45	1856.25	217.32
	17.30	19.76	17.51	114.78	133.40	189.08	1852.06	223.60
3	14.34	18.43	27.10	109.79	136.76	180.89	2424.24	314.86
	17.12	24.14	25.91	113.51	128.13	151.00	2592.78	325.23
	16.12	22.15	30.90	117.00	129.75	179.49	2391.67	281.82
4	17.44	18.23	22.74	138.83	172.51	220.62	1716.80	126.97
	17.24	22.22	19.95	140.09	175.00	204.49	1519.44	123.94
	14.98	19.13	21.13	114.61	159.84	212.83	1718.56	114.97
5	18.84	20.66	31.30	125.84	149.45	197.43	2546.12	246.58
	19.96	19.93	36.35	126.39	149.50	220.16	2558.45	232.23
	19.97	23.48	28.46	128.39	149.71	209.57	2786.70	273.32
6	15.60	19.51	14.78	117.02	136.59	180.30	2102.56	142.01
	15.68	14.18	22.26	115.61	144.68	194.81	1910.84	133.74
	16.04	17.04	16.27	111.04	149.41	195.30	2077.83	146.61
8	11.94	19.17	16.05	120.20	146.02	197.64	2829.80	385.88
	13.33	10.75	15.31	126.67	139.78	195.81	2830.30	362.42
	14.70	14.07	16.02	123.80	141.94	185.72		
10	18.20	20.86	15.84	129.03	147.54	186.65	3021.54	353.74
	18.34	16.29	17.68	122.28	142.86	194.50	3058.38	343.78
	15.58	13.99	17.69	121.87	138.86	197.14	3073.13	372.94
12	13.03	13.49	15.69	113.52	134.93	154.07	1419.91	199.13
	13.42	11.74	16.16	117.14	130.28	167.21	1504.08	220.11
	13.14	10.83	16.45	113.30	122.50	156.65	1372.21	194.73
16	9.03	9.08	6.98	104.24	131.62	161.52	3423.35	387.48
	8.20	8.40	12.17	106.56	130.25	166.96	3336.12	426.62
	10.80	9.05	11.67	109.09	132.66	165.00	3321.86	366.26
				Control				
1	9.68	10.64	13.05	118.39	141.34	212.89	31.76	404.99
	9.13	10.65	10.77	121.25	143.81	216.92	33.94	449.61
	9.19	13.29	9.24	116.82	137.68	225.37	35.81	392.32
8	8.72	13.18	9.49	111.63	143.33	147.15	32.05	235.58
	8.33	9.10	8.72	115.00	144.34	156.89	28.04	235.51
	8.39	8.60	9.17	110.06	132.26	159.54	22.26	267.09
15	7.14	9.10	7.97	95.60	132.64	157.89	41.40	342.23
	8.17	9.08	7.87	111.25	122.59	162.73	36.38	316.26
	8.01	8.93	7.98	108.16	127.98	153.59	36.27	246.14

Appendix 1.7

Experimental data for copper and zinc concentrations (ug/g dry weight) of the caecal tissues and caecal contents of copper supplemented and control rats

Time (weeks)	Caecal tissues Copper	Caecal tissues Zinc	Caecal contents Copper	Caecal contents Zinc
	Copper supplementation			
1	29.22	126.62	3435.14	317.84
	34.11	125.58	3375.44	312.99
	34.70	107.81	3471.33	336.00
2	29.68	128.01	2230.63	211.12
	25.23	127.49	2212.23	215.83
	22.16	116.34	2214.97	212.72
3	49.80	111.25	3082.95	301.16
	43.23	114.90	3216.98	305.13
			3163.66	313.63
4	49.33	128.00	3016.54	329.12
	53.25	127.81	2851.15	313.79
	40.29	117.77	3155.96	337.35
5	38.92	137.06	2493.81	346.39
	42.09	137.76	2489.96	335.05
	32.02	131.00		
6	28.07	115.79	3046.99	332.79
	29.16	113.00	2938.73	339.88
	23.78	116.40		
8	21.21	131.82	3208.70	563.04
	23.05	125.14	3326.75	552.63
	25.91	120.14	3485.32	540.96
10	24.27	126.21	2407.29	272.56
	23.61	131.05	2622.32	274.51
	31.77	121.98	2408.18	265.43
12	19.32	111.44	2067.40	199.20
	17.54	117.65	2242.04	225.91
	21.16	113.59	2227.82	209.37
16	15.78	112.43	2754.52	289.41
	19.61	113.97	2649.11	271.37
	17.07	110.95		
	Control			
1	12.41	132.98	55.40	451.10
	9.39	136.15	54.31	449.61
	10.19	122.24	54.85	398.73
8	6.37	124.20	42.35	363.81
	8.78	122.99	43.34	374.76
	8.09	101.08	42.41	351.25
15	10.01	107.30	45.90	282.27
	9.64	112.05	35.83	218.89

Appendix 1.8

Experimental data of copper and zinc concentrations (ug/g dry weight) of colon tissues and their contents of copper supplemented and control rats.

Time (weeks)	Tissues Copper	Tissues Zinc	Contents Copper	Contents Zinc
	Copper supplementation			
1	17.80	128.80	4385.19	352.83
	14.95	126.65	4453.48	365.13
	21.52	129.14	4296.01	333.18
2	18.47	132.96	3358.90	315.96
	18.02	135.14	3527.58	339.69
	16.83	132.86	3455.91	315.73
3	19.76	139.18	4275.35	398.61
	19.28	126.72	4446.77	402.24
	20.00	129.23	4158.77	393.61
4	22.06	138.51	4497.72	457.91
	18.20	137.43	4204.47	465.30
			4198.23	459.10
5	21.70	144.34	3098.36	406.05
	20.95	140.00	2895.77	401.81
	25.21	143.70	2824.41	392.28
6	16.73	126.97	3660.43	366.04
	17.61	126.22	3705.46	353.93
	17.27	126.68	3443.88	375.00
8	18.35	121.89	3656.43	539.81
	18.96	122.27	3681.82	499.30
	16.31	117.46		
10	12.54	153.23	3749.00	355.14
	14.31	128.79	3613.18	342.54
	14.18	125.10	3702.07	346.59
12	13.62	122.57	3301.45	296.61
	15.33	123.49	3340.59	272.47
	13.87	119.90	3206.05	283.41
16	8.79	141.76	4058.19	428.31
	9.32	131.41	4283.21	418.48
	9.81	130.01	4413.88	427.03
	Control			
1	10.82	134.78	60.15	474.50
	10.09	128.01	57.80	416.18
	9.32	139.86	57.37	387.82
8	9.30	125.58	44.93	394.61
	9.41	125.90	45.82	403.55
	8.15	119.80	50.54	422.38
15	9.78	134.17	50.56	283.67
	9.96	124.14	54.83	300.36
	9.43	130.53	57.60	379.60

