CELLULAR MECHANISMS OF TOXICITY AND TOLERANCE IN THE COPPER-LOADED RAT.

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by

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ABSTRACT.

Copper associated diseases occur in both man and animals although the relationship between copper (Cu) excess and liver damage is poorly understood. Accumulation of Cu in the liver and kidneys of the rat is initially injurious but is followed by recovery and copper tolerance.

The aim of this study is to clarify the intracellular localisation of Cu in relation to cell damage and recovery in the livers and kidneys of copper-loaded rats. Young male Wistar rats were fed a high Cu (1,500ppm) diet for 16 weeks, with appropriate low Cu (10 ppm) controls, their body weights and food intake recorded weekly. They were killed sequentially and livers and kidneys retained for Cu and Zinc (Zn) estimation, histological and ultrastructural evaluation. Blocks of formalin fixed, paraffin-embedded tissue were sectioned and stained with H.E., Rhodanine and Rubeanic acid for copper. Other samples were dried, wet-ashed and analysed for Cu and Zn by AA spectrophotometry. Transmission electron microscopy: tissues were fixed overnight (4 ^{o}C) in Karnovsky fixative, post-fixed in OsO_4, resin embedded, cut and examined in a Hitachi electron-microscope. X-ray electron probe microanalysis: tissues were fixed in a similar manner omitting osmication, sections were collected on Al grids and analysed for their elemental content in an EM fitted with a XEVEX detector.

Initial studies showed that for 5 weeks Cu accumulated in liver and kidneys with accompanying damage. Subsequently copper concentrations fell with cellular recovery. Zn was unaffected. Histochemical copper distribution occurred in the periportal and midzones of the liver and in the proximal convoluted tubules (PCT) of the kidney. Cu localisation was limited to intracytoplasmic granules and renal droplets (α2u globulin). Furthermore histochemical stains showed an imperfect correlation with hepatic copper concentrations. Ultrastructural and microanalytical investigations revealed early hepatocyte and PCT cell nuclear degeneration, coinciding with Cu (S,P,Zn and Ca) uptake, culminating in lysis at 4 weeks. Lysosomes increased in numbers and morphological diversity but maintained their structural integrity and 3 types were identified; Type I contained mainly Cu; Type II included S,P and Cu (putative Cu-metallothionein) and replaced Type I; Type III contained much reduced elemental residues and characterised the recovery period in the liver. Additionally there was swelling of SER and mitochondria and dissaggregation of RER. Cell death was presaged by apoptotic bodies (3-4 weeks) in hepatocyte and Kupffer cells and luminal debris in PCT. Membrane changes occurred in both biliary canalicular and PCT microvilli. Copper content occurred in PCT spherules (α2u globulin). Recovery was characterised by a regression of degenerative changes, decline in lysosomal numbers and unremarkable nuclei; presence of Mallory-body-like structures in liver and apical extrusion of PCT cell content in the kidney.

Lysosomal sequestration of copper is protective whereas Cu entering the nucleus causes irreversible damage. Lysosomal subtypes illustrate stages in the internal reorganisation whereby copper is rendered innocuous and is ultimately discharged from the liver; kidney copper balance is maintained by continuous discharge of copper into PCT lumina.

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GENERAL INTRODUCTION.

A.- Background.

Copper is an essential trace element present in most animal tissues and plays a significant role in a wide variety of physiological processes (Underwood, 1977). A number of metalloenzyme systems contain copper: cytochrome oxidase, the terminal enzyme in the oxidative phosphorylation process (Ambe and Venkatarama, 1959), superoxide dismutase, an antioxidant (Mann and Keilin, 1939), lysyl oxidase involved in collagen synthesis (Underwood,1974) and tyrosinase in melanin production (Lerner et al., 1950). Ceruloplasmin is a circulating copper-containing protein (Kasper and Deutsch, 1963) with many functions: in addition to its amine-oxidase activity ceruloplasmin acts as a copper transport and donor protein (Frieden, 1980). It has been shown to promote mobilization of iron (Frieden, 1970) and is also important in the conversion of haemosiderin to transferrin (Roeser et al., 1970). Metallothionein (MT) is a copper-binding protein considered to be important in the metabolism of copper and zinc and in the storage and detoxification of heavy metals (Kagi and Nordberg, 1979). In addition both ceruloplasmin (Evans et al., 1969) and MT (Cousins, 1983) are acute-phase reactants.

Copper metallothioneins have been isolated from fungi, plants (Rauser and Curvetto, 1980; Lolkema et al., 1984), invertebrates (Olafson et al., 1979) and the liver, kidneys and intestine of several mammals (Kagi and Vallee, 1961; Nordberg et al., 1971) and fish (Noel-Lambert et al., 1978; Overnell and Coombs, 1979).

Typically, metallothioneins are sulphydryl-rich, inducible proteins characterised by a low molecular weight (6500 Daltons), high cysteine content and the capacity to bind a variety of metals including mercury, gold, lead, cadmium, zinc and copper (Winge and Rajagopalan, 1975; Kagi and Nordberg, 1979).

The functions of the metallothioneins are not completely understood, although they are believed to be involved in the detoxification of heavy metals (Kojima and Kagi, 1978), particularly cadmium (Leber and Miya, 1976; Probst et al., 1977), the donation of metals to other metalloproteins during their synthesis (Lerch, 1980), and the maintenance of homeostatic levels of copper and zinc in tissues (Evans, 1973; Bloomer and Lee, 1978; Cousins, 1979).

Several pathological conditions are associated with the accumulation of copper, together with alterations in metallothionein levels; these include Wilson's disease (Evans et al., 1973) and Menkes disease in man (Menkes et al., 1962; Riordan and Jolicoeur-Paquet, 1982), and in animals inherited copper toxicosis in Bedlington Terriers (Twedt et al., 1979; Johnson et al., 1981).

Under natural conditions copper-metallothionein can occur as a major copper-binding protein in both the cytosolic and particulate fractions of the liver (Bremner, 1987) and there is some evidence that copper-metallothionein is incorporated into lysosomes (Goldfischer, 1967; Porter, 1974; Riordan and Richards, 1980). In addition, radioimmunoassay has established the presence of metallothionein in the plasma, urine and the bile of copper-loaded rats (Bremner et al., 1986).

Copper homeostasis is maintained by the retention of adequate reserves of the metal within the liver and the excretion of excess from the bile in most species (Bush et al., 1955; Mahoney et al., 1955; Owen and Hazelrig, 1966). Mean liver copper concentrations vary between species, from 17 ± 1.5 μg/g dry weight tissue in the rat (Haywood, 1985), 193 (range 91-377 μg/g dry weight tissue in the dog (Ludwig et al., 1980), and 50 μg/g wet weight tissue (150-250μg/g dry weight) in the sheep (Soli, 1980).

However, under certain conditions, copper has been shown to accumulate in the liver as the cause, or as the consequence, of liver damage. Ruminants, especially sheep, are considered to be the most susceptible to copper excess (Todd, 1962; Todd, 1969) and copper toxicosis has been a problem in sheep for many years (Soli, 1980). Familial copper storage diseases occur in man, Wilson's disease, (Sternlieb, 1980) and in Bedlington terriers (Hardy et al., 1975) and West Highland White terriers (Thornburg et al.,

1985). Other copper associated diseases in human subjects occur in Primary biliary cirrhosis (Epstein et al., 1981b) and Indian childhood cirrhosis (Portmann et al., 1978). Chronic active hepatitis in dogs (Crawford et al., 1986) and Skye terrier hepatitis (Rutgers et al., 1988), are also associated with excess liver copper.

In contrast, extremely high concentrations of liver copper have been reported without evidence of hepatic injury in the toad (Goldfischer et al., 1980) and the swan (Molnar, 1983). Numerous attempts have been made to reproduce copper toxicosis in rats without success and prolonged parenteral injection of copper salts induced only a mild chronic hepatitis (Wolff, 1960; Wiederanders, et al., 1968). The reason for this became apparent when it was shown that the rat can adapt to tissue damaging doses of copper and become tolerant (Haywood, 1980).

Tolerance to metal intoxication is a recognized concept and has been defined as "the ability to endure the continued increasing administration of a toxicant and the capacity to exhibit less response to a test dose than previously" (Luckey and Venugopal, 1977). Besides rats there are very few reports of a similar phenomenon occurring with copper in animals. An acquired resistance to copper has been recognised in salmon, in which the tolerant state is directly related to the metallothionein content of the liver (McCarter and Roch, 1983, 1984; Roch and Mc Carter, 1984). Copper-tolerant plants, associated with the production and accumulation of metallothionein-like metal complexes have been

described with more frequency in the literature (Rauser, 1984; Lolkema et al., 1984; Robinson and Jackson, 1986).

B.-Causes of copper-associated disease.

Environmental contamination by metal particulates including lead, cadmium, mercury, copper and molybdenum has risen considerably with their increasing use in diverse agricultural, chemical, technological and industrial processes (Schoeder and Nason, 1971; Chappell, 1975). Excess copper from such sources as slurry (pig and poultry residues), molluscicides and herbicides are possibly the main source of toxicosis in sheep and occasionally other farm animals (Rana and Kumar, 1983a).

Acute toxicity has been reported in sheep as a result of prophylactic administration of the copper-calcium complex of ethylene diamine tetra-acetic acid (Ishmael et al., 1969) and copper diethylamine oxyquinoline sulphonate (Mason et al., 1984) for the treatment of copper deficiency; but this is rare and the cumulative form is by far the more common manifestation of the disease in sheep (Soli, 1980).

In man excessive intake of copper may be the cause of Indian childhood cirrhosis (Sharda and Bhandari, 1980), whereby young children absorb high levels of the metal from copper and brass household utensils used in the storage of milk (Tanner et al., 1983). Likewise copper-associated cirrhosis in Bavarian children, indistinguishable from Indian childhood cirrhosis is related to drinking water with a high copper content (Muller-

Hocker et al., 1987).

The nutritional significance of a trace element is determined not only by its specific physiological and biochemical roles in animal metabolism, but also by its capacity to interact with other elements in ways which can be beneficial or harmful in terms of animal health. Copper toxicity can also be due to imbalance of other elements such as zinc, iron, molybdenum, sulphur and cadmium (Rana and Kumar, 1978), of which the interaction of molybdenum and sulphur with copper in ruminants is probably the best understood (Suttle, 1974; Dick et al., 1975). Molybdenum and sulphur complex with copper to form copper-thiomolybdate which acts both within the rumen and systemically to reduce the biological availability of copper (Dick et al., 1975); conversely a lack of molybdenum (Buck and Ewan, 1973) and sulphur can increase the absorption of copper, producing overload (Suttle, 1974). Cadmium-copper antagonism has been reported in lambs (Mills and Dalgarno, 1972; Doyle and Pfanders, 1975) and adult sheep (Lee and Jones, 1976). Iron and zinc likewise interact competitively with copper inhibiting its absorption but facilitating copper uptake when iron and zinc concentrations are low (Van Campen, 1970).

Pyrrolizidine alkaloids are known to be hepatotoxic and also contribute to chronic copper poisoning because of the supposed greater avidity for copper of the altered cells (Bull et al., 1956a). In Australia sheep consuming Heliotropium europeum, a common pasture contaminant containing pyrrolizidine alkaloids,

accumulate excess liver copper and may die of a copper-induced haemolytic crisis (Bull et al., 1956b). Hepatogenous chronic copper poisoning has also been reported in sheep grazing Echium plantaginum another pyrrolizidine-alkaloid-containing plant (Seaman, 1985). Consumption of tansy ragwort (Senecio jacobea) results in increased liver copper concentrations both in rats (Swick et al., 1982a) and rabbits (Swick et al., 1982b) but not apparently in sheep (White et al., 1984), neither have pyrrolizidine alkaloids been associated with copper retention in other species. The effect of pyrrolizidine alkaloids on copper metabolism requires further study.

Copper toxicosis may be genetically determined as in Wilsons's disease in which it is inherited in an autosomal recessive pattern (Sternlieb et al., 1961) and in Bedlington terriers in which it is inherited in a similar manner (Johnson et al., 1980). Familial copper storage disease also occurs in West Highland White terriers although the pattern of inheritance remains unclear (Thornburg et al., 1986). All these diseases are characterised by the progressive accumulation of copper with liver damage culminating in cirrhosis. Several factors have been implicated such as failure of biliary excretion of copper in both Wilson's disease (Frommer, 1974) and Bedlington terrier toxicosis (Su et al., 1982a); also defective lysosomal incorporation of copper (Johnson et al., 1981) and depressed ceruloplasmin synthesis in Wilson's disease (Williams and Lee, 1978).