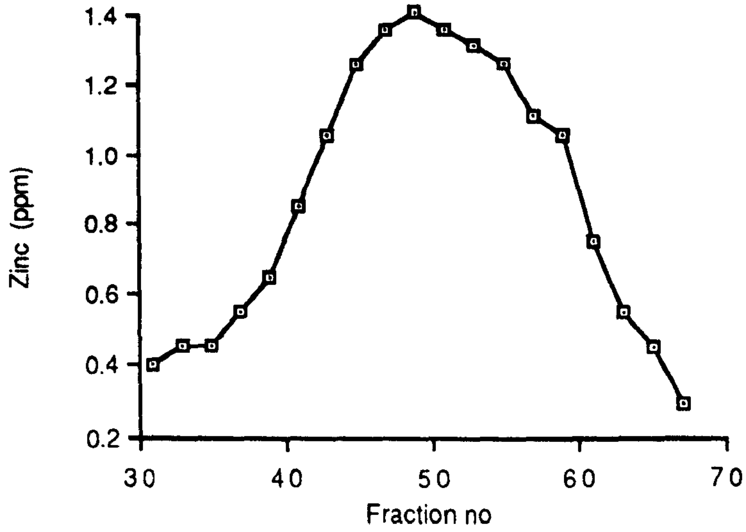
Appendix 1.9

Experimental data of copper and zinc concentrations (ug/g dry weight) of feces of copper supplemented and control rats.

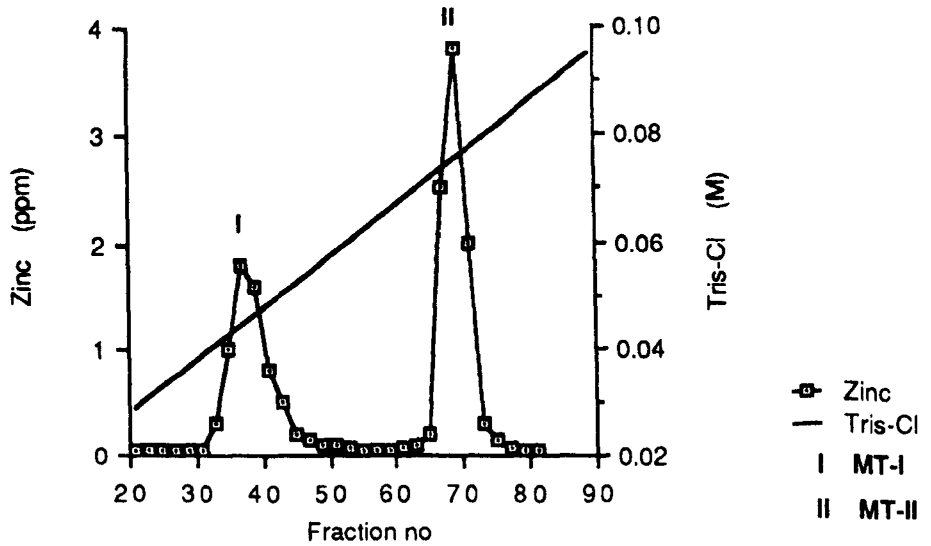
Time (weeks)	Feces Copper	Feces Zinc
	Copper supplementation	
1	4269.47	364.23
	4358.31	343.16
	4503.17	360.66
2	3566.04	318.53
	3593.70	287.22
	3360.41	295.30
3	4038.86	349.09
	3880.40	329.01
	4046.56	332.03
4	4573.88	398.80
	4964.54	401.96
	4510.73	397.72
5	3870.89	395.30
	3854.50	412.72
	3930.38	410.86
6	4376.90	332.32
	4368.18	341.21
	4451.35	344.74
8	4278.51	393.76
	4235.29	329.81
	4061.60	372.02
10	3939.91	351.93
	3980.61	355.11
	4031.67	353.37
12	4195.44	330.34
	4370.18	345.64
	4379.34	360.44
16	3994.72	370.10
	4094.45	378.97
	4167.20	384.51
	Control	
1	49.21	432.03
	50.50	447.03
8	66.00	359.33
	62.14	369.96
	61.41	371.49
15	60.68	390.71
	50.75	314.55
	42.40	261.44

Appendix 2.0

(i) Zinc distribution in LMW protein of rat liver



(ii) Isoproteins from LMW band of rat liver



Appendix 2.1

i. **Coating buffer** (to be prepared on the day of used).
100mM solution of carbonate/bicarbonate buffer, pH 9.6.

Substance	Amount per litre
Sodium hydrogen carbonate ($NaHCO_3$).*	5.880 g
Sodium carbonate (Na_2CO_3).*	3.180 g

ii. **Rinsing buffer** (to be prepared on the day of used).
0.01M phosphate, 0.15M sodium chloride, 0.05% (w/v) Triton X-405, PH 7.4.

Substance	Amount per litre
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4).*	1.150 g
Sodium dihydrogen orthophosphate ($NaH_2PO_4 . 2H_2O$)*	0.297 g
Sodium chloride (NaCl).*	8.760 g
Triton X-405**	0.500 g

iii. **Diluent buffer**
0.02M phosphate, 0.15M sodium chloride, 1.0% (w/v) bovine serum albumin (BSA), 0.01% merthiolate, pH 7.2.

Substance	Amount per litre
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)*	2.044 g
Sodium dihydrogen orthophosphate ($NaH_2PO_4 . 2H_2O$)*	0.874 g
Sodium chloride (NaCl)*	8.760 g
Bovine serum albumin***	10.000 g
Merthiolate***	0.100 g

iv. **Wash solution.**
0.02M phosphate, 0.15 sodium chloride, 0.01% (w/v) merthiolate, 0.5% (w/v) Tween 80, PH 7.8.

Substance	Amount per litre
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)*	2.598 g

Sodium dihydrogen orthophoaphate ($NaH_2PO_4 . 2H_2O$)*	0.265 g
Sodium chloride (NaCl)*	8.760 g
Merthiolate***	0.100 g
Tween 80***	5.000 g

v. **Proxidase conjugate.**

A mixture of 10ml of conjugate buffer with 10ul peroxidase-conjugate rabbit immunoglobulins raised to sheep immunoglobulins (Dako-immunoglobulins, Denmark).

Conjugate buffer.

0.02M phosphate, 0.15M sodium chloride, 0.1% (w/v) bovine serum albumin, 0.01% (w/v) merthiolate, 0.05% (w/v) ANS (8-anilino-napthalene sulphonic acid), pH 7.2.

Substance	Amount per litre
Disodium hydrogen orthophosphate anhydrous (Na_2HPO_4)*	2.044 g
Sodium dihydrogen orthophosphate ($NaH_2PO_4 . 2H_2O$)*	0.874 g
Sodium chloride (NaCl)*	8.760 g
Merthiolate***	0.100 g
Bovine serum albumin***	1.000 g
ANS***	0.500 g

vi. **Working substrate solution.**

A mixture of 0.5ml stock substrate solution in 12 ml of substrate buffer.

a. **Stock substrate solution.**

A 15mg/ml solution of ABTS (2, 2" azinoBis (3-ethylbenzthiazoline) sulfonic acid)*** prepared in distilled water.

b. **Substrate buffer.**

A solution of 2.3% citric acid* containing 50ul/100 ml of hydrogen peroxide*, pH 4.0.

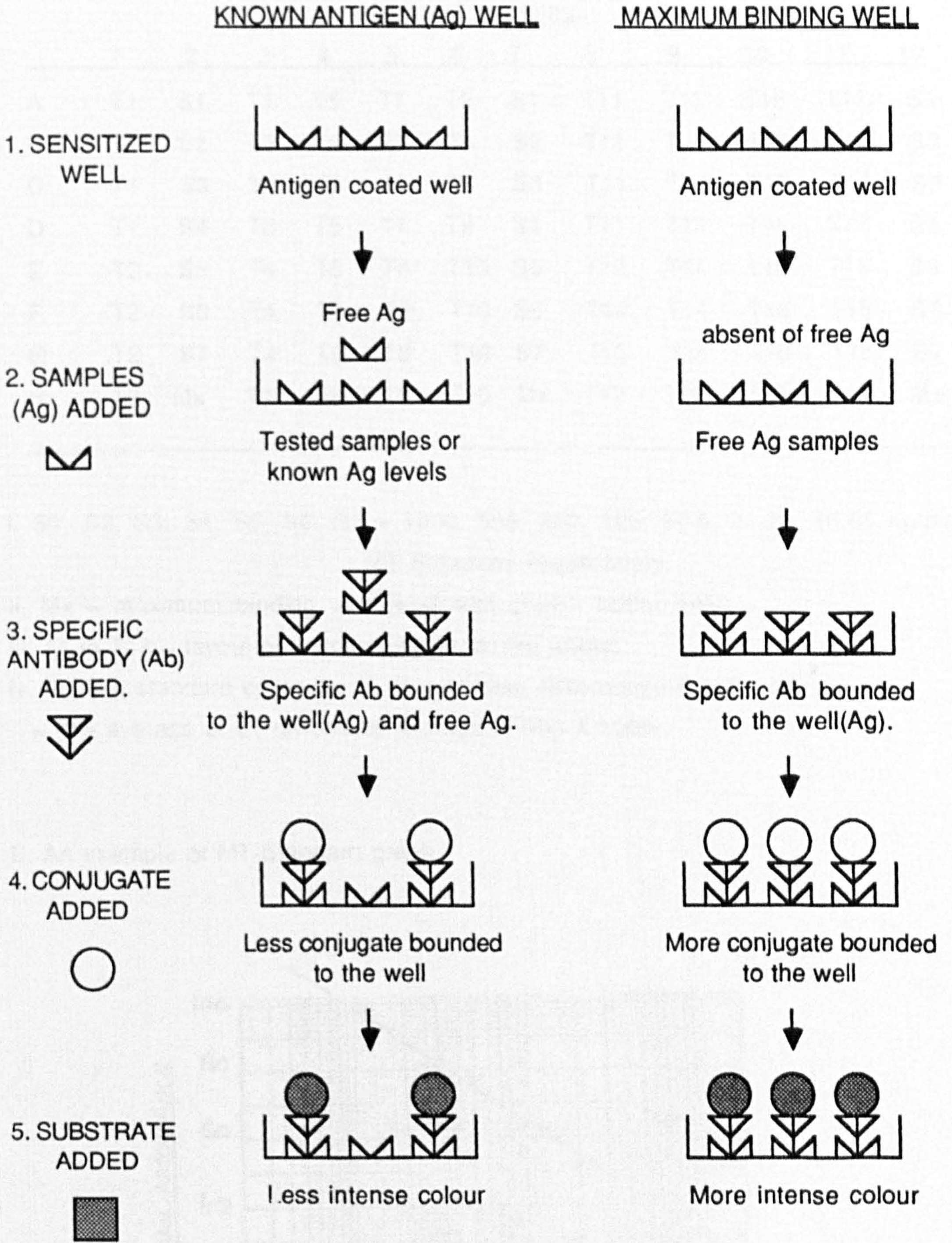
* Analar grade, BDH Chemical Ltd. Poole, England.

** Laboratory reagent,BDH Chemical Ltd. Poole, England.

*** Sigma Chemical Company.

Appendix 2.2

ELISA Technique for Metallothionein.



Appendix 2.3

i. Layout of plates for ELISA.

a. An example of 18 tested samples (4 replications each) with 3 MT-Standard columns.