Chronic cholestatic conditions such as Primary biliary cirrhosis (Epstein et al., 1981a) in man, chronic active hepatitis in Doberman Pinschers (Crawford et al., 1986) and idiopathic cholestasis in Skye terriers (Haywood et al., 1988) may also cause copper overload.

Finally stress factors may be important in precipitating the clinical disease; in the copper-loaded sheep a fall in plane of nutrition, fasting, movement or handling of the flock, undue exertion or exposure to severe weather conditions frequently precipitates the haemolytic crisis (Pierson and Aanes, 1958). Stress possibly associated with whelping and showing has also been incriminated with an acute episode in Bedlington terriers (Herrtage, 1987). Environmental stress also enhances copper toxicity in the crustacean, Carcinus maenas (Depledge, 1987).

The relationship between copper accumulation and liver injury is unclear. There appears to be no correlation between copper content and hepatocellular damage in Wilson's disease (Goldfischer et al., 1980), Indian childhood cirrhosis (Talbot et al., 1985) and Primary biliary cirrhosis (Epstein et al., 1981a). Likewise, in animals liver copper concentration is not necessarily consistent with the severity of the disease in copper-associated disorders in dogs (Herrtage et al., 1987b) and comparatively modest hepatic accumulations of copper may be associated with a haemolytic crisis in sheep (Soli, 1980). Conversely, greatly elevated hepatic copper concentrations have been reported in the swan (Molnar, 1983) and buffalo toad (Goldfischer et al., 1970)

without any pathological consequences and copper-loaded rats can adapt and recover from very high metal burdens (Haywood, 1985; Haywood and Loughran, 1985). It seems probable that the molecular association and intracellular localization of copper are important in determining the toxicity or otherwise of the metal.

C.- Clinico-pathological findings.

a).- **Copper toxicosis in sheep.**

Chronic copper poisoning in sheep occurs after a clinically silent cumulative phase and usually presents as an acute haemolytic episode or crisis, in which copper is released from the liver into the blood stream with haemolysis of erythrocytes (Soli, 1980). Despite liver injury, death usually occurs due to acute renal failure consequent on tubular obstruction by haemoglobin casts (Gopinath and Howell, 1975).

Animals that have suffered a haemolytic crisis have yellow-brown discolouration of the mucous membranes and body fat (Soli and Nafstad, 1976). The liver is yellow-brown and the kidneys appear softer than normal and discoloured (Ishmael et al., 1971). Sometimes subclinical haemolytic episodes are followed by recovery with decreased hepatic copper levels. Fibrosis (Gopinath and Howell, 1975), and early cirrhosis have occasionally been seen in such sheep (Susan Haywood, personal communication).

Changes have also been described in the central nervous system: vacuolation of white matter has been recorded in the

brains of sheep which have died of chronic copper poisoning (Doherty et al., 1969; Ishmael et al., 1971).

Excess copper has been demonstrated in the liver (Gopinath and Howell, 1975) and kidneys (Gooneratne and Howell, 1983), but not in the brain and, terminal neurological disturbances may be ascribed to hepatic encephalopathy (Gooneratne and Howell, 1979; Soli, 1980). Liver copper values between 250-750 ug/g dry weight reflect a moderate overload and copper concentrations above 750 ug/g dry weight in the liver (Clarke and Clarke, 1975) and above 50 ug/g dry weight in the kidney, may be sufficient to explain a copper induced haemolytic crisis (Soli, 1980).

At the onset of the haemolytic crisis there are very marked increases in the activity of serum enzymes lactic dehydrogenase, glutamic-oxalacetic transferase (GOT) (Ross,1964; 1966) and alanine aminotransferase (ALT) (Todd, 1969). There is also a sudden and dramatic increase in the levels of creatine phosphokinase suggesting that changes occur in the muscle cell membrane as well as in the liver and kidney (Thompson and Todd, 1974). Elevation of blood copper occurs prior to and during haemolysis; the ceruloplasmin levels fluctuate during the pre-haemolytic period and increase over two-fold immediately before and are sustained during the haemolytic crisis; serum bilirubin levels and blood urea also increase during the haemolytic period. (Ishmael et al., 1972).

The pattern of histopathological changes was first studied in the livers of experimental sheep dosed with Cu SO_4 (Ishmael et al., 1971). Throughout the pre-haemolytic phase there was progressive swelling of isolated parenchymal cells and single cell necrosis in association with a polymorphonuclear neutrophil leukocyte response in the centrilobular region; with duplication and collapse of the reticulin framework. Nuclear enlargement and vacuolation were also apparent and occasional swollen Kupffer cells with granular cytoplasm were identified. Copper was demonstrated histochemically with Rubeanic acid as fine particles within the parenchymal cell cytoplasm and denser granular deposits within swollen Kupffer cells. Later studies confirmed these findings and furthermore Kumaratilake and Howell (1987), demonstrated the histochemical deposition of copper first in the centrilobular zones extending to mid and periportal zones with progressive copper-loading.

In addition to the changes described, numerous large foci of necrotic liver cells unassociated with polymorphonuclear leukocytes were observed during the haemolytic crisis. An increase in canalicular bile pigment was seen during the haemolytic and post-haemolytic phase, when many of the parenchymal cells showed fatty change. Following haemolysis nuclear enlargement and vacuolation were still present, but focal necrosis was no longer evident. In the portal areas there was fibroblast proliferation, an increase in bile duct numbers and lymphocytic infiltration.

The histopathological changes in the kidneys during the haemolytic crisis were characterised by marked degenerative changes, with necrosis, desquamation, vacuolation and eosinophilia of the epithelial cells of the proximal convoluted tubules. Numerous small eosinophilic, intracytoplasmic granular inclusions were present within the proximal convoluted tubule epithelial cells. These granules stained positively for copper (Gopinath et al., 1974; Gooneratne and Howell, 1983). Many of the cortical tubules were dilated and contained eosinophilic and granular casts. Dilatation was more prominent among distal convoluted tubules. During the post-haemolytic phase, there was residual vacuolation and swelling of the lining cells of the proximal tubules, and some cells also contained brown pigment granules. Many of the distal tubules and occasional collecting tubules in the cortex showed dilatation. Foci of interstitial connective tissue proliferation with limited mononuclear cell infiltration were also evident in the cortex (Ishmael et al., 1971; Gopinath et al., 1974; Gooneratne and Howell, 1983).

b).-Copper associated disease in Human Subjects.

Wilson's disease.

Increasing accumulation of copper, up to 50 times normal values (over 2500µg/g dry weight), accompanied by hepatocellular damage occurs in the livers of young people affected with Wilson's disease (Sternlieb, 1980). This early stage of the disease may be

asymptomatic, although less occasionally, may culminate in an acute haemolytic crisis (Sternlieb and Scheinberg, 1968). The later stages of the disease are associated with cirrhosis and paradoxically some decline in liver copper with the increasing manifestation of neurological signs (Goldfischer and Sternlieb, 1968).

Copper accumulates additionally in the kidney, brain and cornea where it is responsible for the distinctive Kayser-Fleischer rings (Webers et al., 1977).

In the presymptomatic stage the alanine aminotransferase (ALT) levels is the best biochemical indication of liver damage (Levi et al., 1967). Deficiency of ceruloplasmin is seen in 95% of patients with Wilson's disease (Gibbs and Walshe, 1979),copper in the blood is also reduced (Walshe, 1987). Urinary copper is increased (Owen and Ludwig, 1982). Liver copper concentration is greater than 250 ug/g dry weight (Sternlieb, 1972).

Histopathological findings in the livers of Wilson's disease patients with the early disease are characterised by steatosis, prominent nuclear vacuolation and Mallory body formation. A spectrum of histological activity may be present, ranging from marked liver cell degeneration and necrosis to inactive macronodular cirrhosis. The histology closely resembles chronic active hepatitis with prominent piecemeal necrosis and rosette formation (Epstein, 1983a).

Histochemical stains for both copper and copper-associated protein are unreliable in Wilson's disease,

particularly in the earlier asymptomatic state (Goldfischer and Sternlieb, 1968, Sternlieb and Scheinberg,1968, Sternlieb,1972). In the kidney, histopathological changes include degeneration and necrosis of lining cells of tubules and degeneration of glomerular structures (Wolff, 1964).

Primary biliary cirrhosis.

Intra-or extrahepatic disturbances in the secretion of bile may result in the retention of copper by the liver (Fleming et al., 1974). Under such conditions the pathologic process precedes the accumulation of copper which progressively increases with the duration of cholestasis, such as occurs in Primary biliary cirrhosis (Benson, 1979) and other chronic cholestatic conditions (Smallwood et al., 1968).

In spite of hepatic copper concentrations that occasionally equal those seen in Wilson's disease, copper does not affect the central nervous system of patients with Primary biliary cirrhosis. Kayser-Fleischer rings, however, can occur in Primary biliary cirrhosis (Fleming et al., 1977, Frommer et al., 1977) and in other cholestatic syndromes and may raise diagnostic problems (Sternlieb, 1978)

In Primary biliary cirrhosis serum bilirubin values are rarely very high although serum alkaline phosphatase is always markedly elevated (Sherlock, 1987) and alanine aminotransferase (ALT) levels are moderately elevated (MacSween, 1979). There is

also hypercupriuria (Sternlieb, 1980). Serum immunoglobulins of all major classes are elevated, but increase of IgM is most consistent with mean increases in excess of 150% normal levels (MacSween et al., 1972).

The most helpful diagnostic test is the demonstration of serum M-antibody by an immunofluorescent technique (Goudie et al.,1966). This antibody is found in more than 90% of patients and in its absence a diagnosis of Primary biliary cirrhosis should be made reluctantly (MacSween and Berg, 1976).

The early stage of Primary biliary cirrhosis is characterized by a chronic non-suppurative destructive cholangitis and the second stage by proliferation and destruction of ductules (Goldfischer et al., 1980). Mallory bodies are a common finding (Ludwig et al., 1979).

Liver copper content can rise to 714 µg/g dry weight and can be distinguished with different copper stains (Goldfischer et al., 1980). However, rhodanine seems to be more reliable than other copper stains (Ludwig et al., 1979).

Indian childhood cirrhosis.

This disease may be associated with extremely high liver copper concentrations which may reach as high as 4788 µg/g dry weight (Goldfischer et al., 1980). In contrast to Wilson's disease copper accumulation does not occur in the brain or cornea (Sternlieb, 1980). Neither is there a decline in ceruloplasmin or serum copper content (Nayak and Ramalingaswami, 1975).

Biochemical changes in Indian childhood cirrhosis include hyperbilirubinaemia and increase in serum copper and ceruloplasmin (Muller-Hocker et al., 1987).

Histopathological changes vary from those resembling the subacute stage of viral hepatitis, to micronodular cirrhosis or may include fulminant massive necrosis (Popper et al., 1979). In the early stages of Indian childhood cirrhosis there may be mild fatty change and in the established stage of the disease there is severe hepatocellular degeneration characterised by ballooning change, focal necrosis and numerous Mallory bodies (Roy et al., 1971, Popper et al., 1979).

Copper and copper-associated proteins can be stained histochemically, but unlike other copper-overloaded states, the rhodanine stain shows diffuse cytoplasmic copper staining in addition to the more granular lysosomal staining (Popper et al., 1979).

b.-Copper associated disease in Dogs.

Familial copper storage disorders in Bedlington and West Highland White Terriers are usually associated with a clinically silent early period during which copper accumulates with concommitant liver injury culminating in cirrhosis (Robertsonet al, 1983; Thornburg et al., 1986). Occasionally however, stressful events such as whelping may precipitate an acute haemolytic episode similar to that occurring in sheep (Herrtage et al., 1987a).

Liver copper values can be as high as 10,000 ppm dry weight in Bedlington terriers (Twedt et al., 1979) whereas the highest copper value recorded in West Highland White terriers is 3,500 ppm (Thornburg et al., 1986). The later stages of the disease in Bedlington terriers, associated as they are with irreversible cirrhotic changes, nevertheless show an overall decline in liver copper (Twedt et al. 1979). Excess copper has also been demonstrated in the kidneys (Su et al., 1982b) and in the brains of some Bedlington terriers (Su et al., 1982b; Hardy et al., 1975) although other reports have denied this (Herrtage et al., 1987a).

Biochemical findings include increases in alanine aminotransferase (ALT) and serum alkaline phosphatase (SAP) (Twedt et al., 1979; Kelly et al., 1984; Herrtage et al., 1987a); conjugated hyperbilirubinaemia has been reported (Hardy et al., 1975), but is probably more common in advanced disease (Twedt et al., 1979). Urinary copper is also increased (Owen and Ludwig, 1982). Serum copper and blood copper have been found to be normal and ceruloplasmin levels have been reported to be normal or significantly elevated (Su et al., 1982b).

The macroscopic appearance varies from a pale discolouration of the liver with rounded edges and hyperplastic nodules (Robertson et al., 1983), to a firm uniformly shrunken liver with a finely nodular capsular surface and firm consistency (Kelly et al., 1984). Alternatively the presence of extensive yellow nodules typical of post-necrotic cirrhosis may be apparent

(Eriksson, 1983).

Microscopically, there is a spectrum of recognisable changes. In the least affected or subclinical cases there appears a concentration of refractile, light-brown granules in the parenchymal cells of the periacinar (centrilobular) zones, which stain histochemically positive for copper (Haywood, personal communication). This is accompanied by hepatocellular degeneration with associated inflammatory cell infiltration and early fibrosis. More characteristically there is a complete architectural disorganisation of the liver with variable sized nodules of parenchymal cells with focal degeneration and neutrophilic leukocyte accumulations, separated by bands of fibrous connective tissue with portal-central bridging (Eriksson, 1983; Kelly et al., 1984). There are also extensive accumulations of dark brown pigment in Kupffer cells and macrophages which are also positive for iron with Prussian blue stain (Robertson et al., 1983). Mallory bodies have not been identified (Ludwig et al., 1980).

Other observers have divided Bedlington terrier toxicosis into 4 grades with respect to histopathological changes; pigment granules without any further hepatic tissue changes, mild focal hepatitis, periportal hepatitis resembling chronic active hepatitis and finally cirrhosis (Twedt et al., 1979; Ludwig et al., 1980). Kidney changes have not been recorded in this condition.

Certain cholestatic disorders, such as chronic active hepatitis and Skye terrier hepatitis occur whereby copper

retention is secondary to the underlying disease as in Primary biliary cirrhosis in man (Haywood et al., 1988). Copper values are never so elevated as in the storage diseases.

d).-Experimental copper overload in the rat.

Rats fed high copper diets (1000-3000 ppm Cu SO_4) accumulated the metal in their livers to approximately 3,000 µg/g with increasing evidence of hepatocellular disturbance, culminating in extensive liver injury. Subsequently liver copper concentrations declined, regeneration and recovery of the liver occurred and the animals became tolerant. Similar changes have been observed in the kidneys although overall copper concentrations were lower (Haywood, 1980; 1985).

Tolerance persisted throughout the lifetime of the animals maintained on the high copper diets and they were resistant to challenge (Haywood and Loughran, 1985). The kidneys have been shown to excrete copper and it is thought that excess copper may be directed from the liver to the kidneys during the unloading process (Haywood, 1980; Haywood et al., 1985a).

The livers became paler than normal during copper-loading and occasionally exhibited areas of massive necrosis, in particular the right median lobe, but never became cirrhotic; the kidneys did not change in appearance (Haywood, 1980).

Liver copper displayed a non-random distribution with greater concentrations of the metal in the right median lobe and

right lobe possibly as a result of portal streaming. This uneven distribution was reflected by differences in the severity of lesions in different lobes (Haywood, 1981).

Clinical signs were subdued, a haemolytic crisis did not occur and organs other than livers and kidneys were not examined for copper (Haywood, 1980).

Copper toxicity in rats is associated with a rise in blood copper and ceruloplasmin concentrations (Wiederanders and Wasdahl, 1968; Haywood and Comerford, 1980) and the (ALT) activity rose gradually from the first week of copper-overload to reach a maximum at 9 weeks (Haywood and Comerford, 1980). The urinary copper also follows a similar pattern reflecting the kidney copper concentrations (Haywood and Comerford, 1980; Haywood et al., 1985a).

The sequence of events in the liver of the copper-loaded rat, constructed from the histological changes and the analysis of liver copper content, has been divided into three phases (Haywood, 1980). In the first phase, copper accumulated in the liver with little initial effect but, as the copper concentration rose, there were increased signs of cellular disruption, until the copper content reached a maximum and precipitated the second phase, or crisis, which was associated with a severe necrosis. This was followed by a decline in liver copper with evidence of hepatocellular regeneration and recovery.

The sequence of events in the kidney of the male rat follow a similar pattern to those which occur in the liver.

Initially, copper accumulated with little effect, except for eosinophilic granules and droplets which appeared in the cytoplasm of the proximal convoluted tubules. However, when copper concentrations reached their peak, there was severe desquamation and necrosis of the cells of the proximal convoluted tubules which was followed by a phase of tubular regeneration and recovery, and some decline in total kidney copper content (Haywood, 1980).

D.- Pathogenesis.

The mechanisms by which copper exerts its toxic effect on the cell are still unclear. There is considerable evidence to show that copper initially accumulates in lysosomes in the liver. Mc Nary (1963) has shown, using ultrastructural techniques, that the copper in hepatocytes from copper-loaded rats was located in membrane-bound bodies bearing a strong resemblance to lysosomes. Supporting evidence came from the concentration of copper in the heavy mitochondrial and lysosomal fraction (Gregoriadis and Sourkes, 1967) and Goldfischer and Sternlieb (1968), demonstrated that the pericanalicular particulate copper in copper-loaded rats was associated with high acid-phosphatase activity indicating a lysosomal localisation. Moreover, copper has been identified in hepatic lysosomes in Wilson's disease using x-ray microanalysis (Goldfischer and Moskal, 1966) and copper has been recovered from Bedlington terriers and sheep (Gooneratne et al., 1980) in the lysosomal fraction. This has lead to the hypothesis by Lindquist (1968) and supported by Gooneratne et al. (1980), that subsequent

rupture of copper-loaded lysosomes is responsible for the cytotoxic effect of copper. An alternative theory supposes that lysosomes are essentially protective (Sternlieb, 1980; Helman et al., 1985) and furthermore that cytotoxicity occurs as a consequence of nuclear disorganisation (Haywood et al., 1985a). Possible mechanisms of nuclear disruption by copper are the ability of the metal to destabilize DNA (Bryan and Frieden, 1967) and to inhibit RNA polymerase activity (Novello and Stirpe, 1969). Further support for the nuclear theory comes from work carried out by Hardy and Bryan (1975) who demonstrated the avidity of nuclear heterochromatin for copper in copper-laden mice both in vitro and in vivo. Panemangalore et al. (1983) have demonstrated metallothionein bound to copper in the hepatic nuclei of fetal and neonatal rats, and Clarkson et al., (1984) have demonstrated the presence of nuclear metallothionein in mature rats.

The cytotoxicity of copper may also be influenced by its chemical state. Within the liver copper occurs in complexed forms, of which the best characterised is metallothionein. Copper is tightly bound to the metallothionein molecule in the reduced form and the consequent unavailability of the metal suggest that copper in this form is innocuous.

Evaluation of immuno-reactive staining for MT with currently employed copper stains in Wilson's disease, PBC and ICC has shown a failure of correlation of these two methods of copper identification. This has led to suggestions that copper is present in another unreduced, toxic form (Elmes and Jasani, 1987).

The relationship between the chemical binding of copper and its organic complexes with its intracellular localisation awaits further clarification.

Copper tolerance in the rat may be associated with the redirection of copper from the liver to the kidney and its tubular excretion as a cuproprotein complex, probably metallothionein (Haywood et al., 1985b).

F.-Diagnosis.

The investigation of copper toxicosis utilizes both histochemical methods and atomic absorption spectrophotometry. Histochemical methods include rhodanine (Lindquist, 1969; Irons et al., 1977), rubeanic acid and orcein staining (Uzman 1956; Goldfischer and Sternlieb, 1968).

Rubeanic acid and 5-p-dimethylbenzidine rhodanine both form coloured complexes with copper (Pearse, 1972). Rhodanine reacts specifically with cupric copper by yielding a red precipitate in fixed, paraffin-embedded liver, but does not react with zinc or iron. It also reacts with silver, mercury, gold, platinum and palladium but these metals rarely are found in the mammalian liver (Irons et al., 1977; Lindquist 1969). Orcein stains a "copper-associated protein" rich in sulphydryl groups probably metallothionein (Salaspuro and Sipponen, 1976).

1985) and antibody to metallothionein (Vander Mallie and Garvey, 1979) in patients with Wilson's disease, Indian childhood cirrhosis and Primary biliary cirrhosis (Elmes and Jasani, 1987).

There are several microanalytic techniques for quantitative measurement of copper, including spectrophotometric methods (Committee on Medical and Biologic Effects of Environmental Pollutants, 1977), atomic absorption (Evenson and Anderson, 1975), and neutron activation analysis via ^{64}Cu or ^{66}Cu (Battistone et al., 1970; Behne et al., 1977). Adequacy of the sample size is particularly important because of the non-homogeneous distribution of copper in the liver (Sternlieb, 1980). Contamination by traces of copper from instruments and glassware should be avoided (Versieck et al., 1973).

Atomic absorption spectrophotometry which utilizes the specific absorptive properties of particular metals towards monochromatic light of characteristic wavelength for the metal with subsequent emission of radiation in spectral lines, is a very sensitive method (Luckey and Venugopal, 1977).

G.- Treatment: Chelating agents.

A key factor in the treatment of copper toxicosis is the induction of negative copper balance in the body by promoting removal of excess copper from the tissues especially from the liver (Gooneratne, 1986). To achieve this, a variety of copper chelating agents have been used.

Penicillamine is known to complex copper with subsequent excretion of a biologically inactive form (Freyer and Walshe, 1963; Chromy and Heyrovsky, 1966). D-penicillamine binds to copper and promotes urinary excretion (Twedt et al., 1987) and has been effective in the treatment of Wilson's disease in the early stages (Epstein, 1983b). In primary biliary cirrhosis the drug has been shown to cause biochemical improvement and improved survival (Jain et al., 1977; Epstein et al., 1979; Epstein et al., 1981b). In addition to its copper chelating effect, D-penicillamine has a potent immunological action, and it is likely that in auto-immune cholestatic liver diseases, like Primary biliary cirrhosis, improvement can be ascribed to its immunological action, rather than metal chelation (Fleming et al., 1978; Epstein et al., 1981b). Penicillamine has also been effective in the treatment of some cases of Bedlington terrier toxicosis (Twedt et al., 1979). The disadvantages of penicillamine are its slow mobilization of hepatic copper and the side effects including renal damage, skin rashes, blood dyscrasias, nausea and vomiting (May et al., 1982).

EDTA (ethylenediamine tetraacetate) has the property of forming stable complexes with many metals and has been successfully used in the treatment of lead and vanadium poisoning (Sidbury et al., 1953; Bessman et al., 1954). Rana and Kumar (1983b) reported a protective effect of EDTA against copper poisoning.

Tetramine compounds (2,2,2-tetramine and 2,3,2-tetramine) induce prolonged cupruresis (Borthwick et al., 1980) and have

proved to be effective copper chelators in patients with Wilson's disease (Harden et al., 1977) and in rats (Borthwick et al., 1980) and dogs (Allen et al., 1987; Twedt et al., 1988).

Gooneratne et al., (1986) were the first to report that tetrathiomolybdate (TTM) when injected intravenously lowered liver copper levels and prevented chronic copper poisoning in sheep. These authors demonstrated that TTM increased the excretion of copper via bile, urine and faeces. However, investigations of the mechanism of action of TTM given to copper-loaded sheep, has shown that this compound facilitates the excretion of copper by the biliary route alone (Youquiang, 1987) in contrast to the aforementioned chelators. This obviously would be contraindicated in cholestatic disorders but might be the vehicle of choice if renal disease was a complicating factor.