Microwell modules

	1	2	3	4	5	6	7	8	9	10	11	12
A	T1	**S1**	T3	T5	T7	T9	**S1**	T11	T13	T15	T17	**S1**
B	T1	**S2**	T3	T5	T7	T9	**S2**	T11	T13	T15	T17	**S2**
C	T1	**S3**	T3	T5	T7	T9	**S3**	T11	T13	T15	T17	**S3**
D	T1	**S4**	T3	T5	T7	T9	**S4**	T11	T13	T15	T17	**S4**
E	T2	**S5**	T4	T6	T8	T10	**S5**	T12	T14	T16	T18	**S5**
F	T2	**S6**	T4	T6	T8	T10	**S6**	T12	T14	T16	T18	**S6**
G	T2	**S7**	T4	T6	T8	T10	**S7**	T12	T14	T16	T18	**S7**
H	T2	**Mx**	T4	T6	T8	T10	**Mx**	T12	T14	T16	T18	**Mx**

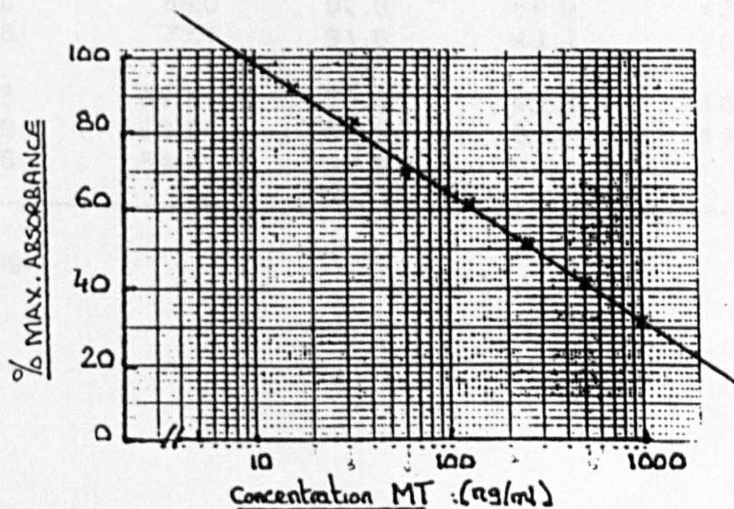
i. S1, S2, S3, S4, S5, S6, S7 = 1000, 500, 250, 125, 62.5, 31.25, 16.65 ng/ml of MT-Standard respectively.

ii. Mx = maximum binding well (well with diluent buffer only)

iii. T1 to T18 = layout of tested samples for the assay.

iv. For the standard curve the % of maximum absorbance (i.e. for SI) = (the average of S1 divided by average of Mx) X 100%.

ii. An example of MT-Standard graph.



Appendix 2.4

Experimental data of
metallothionein concentration in the soluble fractions of gastrointestinal tract of the control and copper supplemented rats.

Time (weeks)	Metallothionein concentration in the soluble fractions of gastrointestinal tract (μg/g wet weight)					
	Stomach	Small intestine			Caecum	Colon
		Proximal	Middle	Distal		
		Control				
1	-	57.2	51.0	51.9	12.1	19.8
		47.5	61.6	61.6	10.5	18.3
		55.0		57.2		
8	9.4	52.8	55.9	52.8	9.4	19.8
	7.2	51.0	57.2	54.1	8.4	18.2
	9.3	58.9				
15	8.3	48.4	51.5	48.4	10.1	19.8
	13.2	52.8	66.0	55.0	8.8	16.5
	10.5	51.4	57.8	62.0		21.4
		Copper supplementation				
1	23.1	59.4	114.4	70.4	28.0	20.4
	21.0	52.8	102.1	74.8	21.5	19.1
		52.8	114.4			
5	28.3	83.6	114.4	88.0	20.9	26.4
	26.4	52.8	103.7	92.4	17.9	19.8
		58.2	114.4			
10	35.2	114.4	83.6	44.0	13.7	19.8
	39.0	88.0	92.0	66.0	13.8	18.7
	32.6	70.4	61.6	48.4	10.5	20.0
16	27.5	88.8	70.4	43.6	10.1	20.3
	33.0	66.0	92.0	63.8	15.0	15.8
	22.6	84.5	96.6			

(-) sample missing.

Appendix 3.0.

Body weight of copper supplemented and control rats

Time (weeks)	Body weight, g (mean ± SEM)	
	Copper supplementation	Control
0	368 ± 5 (20)	364 ± 5 (12)
1	376 ± 5 (20)	392 ± 4 (12)
2	397 ± 5 (16)	420 ± 7 (8)
3	419 ± 6 (16)	442 ± 7 (8)
4	423 ± 8 (12)	465 ± 7 (8)
5	432 ± 9 (12)	489 ± 9 (8)
6	440 ± 8 (8)	509 ± 13 (4)
7	447 ± 8 (8)	528 ± 12 (4)
8	458 ± 9 (8)	539 ± 11 (4)
9	469 ± 9 (8)	552 ± 12 (4)
10	480 ± 10 (8)	568 ± 12 (4)
11	485 ± 16 (4)	584 ± 13 (4)
12	489 ± 18 (4)	593 ± 12 (4)
13	490 ± 18 (4)	598 ± 11 (4)
14	493 ± 17 (4)	603 ± 9 (4)
15	492 ± 18 (4)	606 ± 9 (4)
16	493 ± 18 (4)	609 ± 9 (4)

Figure in brackets is the number of rats.

Appendix 3.1

Food consumption of copper supplemented and control rats

Time (weeks)	Food consumption, g (mean ± SEM)	
	Copper supplementation	Control
1	563 ± 8 (5)	801 ± 2 (3)
2	732 ± 21 (4)	824 ± 23 (2)
3	773 ± 28 (4)	835 ± 11 (2)
4	720 ± 23 (3)	829 ± 12 (2)
5	696 ± 25 (3)	851 ± 6 (2)
6	722 ± 27 (2)	830 (1)
7	786 ± 26 (2)	848 (1)
8	813 ± 26 (2)	837 (1)
9	848 ± 27 (2)	863 (1)
10	892 ± 27 (2)	874 (1)
11	923 (1)	881 (1)
12	947 (1)	881 (1)
13	980 (1)	810 (1)
14	960 (1)	843 (1)
15	850 (1)	835 (1)
16	835 (1)	834 (1)

Figure in brackets is the number of groups of 4 rats each.