An alternative method to the use of chelating agents to mobilise copper has been to use its natural antagonist zinc. Dick (1954) first reported the use of zinc supplementation to decrease copper absorption in sheep. Since then, high doses of zinc have been used successfully in the treatment of Wilson's disease (Hoogenraad and Van den Hamer, 1983; 1984). It is postulated that zinc induces the synthesis of intestinal metallothionein, which in turn sequesters copper so that it is unavailable for transfer into the portal circulation and is sloughed with intestinal cells into the lumen. Copper absorption may also be blocked competitively by the elevated zinc levels (Lipsky and Gollan, 1987). These combined actions may account for the elevated faecal copper levels observed

in zinc-treated patients (Brewer et al., 1983).

Medication may initially be necessary to reduce copper excess; therapeutic intervention must ultimately seek to promote natural methods of detoxification whenever possible.

AIMS AND OBJECTIVES .

The aim of the present study is to clarify the pathogenesis of copper-induced damage in the liver and kidneys of copper-loaded rats and to increase understanding of the subsequent adaptation to copper that takes place in this species; with particular emphasis on the role of the kidney and the mode of renal tubular excretion of copper.

The specific objectives are:

1. To study the morphological changes in the liver and kidneys of copper-loaded rats at the cellular and subcellular level.

2. The intracellular localization of Copper and its complexes in these organs using histochemical and microanalytical methods.

CHAPTER I.

CELLULAR MECHANISMS OF TOXICITY AND TOLERANCE IN THE COPPER-LOADED RAT. I. EFFECT OF HIGH DIETARY COPPER ON GROWTH RATE, LIVER AND KIDNEY COPPER ACCUMULATION AND ASSOCIATED PATHOLOGICAL CHANGES.

1.- INTRODUCTION.

The most widely used criteria of the toxicity of a substance in animals are the reduction in body weight gain, the detection of gross and histopathological changes in tissues and an increase in mortality (Barnes and Dentz, 1954).

High levels of copper have been shown to inhibit growth in rats (Boyden et al., 1938; Wolff, 1960; Rana and Kumar, 1983a). A depressed growth rate has also been observed in copper-loaded rats, not apparently associated with a lowered food intake (Haywood, 1979).

The rat is less susceptible to cumulative copper poisoning than the sheep and the pig (Erikkson,1983). Although extremely high dietary concentrations of copper can lead to irreversible clinical deterioration and death (Haywood, 1985), rats usually adapt to tissue-damaging doses and animals fed high copper diets initially accumulate the metal in the liver and kidneys in toxic concentrations, but subsequently unload and become tolerant to continued dosing (Haywood, 1985; Haywood and Loughran, 1985; Haywood et al., 1985 a,b).

Copper accumulates more readily in the liver of the male Wistar rat and they are also less resistant than the females to the toxic effects of the metal (Haywood, 1979). Similar sex differences have been reported in three different strains of rats

(Nederbragt, 1985). Strain-related differences in the pattern of biliary excretion and hepatic distribution have also been recognised in rats, the strain WAG/Cpb being the most susceptible (Nederbragt and Lagerwerf, 1986).

The sequence of events in the liver and kidneys during continuous copper loading of the rat has been divided into three phases:cumulative, crisis and recovery (Haywood, 1980). The first phase is associated with the gradual accumulation of copper in the liver and kidneys and some signs of cellular disturbance; it culminates in a crisis characterised by maximal liver and kidney copper concentrations and severe cellular disruption. The final phase is one of regeneration and healing in which liver copper concentrations decline and the animal demonstrates its tolerance to the metal.

The right compartment of the rat liver accumulates more copper than the left (Haywood, 1981), probably because the greater proportion of copper absorption occurs from the intestine with maximal absorption taking place from the upper part of the duodenum (Owen, 1964). This is in contrast to liver copper in neonatal children in which more accumulates in the left lobe (Faa et al., 1987).

Differences in the intralobular deposition of copper in the liver and its pathogenetic significance have not been sufficiently appreciated. Primary biliary cirrhosis, a cholestatic disease in man, has a periportal deposition of copper whereas in Wilson's disease it is more diffuse and without a particular

lobular pattern (Goldfischer et al., 1980). By way of contrast, in copper storage diseases in the Bedlington and West Highland White terriers copper has been identified almost exclusively in the centrilobular zones, (Twedt et al., 1979; Erikkson, 1983; Thornburg et al., 1986). A similar distribution of copper has been reported in copper toxicosis in sheep (Ishmael et al., 1971; Gooneratne et al., 1980; Kumaratilake, 1984).

In the copper-loaded rat kidney, the metal accumulates in the proximal convoluted tubules (Haywood, 1979; Haywood et al., 1985b), a similar distribution has been reported in Wilson's disease (Wolff, 1964) and in chronic copper poisoning in sheep (Gopinath et al., 1974).

Elevated hepatic copper can be identified for diagnostic purposes by either histochemical means or atomic absorption spectrophotometry. Histochemical methods, which include rubeanic acid, rhodanine and orcein for copper-associated protein, are faster and less costly (Goldfischer et al., 1980), and rhodanine has been shown to provide a semiquantitative analysis when compared with atomic absorption values in Bedlington Terriers (Hardy et al., 1975; Twedt et al., 1979; Ludwig et al., 1980).

However, in Wilson's disease in young patients with very high liver copper concentrations copper staining is unreliable (Goldfischer and Sternlieb, 1968; Goldfischer et al., 1980). Also in the copper-loaded rat there is evidence that stainable copper does not always compare with total copper concentration measured by spectrophotometric analysis (Haywood and Loughran, 1985;

Haywood et al., 1985b). Immunocytochemical methods for the detection of metallothionein have likewise shown a lack of correlation with histochemical staining (Elmes and Jasani, 1987).

Several chemically similar elements are known to interacts with copper, resulting in competition for various binding sites within specific metabolic systems (Evans, 1973). Van Campen and Scaife (1967) demonstrated that zinc interact with copper absorption either in or on the intestinal mucosa. Evans et al. (1970) isolated and purified metallothionein from bovine duodenum and demonstrated that cadmium and zinc displace copper from sulfhydryl binding sites on the protein. It may be that an overwhelming influx of copper creates an artificially induced zinc deficiency which may be responsible at least in part for the clinical signs.

The aims of this study were:

a) to measure the effect of copper-overload on the growth rate and food intake of rats,

b) to study the pattern of copper deposition and associated changes within the livers and kidneys of copper-loaded rats

c) to quantify stainable copper in these organs and relate this to their copper concentration,

d) to monitor zinc concentration and attempt to correlate any deviations with copper content.

2.- MATERIALS AND METHODS.

a.- Animals:

Male, 6 weeks-old, Wistar rats of uniform weight were randomly allocated to groups of four, caged and fed a pelleted diet (Labsur Animal Diet, Agriculture South Ltd) with a copper content of 1,500 mg/kg (range 1,300-1,800), in the form of $CuSO_4$ (Appendix 1). Tap water and food were always available. Control animals, similarly caged were fed the unsupplemented diet with a copper content of 10 mg/kg. The animals were regularly observed and weighed at weekly intervals; the food was weighed and consumption per group recorded weekly. One group of rats on the supplemented diet were killed at each interval of 1,2,3,4,5,6,8,12 and 16 weeks. Five groups of 4 control rats were included at 1,4,8,12 and 16 weeks. Prior to killing, the rats were placed in a polythene chamber in which the air was gradually replaced by CO_2. After 20-30 seconds respiration was depressed or had ceased altogether, the animals were unconscious. The rats were removed from the chamber and killed by cervical dislocation. The livers and kidney were removed immediately for histology, copper and zinc analysis, the liver being sampled from the right median lobe for these purposes (Haywood, 1981).

b.-Copper and Zinc analysis.

All glassware was soaked before use for 48 hours in 0.1M

nitric acid followed by three 24 hours washes each in deionized water, rinsed, drained and dried in a hot air oven at 70 °C.

Triplicate samples of approximately 0.3 g. of liver and kidney cortex were removed with plastic knives, oven dried at 70°C for 3 days in plastic containers, weighed and digested in Aristar grade 70% nitric and 70% perchloric acids (2:1) in Pyrex glass test tubes using the following procedure: 70°C for 30 min., 150°C for 30 min., 250°C for 1 hour and 300°C for 1 hour in a heating block (Tecam, Dri-Block DB-4); each batch of 24 included three recoveries. It was found advisable to cover the boiling tubes with glass marbles to minimise loss. The digest was made up to 10 ml with deionized water and diluted further if required; the copper and zinc content was recorded in an Atomic Absorption Spectrophotometer (Instrumentation Laboratory Inc. U.S.A.).

Precinorm "U" Q.C. serum (BCL) was used to monitor performance of the spectrophotometer. Standardisation for copper was with solutions containing 2 and 4 ppm copper in 0.1 M HCl prepared from 15.7 mmols/l cupric nitrate stock solution (BDH). Standardisation for zinc was with solutions containing 0.5 and 1 ppm zinc in 0.1 M HCl prepared from 15.3 mmol/l zinc nitrate stock solution (BDH).

Liver and kidney metal concentrations are expressed as the mean and the standard error of the mean, in µg/g of tissue on a dry weight basis.

Statistical analyses employed Student's t-test and the Pearson-Moment coefficient correlation.

c.- Histology.

Preparation of tissues:

Transverse blocks (2-3mm) of liver were taken at approximately 0.5 mm from the ventral border of the right median lobe fixed in 10% freshly prepared formalin. After fixation, tissues were dehydrated in alcohol, cleared with xylene and embedded in paraffin wax using an automatic tissue processor (Elliot, Liverpool, UK). Five um sections were cut on a Wetzlar microtome (Leitz, W. Germany).

Blocks of kidney cortex were taken for histology and were processed as described for the liver.

Staining methods:

Haematoxylin Eosin: All sections were routinely stained with haematoxylin and eosin (H and E) (Pearse, 1972). Details of the staining procedure are given in Appendix 2.

From all the H and E liver sections the total area of the tissue was measured using a digitased tablet linked to a VIDS III image analysis programe running on a IBM compatible computer (COMCEN). The sections were examined using a 40x objective and the cellular changes recorded in number of hyperchromatic hypertrophic parenchymal cells per high power field and number of inflammatory foci per mean total area measured previously (69.6±12 mm^2.

Necrosis was identified in single cells and focal aggregates and the severity graded on the following scale:

mild = +

moderate = ++

severe = +++

d. -Histochemistry.

Sections of liver and kidney prepared as described before, were stained for copper with Rubeanic acid and Rhodanine; and with Orcein to demonstrate the presence of copper-associated protein.

Rubeanic acid: A modified method of Uzman (1956) was used, paraffin sections were deparaffinized and hydrated in distilled water and then placed in a solution of 0.1 g. rubeanic acid (dithioxamide) in 100 ml. of 70 ethyl alcohol. After 20 minutes, 0.2 g. of sodium acetate per 100 ml of solution was added and allowed to settle to the bottom. Tissue sections were incubated in the mixture at room temperature for 72 hours. Following incubation, sections were rinsed in two changes of 70% ethyl alcohol for a total of three hours. This was followed with a final rinse of 100% ethyl alcohol for 24 hours. Slides were counterstained lightly (30 seconds) in alternatively neutral red or methanil yellow, dehydrated, cleared and permanently mounted (Thornburg et al., 1985).

Rhodanine: The modified method of Okamoto and Utamara was used (Lindquist, 1969), paraffin sections were deparaffinized and

hydrated in distilled water then incubated in Rhodanine working solution at 37°C for 18 hours, rinsed in several changes of distilled water and stained with diluted haematoxylin for 10 minutes, rinsed in distilled water and then quickly rinsed with Borax solution, rinsed well in distilled water, dehydrated, cleared and mounted.

Orcein: this method was used to demonstrate copper-associated protein (Shikata et al., 1974). Sections were dewaxed, hydrated and treated with acid permanganate for 10 minutes, rinsed in water and decolorised in 2% oxalic acid, rinsed in distilled water, stained in orcein for 4 hours at room temperature, rinsed in water, differentiated in 1% HCl in 70% alcohol, dehydrated through alcohols, cleared in xylene and mounted.

Grading system:

In the liver copper stained slides were graded using a 1 to 4 point system based on the number and size of copper-containing granules within the cytoplasm of the hepatocytes, according to their zonal distribution,

0 = absence of granules

\+ = few very small granules

++ = many small granules

+++ = numerous small to medium size granules

++++ = cells saturated with small to medium size granules.