Appendix 3.2.

Liver copper and zinc concentrations of copper supplemented and control rats

Time (weeks)	mean ± SEM (ug/g dry weight)	
	Copper	Zinc
	Copper supplementation	
1	326.1 ± 58.2* (4)	141.5 ± 9.5 (4)
3	1134.8 ± 111.8* (4)	123.3 ± 4.2 (4)
5	2388.0 ± 267.9 (4)	154.9 ± 11.5 (4)
10	2319.8 ± 301.1 (4)	135.1 ± 10.5 (4)
16	2023.2 ± 166.2 (4)	117.7 ± 7.6 (4)
	Control	
1	21.2 ± 1.4* (4)	129.5 ± 4.9 (4)
5	19.5 ± 1.0* (4)	133.6 ± 6.8 (4)
16	21.7 ± 0.4* (4)	127.3 ± 4.1 (4)

Figure in brackets is the number of rats. (*) $p<0.05$.

Appendix 3.3

3.3.i. Cacodylate buffered glutaraldehyde (2.5%).

A mixture of 10 ml of 25% glutaraldehyde (TAAB) and 0.2M sodium cacodylate buffer (35-50 ml) in a distilled water to a volume of 100 ml, pH 7.3. The buffer was prepared by weighing 8.5612 g of sodium cacodylate diluted in distilled water and adjusted to pH 7.3 using 0.1N hydrochloric acid and make up to 200 ml of distilled water

3.3.ii. Cacodylate sucrose wash.

A solution of 0.08 M cacodylate buffer (3.4224 g) containing 0.18 M sucrose (12.32 g) in 200 ml distilled water, pH 7.3.

3.3.iii. Zetterquist's buffered isotonic osmic acid.(1 %).

A mixture of 2% osmium tetrachloride (25 ml) in an isotonic solution (3.4 ml) buffered in sodium acetate and sodium barbitone (10 ml) and 0.1 M hydrochloric acid (10 ml) in distilled water to a final volume of 50 ml.

a Isotonic solution.

A mixture of sodium chloride (8.05 g), potassium chloride (0.42 g) and calcium chloride dihydrate (0.18 g) in 100 ml distilled water.

b. Buffer.

A solution of sodium acetate trihydrate (1.942 g) and barbitone sodium (2.942 g) in 100 ml distilled water.

3.3.iv. Epon resin.

A medium formula resin was prepared by mixing of 50 ml Epon 812 (EM Scope Equipment Limited) with 40 ml DDSA (dodecenyl saccinic anhydride hardener), 10 ml MNA (methyl nedic anhydride) and 2 ml DMP 30 (2,2,6-tri dimethyl amino methyl) (Taab Lab). The mixture was then gently agigated for 30 minutes and left to stand to allow any air bubble to spread out.

3.3.v. Staining solutions.

a. Toluidine blue (0.1%).

A solution of 0.1g toluidine blue (EM Scope) diluted in distilled water (100 ml).

b. Uranyl acetate (2%).

An aqueous solution of 2% uranyl acetate was prepared by mixing 2 g of uranyl acetate and 0.69 g maleic acid (Taab Lab.) in 100 ml distilled water.

c. Lead citrate (Standard method).

Lead nitrate (1.33 g) was first dissolved in 30 ml of distilled water. Sodium citrate (1.76 g) was then added and mixed for about 30 minutes before 1 N of NaOH (8.0 ml) was added and the pH was adjusted to 12.0 and the solution was make into 50 ml with distilled water.

3.3.vi. Tissue processing program using an Automatic Lynx Microscopy Tissues Processor.

Each sample was placed into an individual compartment of mesh plastic baskets The baskets were automatically agigated and the temperature maintained at 20°C throughout the procedure.

Vial no.	Time(hrs:mins)	Solutions (15 ml)
1	01 : 30	1% osmium tetrachloride
2	00 : 10	cacodylate buffer
3	00 : 10	cacodylate buffer
4	00 :10	cacodylate buffer
5	01 : 30	2% uranyl acetate
6	00 : 10	50% ethyl alcohol
7	00 : 10	70% ethyl alcohol
8	00 : 10	70% ethyl alcohol
9	00 : 10	90% ethyl alcohol
10	00 : 10	90% ethyl alcohol
11	00 : 10	100% ethyl alcohol
12	00 : 10	100% ethyl alcohol
13	00 : 10	100% ethyl alcohol
14	00 : 10	Absolute acetone
15	00 : 10	Absolute acetone
16	00 : 10	Absolute acetone
17	Overnight	50% resin in acetone
18	02 : 00	100% resin
19	03 : 00	100% resin

Appendix 4.0.a

Body weight of control and dietary supplemented rats

Time (weeks)	Body weight, g (mean ± SEM)			
	Control	Copper	Zinc	Copper + Zinc
0	329 ± 8 (12)	331 ± 6 (16)	392 ± 3 (16)	386 ± 3 (16)
1	365 ± 11 (8)	345 ± 6 (12)	419 ± 4 (12)	391 ± 3 (12)
2	388 ± 11 (8)	361 ± 7 (12)	444 ± 5 (12)	407 ± 4 (12)
3	408 ± 10 (8)	378 ± 8 (12)	462 ± 5 (12)	422 ± 5 (12)
4	426 ± 9 (8)	387 ± 13 (8)	477 ± 6 (8)	424 ± 4 (8)
5	443 ± 10 (8)	398 ± 12 (8)	493 ± 6 (8)	428 ± 5 (8)
6	459 ± 12 (4)	392 ± 11 (4)	494 ± 6 (4)	443 ± 10 (4)
7	472 ± 11 (4)	409 ± 11 (4)	509 ± 8 (4)	446 ± 9 (4)
8	484 ± 10 (4)	420 ± 23 (4)	515 ± 7 (4)	442 ± 8 (4)
9	491 ± 9 (4)	425 ± 14 (4)	522 ± 8 (4)	445 ± 9 (4)
10	498 ± 10 (4)	427 ± 13 (4)	524 ± 8 (4)	450 ± 10 (4)
11	508 ± 10 (4)	439 ± 13 (4)	540 ± 8 (4)	455 ± 9 (4)
12	515 ± 10 (4)	440 ± 13 (4)	553 ± 8 (4)	465 ± 11 (4)
13	525 ± 12 (4)	447 ± 13 (4)	562 ± 9 (4)	477 ± 11 (4)
14	531 ± 12 (4)	450 ± 13 (4)	565 ± 9 (4)	484 ± 12 (4)
15	536 ± 11 (4)	458 ± 14 (4)	568 ± 9 (4)	490 ± 14 (4)
16	547 ± 12 (4)	463 ± 14 (4)	575 ± 8 (4)	500 ± 15 (4)

Figure in brackets is the number of rats.

Appendix 4.0.b

Body weight of control and dietary supplemented rats

Time (weeks)	Body weight, g (mean ± SEM) Zinc (s/c) 20 hours prior to dietary supplementation			
	Control	Copper	Zinc	Copper + Zinc
0	354 ± 10 (16)	364 ± 8 (16)	396 ± 3 (16)	331 ± 5 (16)
1	379 ± 11 (12)	374 ± 9 (12)	419 ± 3 (12)	341 ± 6 (12)
2	392 ± 10 (12)	387 ± 9 (12)	441 ± 4 (12)	361 ± 7 (12)
3	412 ± 10 (12)	400 ± 10 (12)	462 ± 4 (12)	377 ± 8 (12)
4	417 ± 15 (8)	395 ± 14 (8)	479 ± 7 (8)	399 ± 10 (8)
5	433 ± 15 (8)	496 ± 12 (8)	497 ± 7 (8)	413 ± 11 (8)
6	416 ± 22 (4)	387 ± 21 (4)	490 ± 10 (4)	414 ± 18 (4)
7	430 ± 20 (4)	399 ± 24 (4)	501 ± 10 (4)	424 ± 18 (4)
8	442 ± 20 (4)	405 ± 22 (4)	507 ± 9 (4)	433 ± 18 (4)
9	451 ± 20 (4)	410 ± 22 (4)	515 ± 9 (4)	438 ± 18 (4)
10	456 ± 19 (4)	414 ± 22 (4)	519 ± 9 (4)	446 ± 20 (.4)
11	467 ± 19 (4)	423 ± 24 (4)	531 ± 12 (4)	454 ± 20 (4)
12	473 ± 21 (4)	430 ± 23 (4)	539 ± 12 (4)	461 ± 19 (4)
13	484 ± 20 (4)	443 ± 26 (4)	545 ± 10 (4)	467 ± 20 (4)
14	490 ± 20 (4)	442 ± 25 (4)	548 ± 11 (4)	473 ± 20 (4)
15	497 ± 20 (4)	450 ± 26 (4)	553 ± 11 (4)	476 ± 22 (4)
16	507 ± 22 (4)	459 ± 26 (4)	557 ± 11 (4)	483 ± 23 (4)

Figure in brackets is the number of rats.

Appendix 4.1.a

Food consumption of control and dietary supplemented rats

Time (weeks)	Food consumption, g (mean ± SEM)			
	Control	Copper	Zinc	Copper + Zinc
1	739 ± 23 (3)	557 ± 14 (3)	908 ± 12 (3)	564 ± 11 (3)
2	760 ± 19 (2)	709 ± 67 (3)	898 ± 11 (3)	733 ± 20 (3)
3	781 ± 10 (2)	734 ± 52 (3)	866 ± 19 (3)	715 ± 24 (3)
4	815 ± 23 (2)	668 ± 35 (2)	857 ± 15 (2)	692 ± 29 (3)
5	807 ± 19 (2)	636 ± 4 (2)	854 ± 18 (2)	700 ± 86 (2)
6	802 (1)	659 (1)	864 (1)	741 (1)
7	817 (1)	726 (1)	826 (1)	684 (1)
8	782 (1)	666 (1)	881 (1)	626 (1)
9	780 (1)	642 (1)	803 (1)	659 (1)
10	840 (1)	698 (1)	831 (1)	677 (1)
11	816 (1)	667 (1)	850 (1)	687 (1)
12	784 (1)	644 (1)	866 (1)	695 (1)
13	818 (1)	632 (1)	843 (1)	689 (1)
14	789 (1)	621 (1)	808 (1)	669 (1)
15	744 (1)	611 (1)	794 (1)	709 (1)
16	798 (1)	. 637 (1)	805 (1)	719 (1)

Figure in brackets is the number of groups of 4 rats each.

Appendix 4.1.b

Food consumption of control and dietary supplemented rats

Time (weeks)	Food consumption, g (mean ± SEM) Zinc (s/c) 20 hours prior to dietary supplementation			
	Control	Copper	Zinc	Copper + Zinc
1	651 ± 25 (3)	526 ± 20 (3)	821 ± 38 (3)	549 ± 27 (3)
2	688 ± 20 (3)	689 ± 41 (3)	843 ± 18 (3)	665 ± 13 (3)
3	721 ± 16 (3)	659 ± 19 (3)	835 ± 25 (3)	690 ± 22 (3)
4	735 ± 19 (2)	646 ± 54 (2)	849 ± 57 (2)	677 ± 13 (2)
5	736 ± 1 (2)	587 ± 40 (2)	820 ± 54 (2)	663 ± 6 (2)
6	740 (1)	544 (1)	812 (1)	643 (1)
7	763 (1)	587 (1)	754 (1)	635 (1)
8	743 (1)	549 (1)	811 (1)	656 (1)
9	749 (1)	586 (1)	777 (1)	669 (1)
10	771 (1)	616 (1)	802 (1)	710 (1)
11	772 (1)	584 (1)	799 (1)	669 (1)
12	730 (1)	602 (1)	799 (1)	667 (1)
13	766 (1)	628 (1)	764 (1)	670 (1)
14	741 (1)	587 (1)	792 (1)	670 (1)
15	725 (1)	580 (1)	796 (1)	643 (1)
16	760 (1)	621 (1)	785 (1)	671 (1)

Figure in brackets is the number of groups of 4 rats each.