+++/0 = considerable individual variation, many cells being negative for copper.

e.-Photography.

Sections were photographed on an Ultraphot II photomicroscope (Carl Zeiss, Oberkochen/Wuertt, W. Germany).

3.- RESULTS.

A.-Growth rate and clinical observations.

All animals on the copper-supplemented diet showed a marked reduction in growth rate compared with controls (Fig.1.1, Appendix 3). Mean body weight rose from 173.2±0.4 g. and 216 ± 0.79 in the copper supplemented and unsupplemented groups respectively ($p > 0.05$) at the beginning of the trial to 377±14.4 g. and 555.5 ± 8.09 at 16 weeks by which time the weight of the copper-loaded group approximated only 70% of these of the control group. For the first few weeks the growth rate of the copper supplemented group was markedly interrupted and body weights fluctuated considerably. By 5 weeks, when higher copper concentrations were attained, body weights of the copper-loaded animals (279.6±1.5 g.) were significantly higher ($p<0.05$) than at the start and thereafter maintained a steady gain. Control animals maintained an uninterrupted growth curve throughout the trail period compared with the copper overloaded group.

The animals were at all times active and sleek, no deaths occurred in either the supplemented or unsupplemented groups.

B.-Food consumption

Groups of animals on both copper supplemented and unsupplemented diets showed considerable variations in food consumption throughout the trial period; however the copper fed animals showed overall a higher average food intake per week than

the controls (Appendix, 4). Food consumption of the copper supplemented group and non-supplemented groups were similar ($p > 0.05$) at week 1, after which intake increased markedly during the first 3 weeks in the copper-supplemented group (Fig.1.2).

C.-Liver copper concentration. (Fig. 1.3) (Appendix 5).

The liver copper concentrations rose to 2,900±60 µg/g at 5 weeks and remained high until week 8 (2,958±134 µg/g) after which it began to fall and at week 16 (1,930 ± 91 µg/g) the liver copper concentration was significantly less than at week 8 ($p < 0.05$).

The liver copper content in control rats varied from 19±0.4 to 29±2 µg/g.

D.-Kidney copper concentration.

Renal copper concentration initially rose more slowly (Fig.1.4, Appendix 6), being 90±7 µg/g dry weight tissue at week 1, reaching a maximum of 1,003±117 µg/g dry weight tissue at 6 weeks, falling in the succeeding weeks to 830±66 at 16 weeks.

Control values varied from 22±1 µg/g at the first week to 47±1 µg/g dry weight tissue at 16 weeks.

E.-Liver and kidney zinc concentration.

Liver zinc concentrations in copper supplemented groups ramained unchanged (128±29 µg/g) and did not differ significantly ($p > 0.05$) from liver zinc concentrations in the control groups (127±12 µg/g). (Fig.1.5; Appendix 7).

Kidney zinc concentrations in copper-supplemented groups likewise remained unchanged (111±20 µg/g) and did not differ significantly ($p > 0.05$) from kidney zinc concentrations in the control groups (111±14 µg/g). (Fig.1.6; Appendix 8).

Kidney zinc concentration in the copper-loaded group supplemented groups did not vary from the control values (Figs.1.7, 1.8).

F.- Gross changes in the liver and kidney.

At week 5 the livers were paler and larger , otherwise no remarkable changes were seen in the livers and kidneys at any sampling point.

G.-Histological Changes in the liver.

The following changes are recorded in Table 1.

Week 1: Minimal changes were observed in the livers of the copper-supplemented groups and consisted of a few individual necrotic cells and mononuclear cells with no particular zonal distribution.

Week 2: Occasional foci of hypertrophied parenchymal cells with enlarged nuclei and homogeneous intensely stained cytoplasm

appeared in the periportal zones, associated with minimal mononuclear cell response.

Week 3: Inflammatory foci were clearly established (Fig.1.9) although limited mainly to the periportal zones. These lesions consisted of hypertrophied hyperchromatic parenchymal cells undergoing necrosis with an associated inflammatory response of polymorphonuclear neutrophilic leukocytes and mononuclear cells.

Week 4: Abundant hypertrophic hyperchromatic parenchymal cells occupied the outer (periportal and midzones) (Fig.1.10). Multiple foci of inflammatory cells were present.

Weeks 5 to 6: Hyperchromatic, hypertrophic cells now occupied the outer zones; their nuclei were often darkly stained and appeared degenerate. Inflammatory foci were numerous and tended to coalesce.

In two individuals there was more extensive necrosis and a correspondingly marked cellular inflammatory reaction mainly in the outer zones (Fig 1.11). Bile duct hyperplasia and mitotic figures in the hepatic parenchyma were noticeable.

Weeks 8 to 12: The hyperchromatic hypertrophic cells were still numerous although degenerative and inflammatory changes had lessened.

Week 16: The inflammatory changes had regressed to control levels although the livers of copper-supplemented rats were distinguished by the persistence of enlarged deeply staining hepatocytes, the presence of hyaline remnants in the periportal areas (Fig.1.12) and bile duct hyperplasia.

Control livers contained small areas of non specific periportal inflammation.

H.- Histochemistry of the liver.

As seen in the Table 2 copper could be demonstrated initially after 2 weeks of copper-loading in the hepatocyte of all zones of the hepatic lobules. This sparse distribution of copper remained constant within the central zone throughout the trial period. Meanwhile copper staining increased in intensity in the two outer zones achieving maximun intensity at 5 weeks (Fig.1.13). At this time, the outer zone, and in particular the midzone, appeared saturated with small to medium size granules of copper, whereas the central zone still contained very little copper (Figs. 1.14). The metal was identified only in copper containing granules within the cytoplasm of the hepatocytes. Up until 5 weeks there was a remarkable uniformity of staining between individual livers at any sampling point, whereas at this time (5 to 6 weeks) differences appeared in the response; although 6 livers demonstrated maximum staining intensity the other two displayed diminished copper-staining in the outer zones (Fig.1.15). This loss of staining

intensity was very variable between individual cells, some of which apparently contained no copper whereas others remained saturated (Fig.1.16). Subsequently the livers from all animals killed displayed a similar progressively diminishing intensity of copper-staining.

There were no differences between the sensitivity of rubeanic acid with the different counterstains and rhodanine, although orcein appeared of little use in this trial.

Up to 5-6 weeks there was a good correlation (r=0.78) between quantitative copper determination and the light microscopic grading of copper-stained sections of liver tissue. However, from 8 to 12 weeks of copper-overload, despite the very marked loss of overall staining intensity and persistently high copper concentrations, no comparison could be made since the grading system no longer strictly applied in a quantitative fashion.

No copper was detected in the livers of control animals.

I. Histological and histochemical changes in the kidney.

Week 1 to 2: All the kidneys appeared normal. Copper could not be demonstrated in sections stained with either of the copper stains.

Week 3 to 4: There was some variation in cytoplasmic and nuclear size in the epithelial cells of the proximal convoluted tubules (Fig. 1.17). Small eosinophilic granules and droplets were present

in the cytoplasm of the proximal tubules; these stained positive for copper. By week 4 the small copper positive granules were more abundant (Fig.1.18).

Week 5 to 6: The cytoplasmic granules were variably sized and numerous, and the presence of eosinophilic droplets was also more marked in the lumen of the proximal convoluted tubules (Fig.1.19). Desquamation of the epithelial cells of the proximal convoluted tubules, individual necrosis and regenerative activity characterised by mitotic figures and considerable variation in cytoplasmic and nuclear size, were prominent features during this time (Fig. 1.20). Rubeanic acid and rhodanine demonstrated the presence of copper in particulate and droplet form. However, there was variation in the intensity of the staining of the droplets (Fig. 1.21).

Weeks 8 to 12: There was still variation in cytoplasmic and nuclear size and presence of a few mitotic figures. The eosinophilic droplets were smaller.

Week 16: Reconstruction of the proximal convoluted tubules was apparently complete. Small eosinophilic droplets were still numerous within the cytoplasm of the proximal tubule cells and extrusion and detachment of droplet-filled copper-positive cells was a prominent feature (Fig.1.22). Little particulate copper could be demonstrated with rubeanic acid and rhodanine stains.

The histological appearance of the control kidneys was unremarkable and a few eosinophilic droplets were seen in the proximal epithelial cells, which did not stain for copper (Fig.1. 23).

Table 1. Pathological changes in copper-loaded and control rats.

Time (weeks)	No. hypertrophic hyperchromatic cells per high power field	Necrosis	No. Inflammatory foci per slide *
1	1	+	10
2	1-2	+	10-20
3	1-3	++	20-30
4	3-6	+++	30-40
5-6	6	+++	Diffuse
8	6	++	Diffuse
12	6	++	10-20
16	6	-	10
Control**	1	-	10

* mean area= 69.56 ± 12 mm²

** sum of 5 groups of 4 rats each.

Evaluation is based on examination of livers from 4 treated rats at each time interval.

Table 2. Hepatic copper concentration and histochemical grading with rubeanic acid and rhodanine staining in copper-loaded rats.

Time (weeks)	Copper concentration (μg/g) *	Histochemical grade		
		Central zone	Midzone	Periportal zone
1	411 ± 40	0	0	0
2	943 ± 35	+	+	+
3	1,478 ± 60	+	++	++
4	1,968 ± 64	+	+++	+++
5**	2,900 ± 60	+	++++	+++
6**	2,888 ± 57	+	++++	+++
8	2,958 ±134	+	+++/0	+++/0
12	2,646 ±108	+	+++/0	+++/0
16	1,930 ± 91	+	++/0	++/0

* mean ±SE of mean (n=4)

** one rat had copper granules varying from +++/0 in the outer zones.

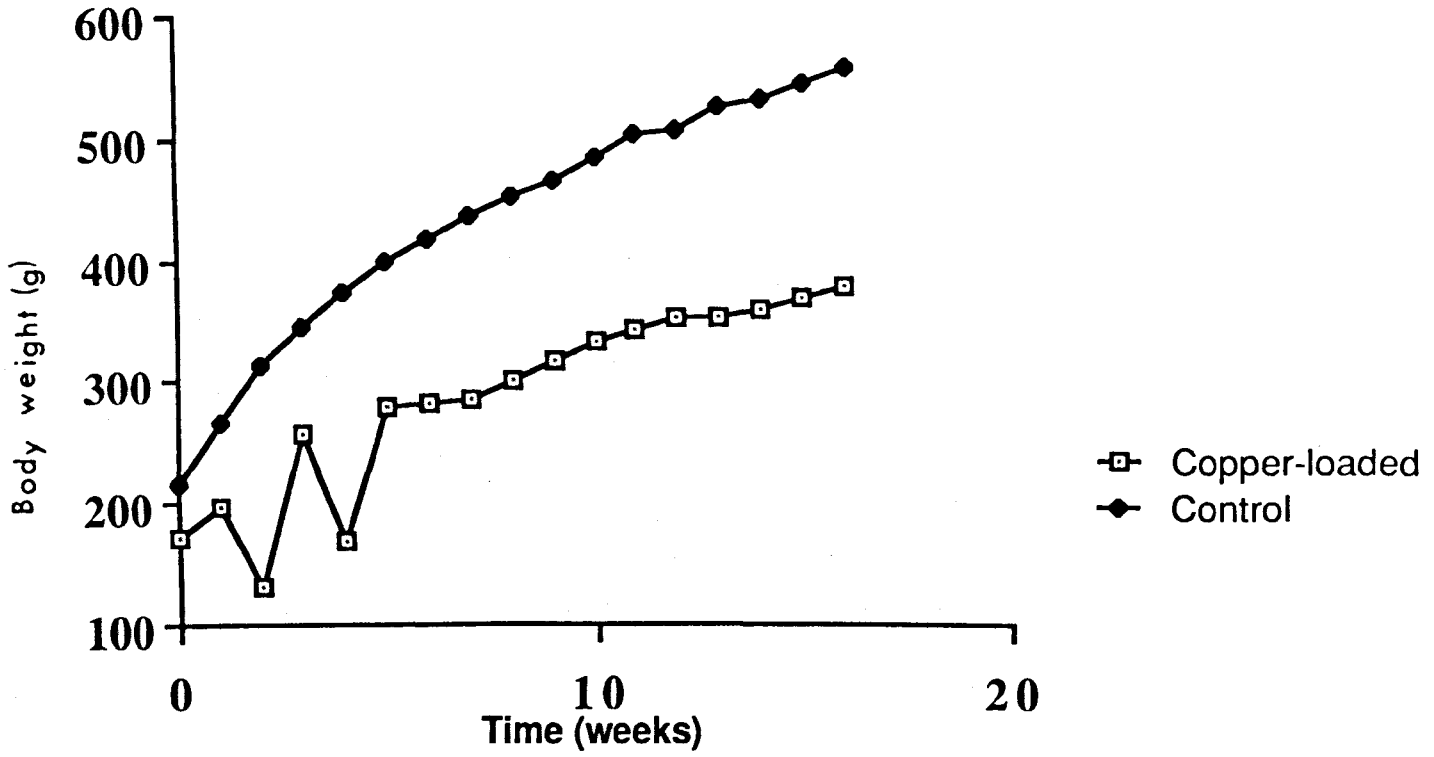


Figure 1.1. Growth rates of young male rats on copper-supplemented compared with control diets for 16 weeks.