Appendix 5.0

Body weight of control, copper and zinc supplemented rats

Time (weeks)	Body weight, g (mean ± SEM) Dietary supplementation Control	Copper	Zinc
0	342 ± 5 (16)	346 ± 5 (16)	344 ± 5 (16)
1	378 ± 6 (16)	359 ± 5 (16)*	380 ± 5 (16)
2	404 ± 8 (12)	384 ± 6 (12)*	403 ± 7 (12)
3	431 ± 7 (12)	408 ± 6 (12)*	427 ± 7 (12)
4	460 ± 5 (12)	429 ± 5 (12)*	458 ± 6 (12)
5	476 ± 6 (12)	439 ± 4 (12)*	477 ± 6 (12)
6	490 ± 11 (4)	443 ± 5 (4)*	489 ± 13 (4)
7	505 ± 11 (4)	448 ± 2 (4)*	505 ± 16 (4)
8	514 ± 9 (4)	468 ± 2 (4)*	517 ± 17 (4)
9	526 ± 9 (4)	469 ± 3 (4)*	528 ± 16 (4)
10	538 ± 9 (4)	480 ± 2 (4)*	539 ± 17 (4)
11	554 ± 8 (4)	492 ± 2 (4)*	550 ± 17 (4)
12	563 ± 10 (4)	496 ± 2 (4)*	562 ± 19 (4)
13	573 ± 10 (4)	498 ± 2 (4)*	566 ± 19 (4)
14	581 ± 11 (4)	509 ± 2 (4)*	573 ± 21 (4)
15	581 ± 11 (4)	514 ± 2 (4)*	578 ± 21 (4)
16	588 ± 11 (4)	522 ± 4 (4)*	583 ± 22 (4)

Figure in bracket is the number of rats. (*) $p<0.05$.

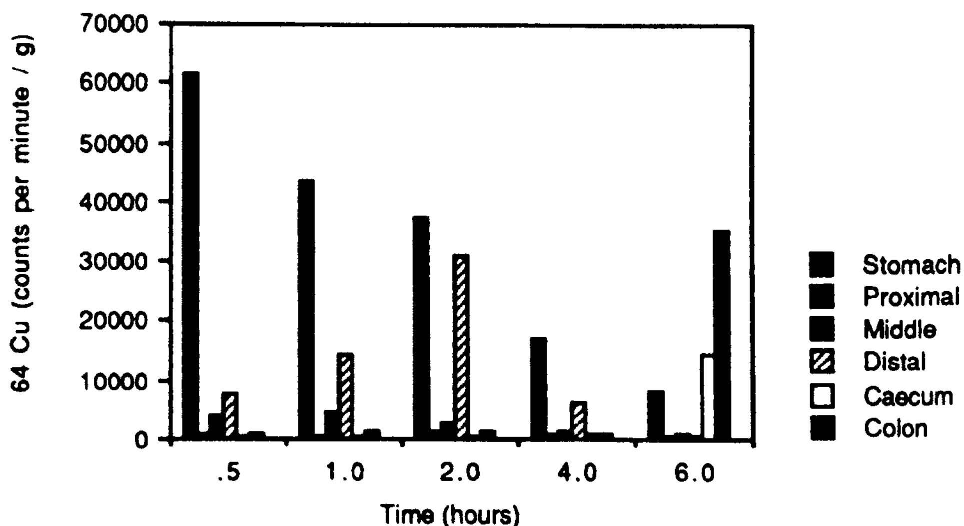
Appendix 5.1

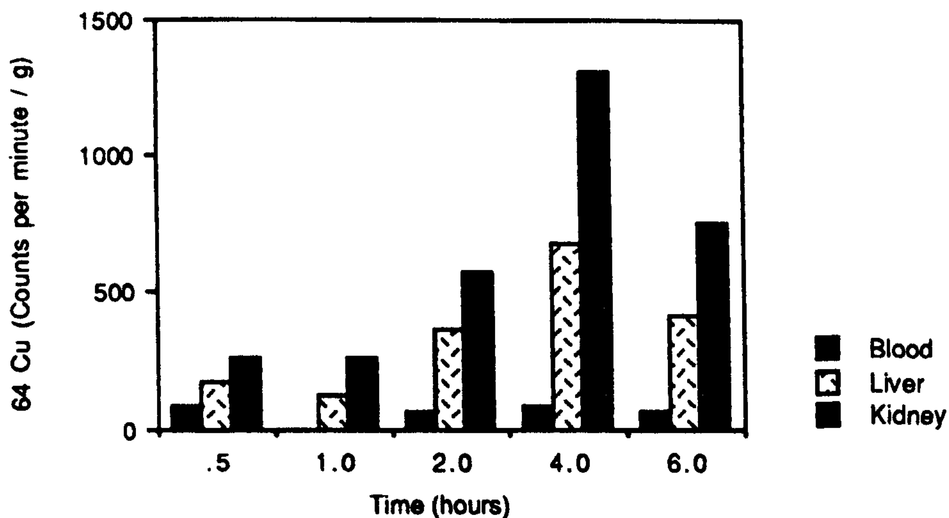
Food consumption of control, copper and zinc supplemented rats

Time (weeks)	Food consumption, g (mean ± SEM) Dietary supplementation Control	Copper	Zinc
1	739 ± 21 (4)	573 ± 23 (4)	800 ± 11 (4)
2	787 ± 23 (3)	788 ± 21 (3)	840 ± 1 (3)
3	787 ± 16 (3)	700 ± 12 (3)	791 ± 4 (3)
4	820 ± 4 (2)	733 ± 14 (2)	823 ± 6 (2)
5	828 ± 4 (2)	734 ± 14 (2)	830 ± 4 (2)
6	800 (2)	711 (1)	815 (1)
7	808 (1)	768 (1)	821 (1)
8	831 (1)	843 (1)	867 (1)
9	806 (1)	793 (1)	846 (1)
10	823 (1)	802 (1)	841 (1)
11	810 (1)	803 (1)	840 (1)
12	814 (1)	817 (1)	847 (1)
13	834 (1)	865 (1)	835 (1)
14	840 (1)	875 (1)	824 (1)
15	862 (1)	925 (1)	873 (1)
16	872 (1)	898 (1)	873 (1)

Figure in bracket is the number of groups of 4 rats each.