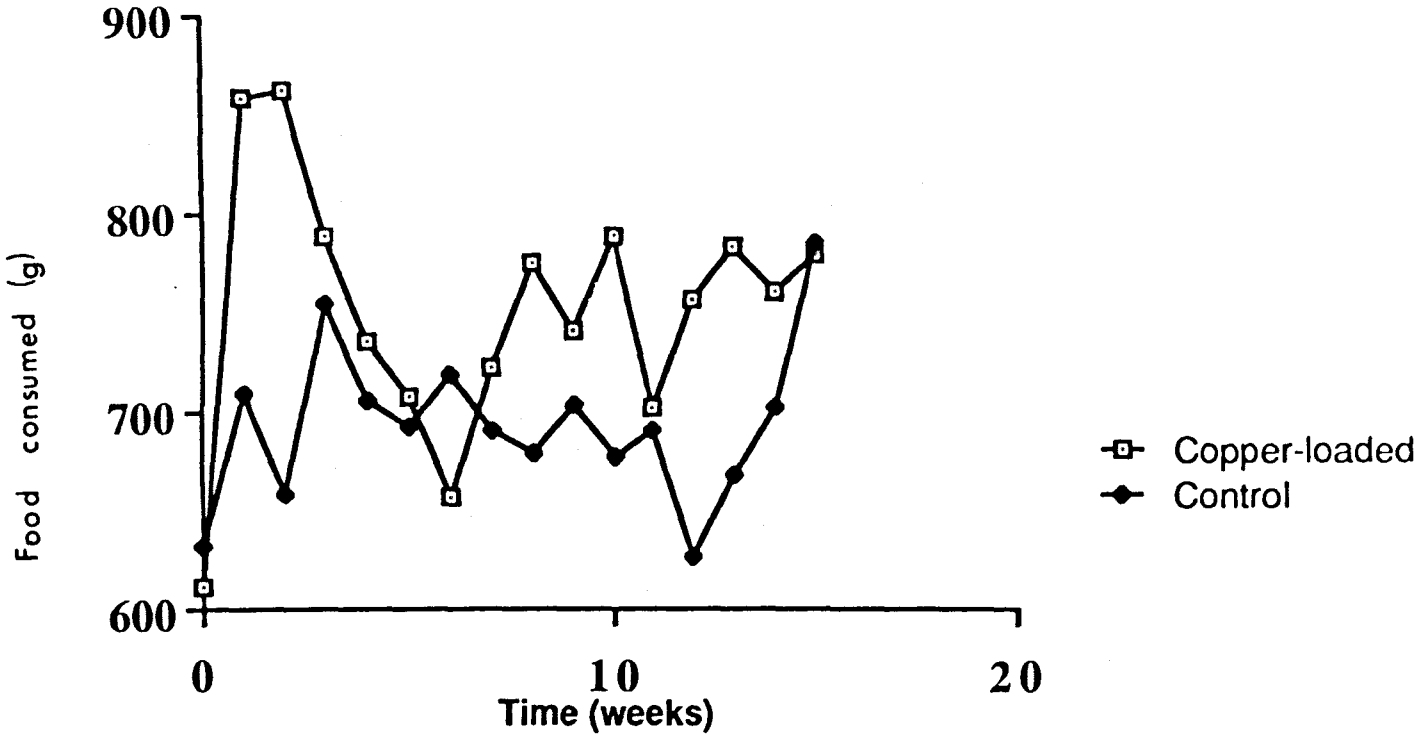


Figure 1.2. Food consumption per group of copper-supplemented and control weanling rats during a period of 16 weeks.

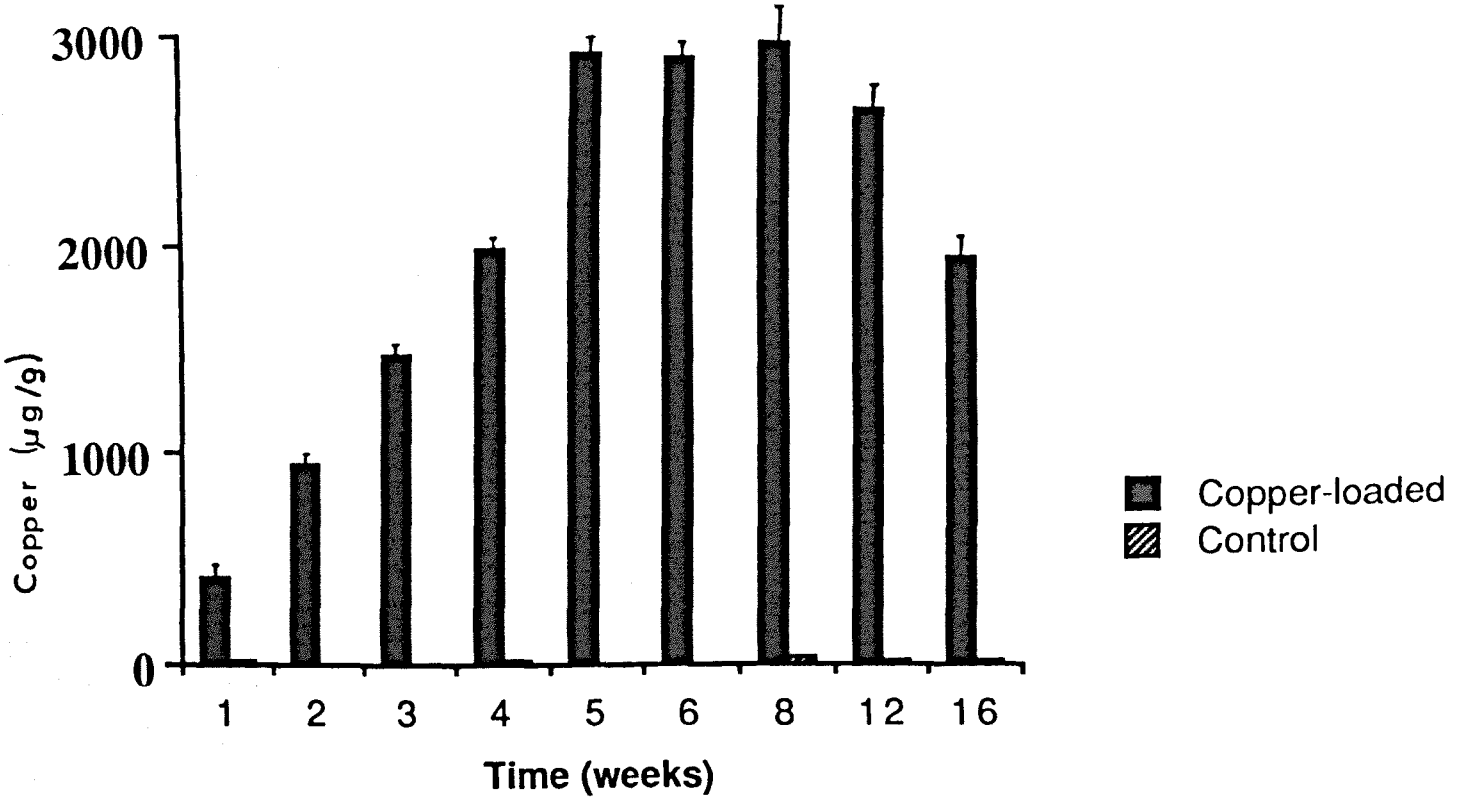


Figure 1.3. Copper concentrations in livers of rats on 1,500 mg/kg copper for 16 weeks. Mean and standard error of the mean. N=4.

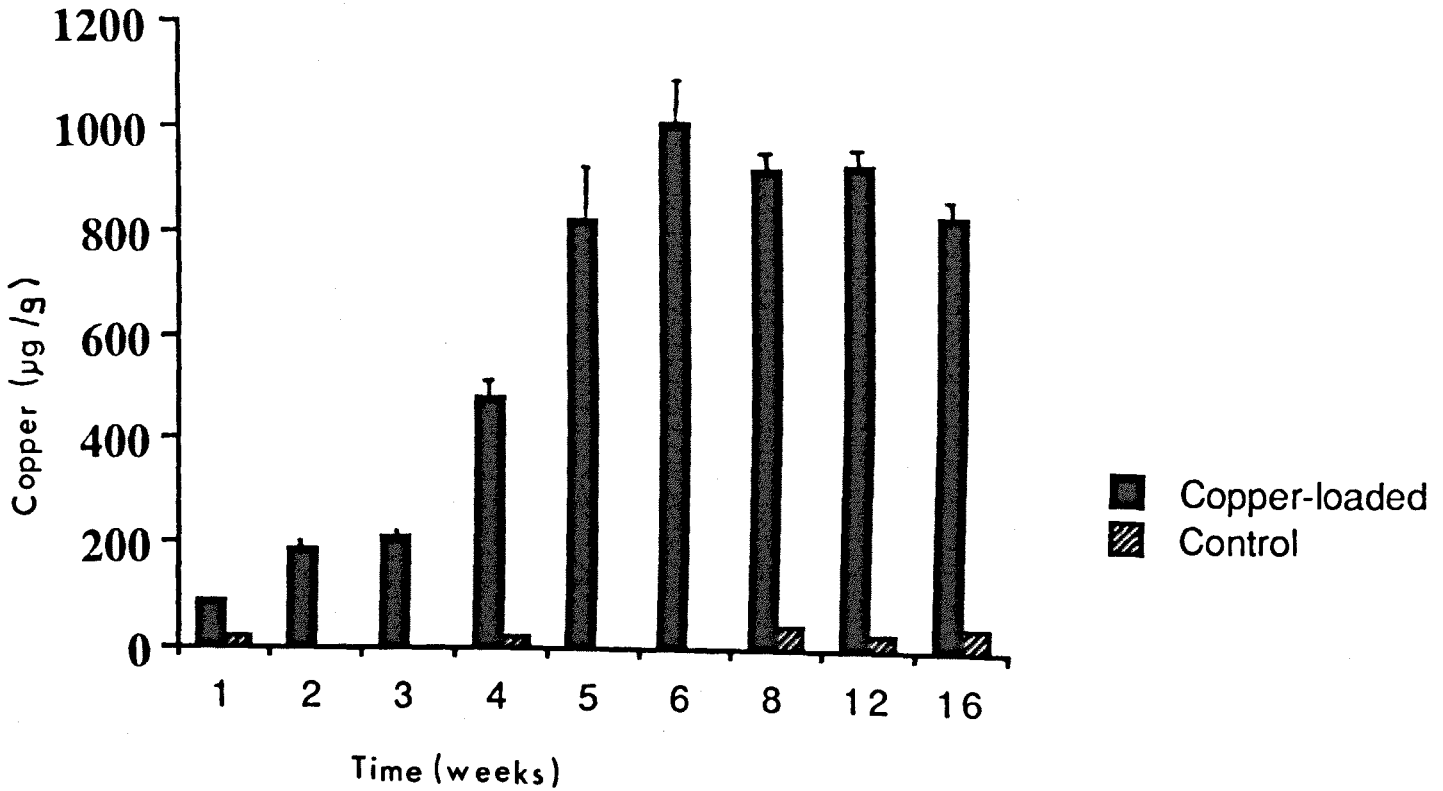


Figure 1.4. Copper concentrations in kidneys of rats on 1,500 mg/kg copper for 16 weeks. Mean and standard error of the mean. N=4.

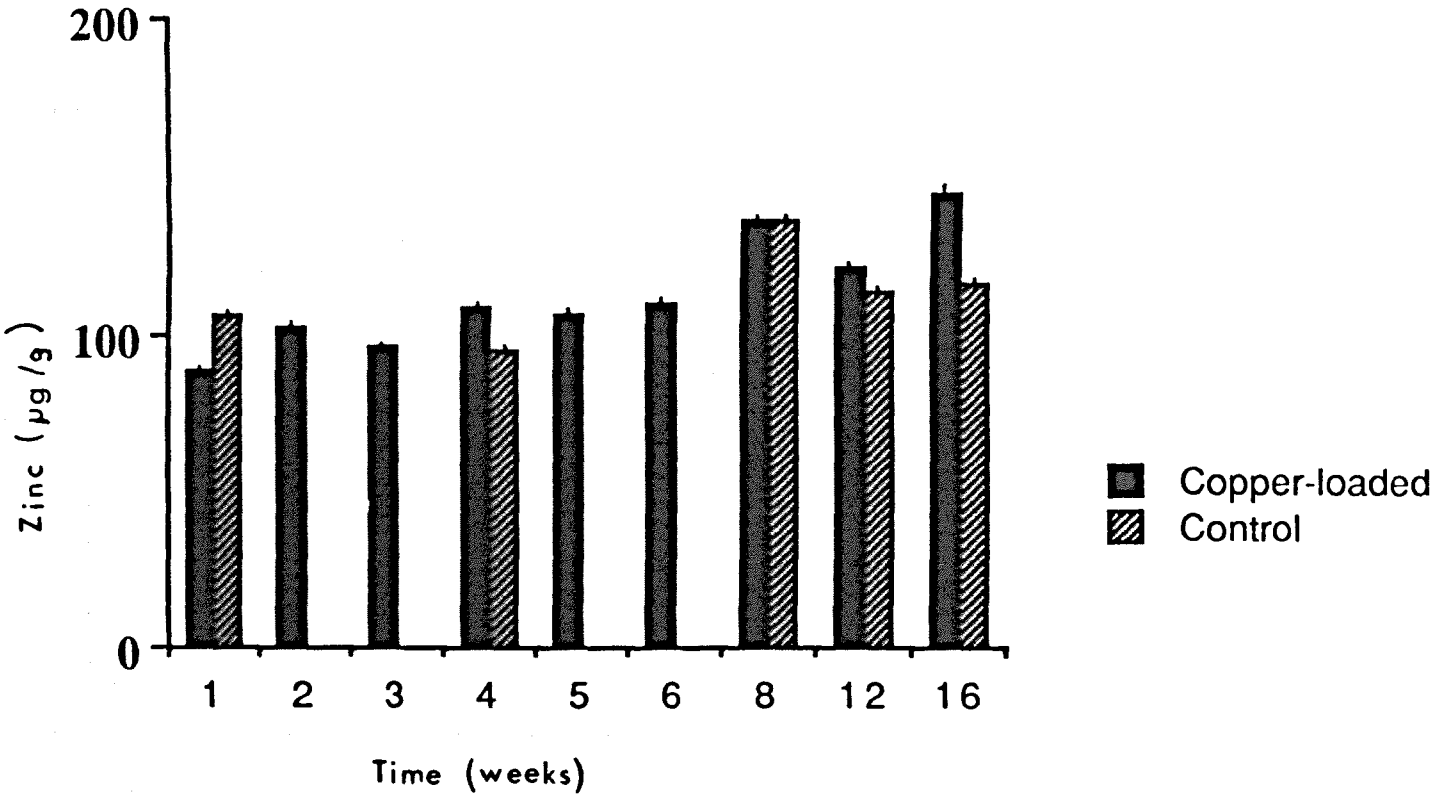


Figure 1.5. Zinc concentrations in livers of rats on 1,500 mg/kg copper for 16 weeks. Mean and standard error of the mean. N=4.

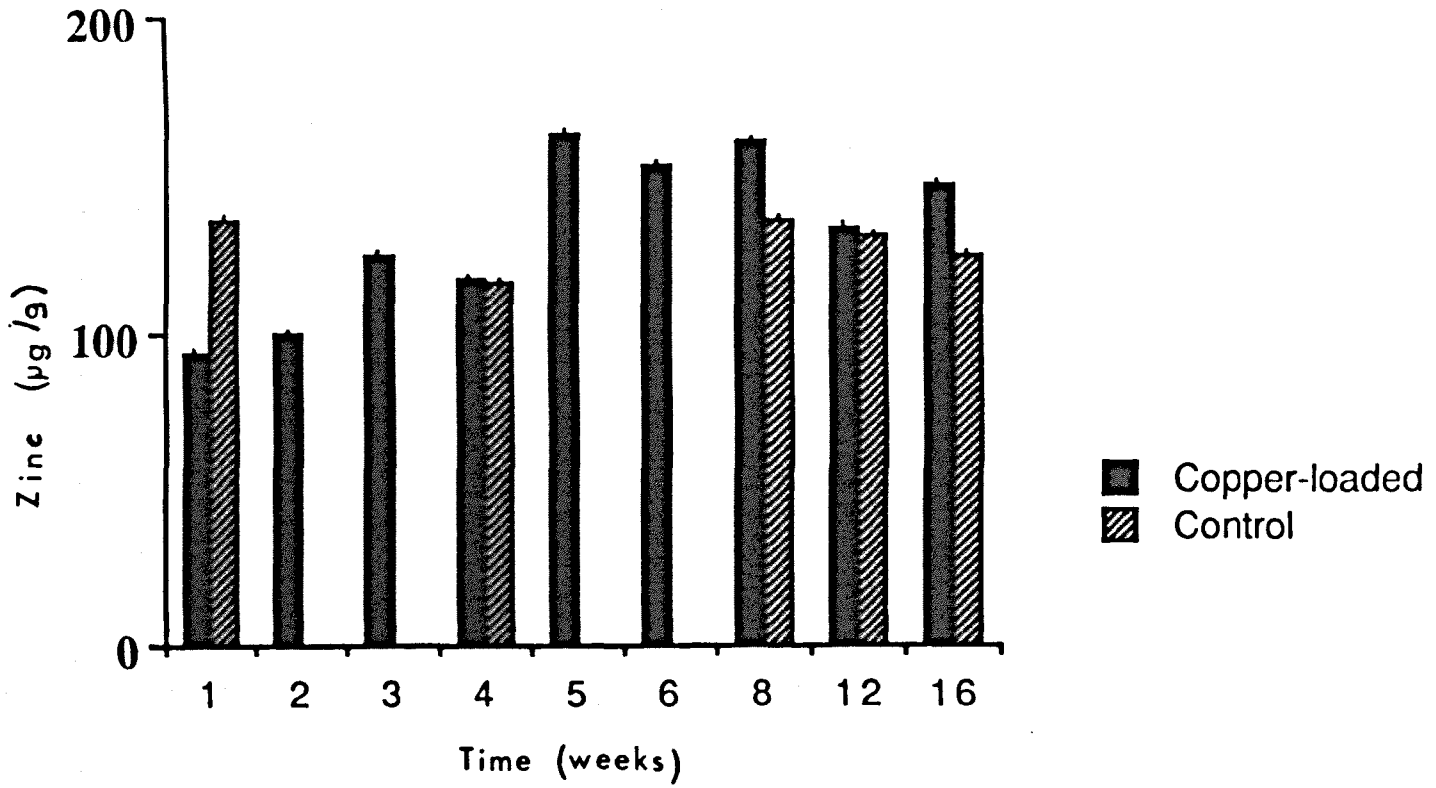


Figure 1.6. Zinc concentrations in kidneys of rats on 1,500 mg/kg copper for 16 weeks. Mean and standard error of the mean. N=4.

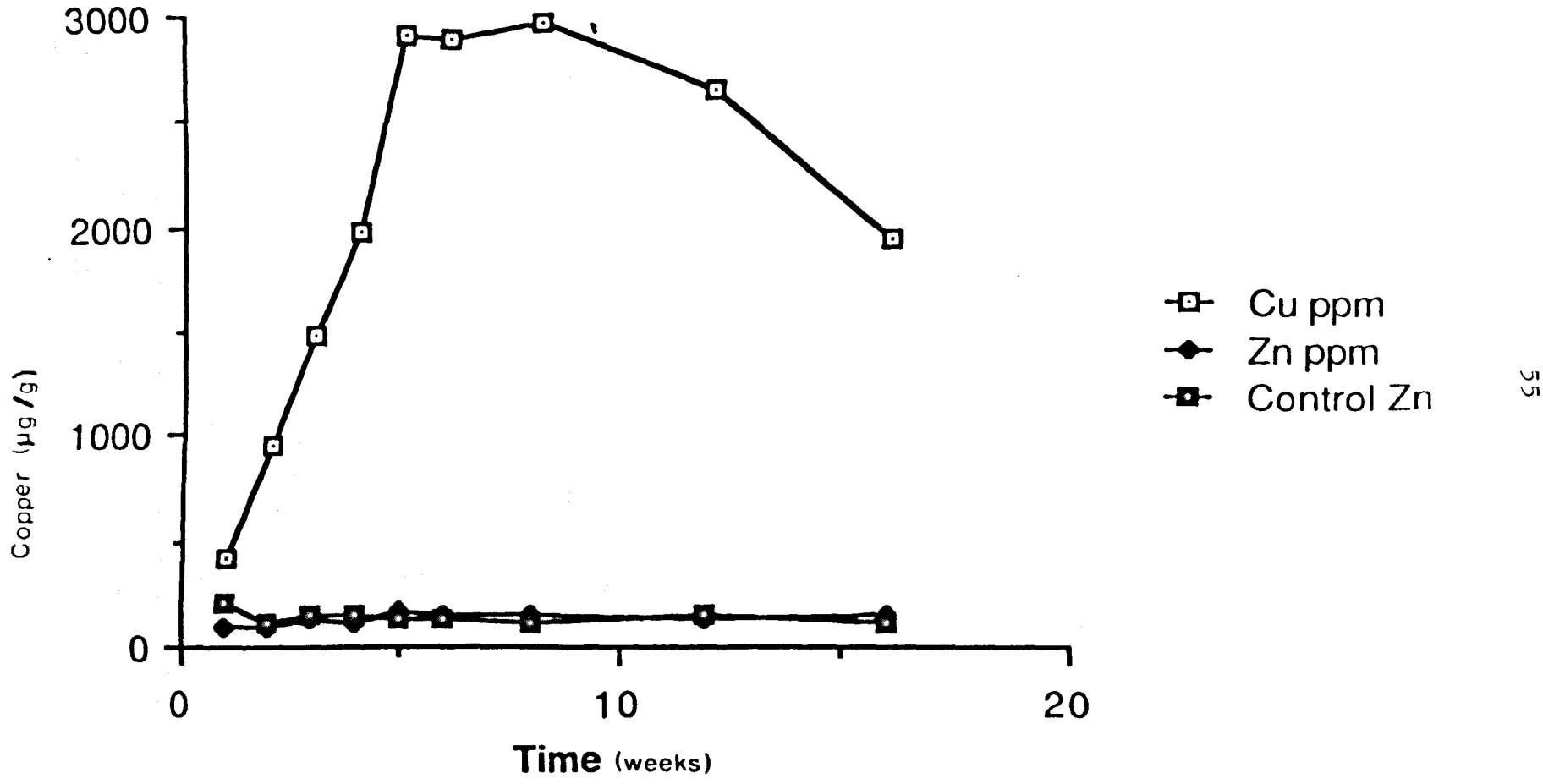


Figure 1.7. Copper and zinc concentrations in livers of rats on 1,500 mg/kg copper for 16 weeks.