Appendix 5.2





Preliminary study. The distribution of ^{64}Cu in the gastrointestinal subdivisions, blood, liver and kidney at various intervals of time after intragastric administration of 1.0 ml of ^{64}Cu solution containing 20μg copper as anhydrous copper sulphate with initial specific activity of 1.4 milicuries per 4.98 mg of copper in 14-week-old Wistar rats. Values are mean of 2 rats.

Appendix 5.3

Liver and kidneys wet weights of control and copper or zinc supplemented rats

Time (wks)	Dietary supplementation	Total weight (g) Liver	Total weight (g) Kidneys	Total weight (% of body weight) Liver	Total weight (% of body weight) Kidneys
	Control	9.36 ± 0.30	2.09 ± 0.11	2.51 ± 0.04	0.56 ± 0.03
1	Copper	10.51 ± 0.24*	2.21 ± 0.11	2.89 ± 0.07*	0.61 ± 0.03
	Zinc	9.78 ± 0.52	2.14 ± 0.07	2.57 ± 0.12	0.56 ± 0.02
	Control	9.50 ± 0.49	2.09 ± 0.10	2.34 ± 0.06	0.52 ± 0.02
3	Copper	9.97 ± 0.30	2.04 ± 0.03	2.58 ± 0.02*	0.53 ± 0.01
	Zinc	9.91 ± 0.53	2.08 ± 0.09	2.48 ± 0.11	0.52 ± 0.02
	Control	11.41 ± 0.13	2.29 ± 0.08	2.36 ± 0.02	0.47 ± 0.02
5	Copper	12.14 ± 0.50	2.37 ± 0.07	2.73 ± 0.09*	0.53 ± 0.02*
	Zinc	11.16 ± 0.24	2.34 ± 0.06	2.33 ± 0.03	0.49 ± 0.02
	Control	13.41 ± 0.70	2.95 ± 0.19	2.28 ± 0.10	0.50 ± 0.03
16	Copper	12.37 ± 0.29	2.61 ± 0.08	2.37 ± 0.05	0.50 ± 0.03
	Zinc	12.31 ± 0.79	2.63 ± 0.21	2.11 ± 0.07	0.45 ± 0.02

Each value is the mean and standard error of mean of 4 rats. (*) $p<0.05$.

Appendix 5.4

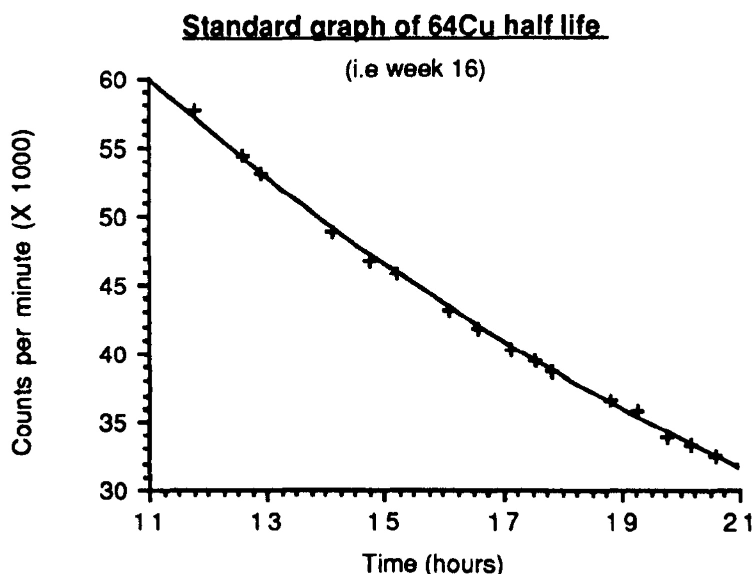
The distribution of ^{64}Cu in the blood

after 4.0 hours intragastric administration of the isotopes

Time (weeks)	Blood per ml, counts per minute (% of dose) ($\times 10^2$) Dietary supplementation: Control	Copper	Zinc
1	24.6 ± 0.8	25.1 ± 1.5	24.0 ± 1.5
3	5.1 ± 0.3	5.5 ± 0.2	5.7 ± 0.4
5	5.8 ± 0.6	5.2 ± 0.3	6.5 ± 0.1
16	6.8 ± 0.4	5.7 ± 0.3*	6.5 ± 0.5

Each value is the mean and standard error of mean of 4 rats. (*) $p<0.05$.

Appendix 5.5



Appendix 5.6

Distribution of ^{64}Cu in the blood, liver and kidney of control, copper and zinc supplemented rats after 4 hours intragastric administration of the isotopes

Time (wks)	Dietary supplementation	^{64}Cu absorbed, counts per minute (% of absorbed dose) Blood	Liver	Kidneys
	Control	12.6 ± 0.4	5.1 ± 0.3	2.2 ± 0.1
1	Copper	21.5 + 1.2*	6.8 ± 0.5*	2.9 ± 0.1*
	Zinc	13.9 ± 0.9	6.1 ± 0.5	2.5 ± 0.1
	Control	2.0 ± 0.1	2.0 ± 0.2	0.9 ± 0.1
3	Copper	2.4 ± 0.1*	4.1 ± 0.9*	0.7 ± 0.1
	Zinc	2.7 ± 0.1*	2.6 ± 0.3	1.0 ± 0.2
	Control	2.6 ± 0.3	1.5 ± 0.1	0.9 ± 0.1
5	Copper	2.6 ± 0.1	2.1 ± 0.2*	0.6 ± 0.1
	Zinc	3.7 ± 0.1	1.9 ± 0.1*	1.1 ± 0.2
	Control	3.9 ± 0.3	2.2 ± 0.1	1.1 ± 0.0
16	Copper	4.0 ± 0.2	4.0 ± 0.6*	0.9 ± 0.2
	Zinc	4.6 ± 0.3	3.3 ± 0.1*	1.4 ± 0.3

Each value is the mean and standard error of mean of 4 rats. (*) $p<0.05$.