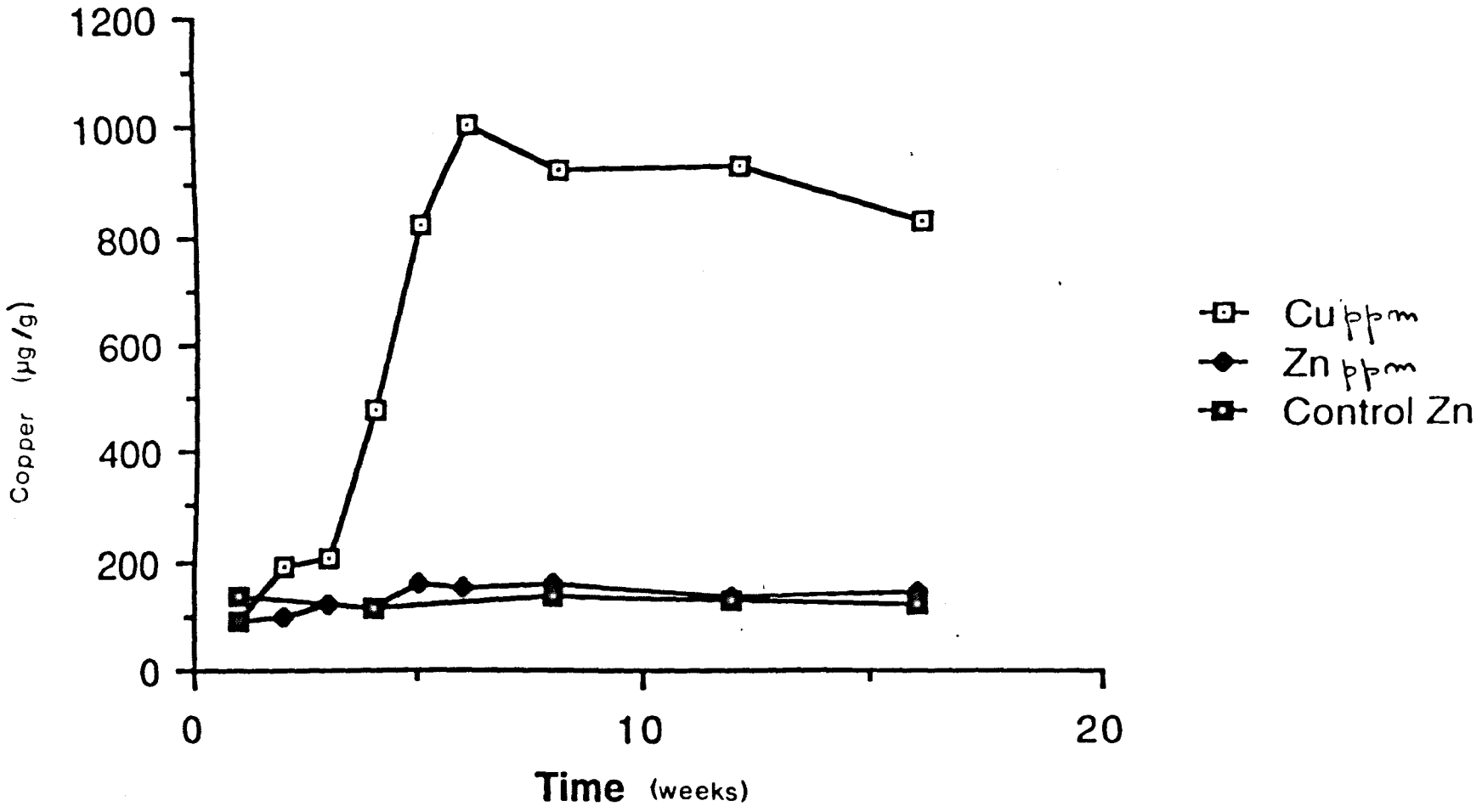


Figure 1.8. Copper and zinc concentrations in kidneys of rats on 1,500 mg/kg copper for 16 weeks.

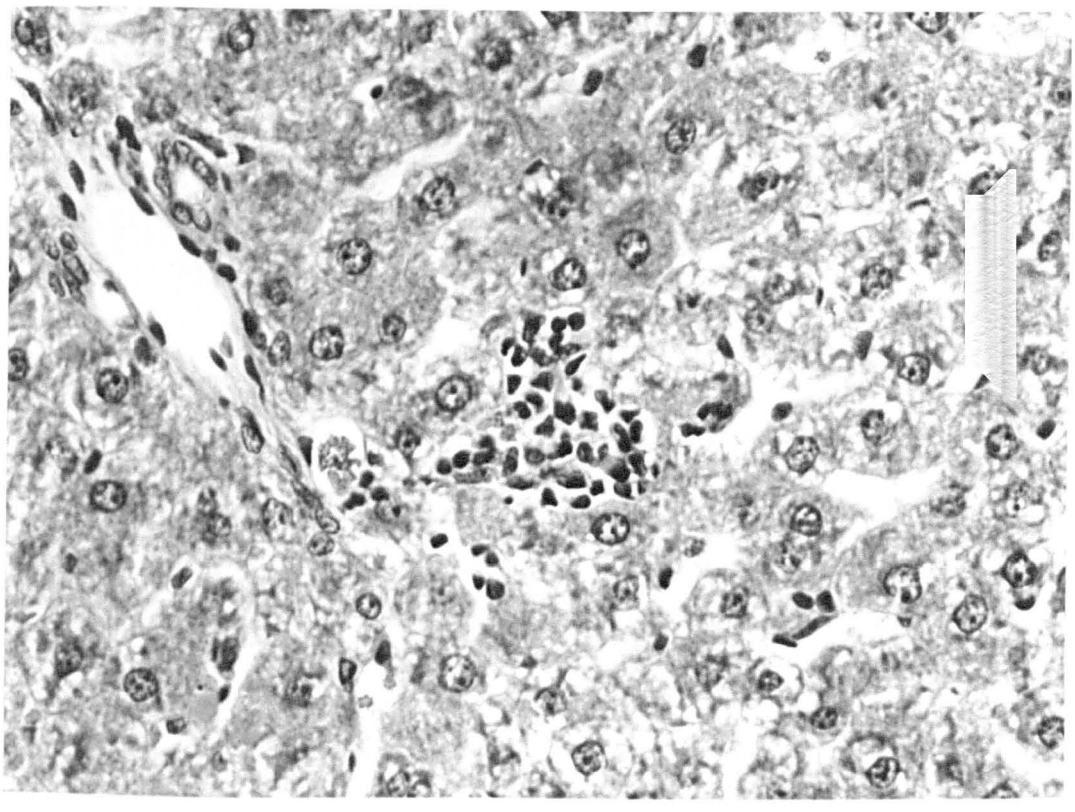


Figure 1.9. Week 3. Copper-loaded rat liver. Periportal inflammatory cell accumulation. H.E. x660

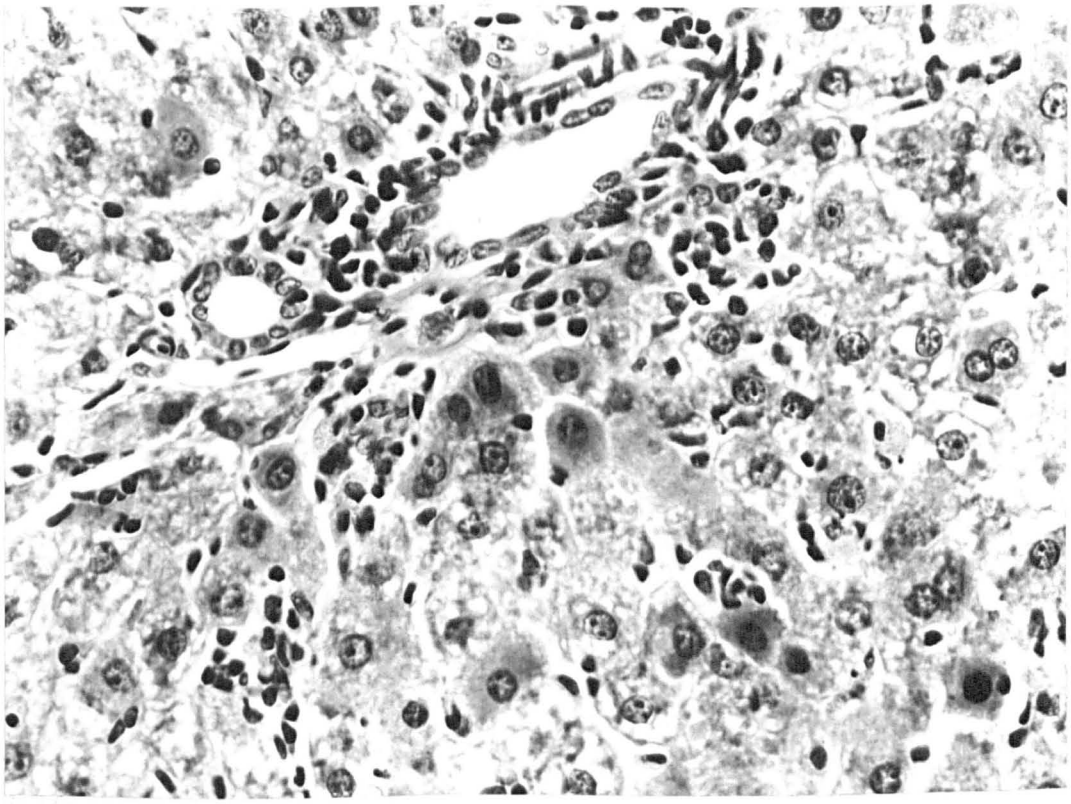


Figure 1.10. Week 4. Copper-loaded rat liver. There are numerous hypertrophic and hyperchromatic hepatocytes in the periportal zone. H.E. x440

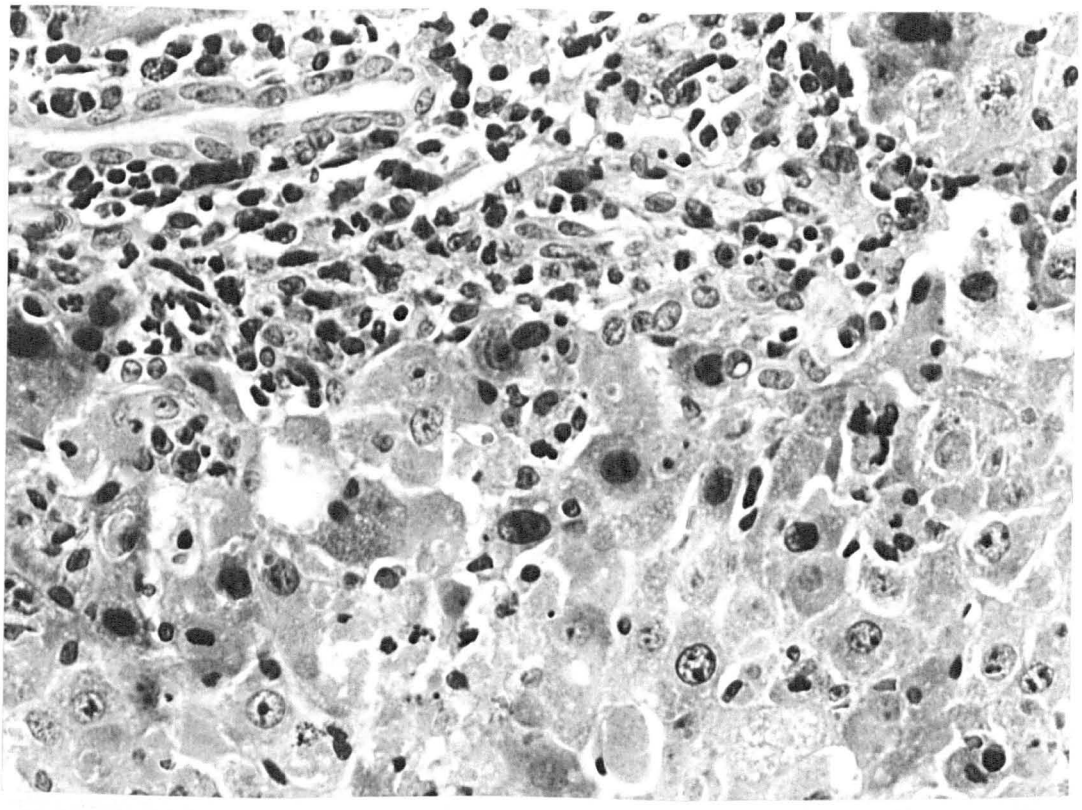


Figure 1.11. Week 6. Copper-loaded rat liver. Extensive necrosis and inflammatory changes in adjacent portal tracts. H.E. x440.

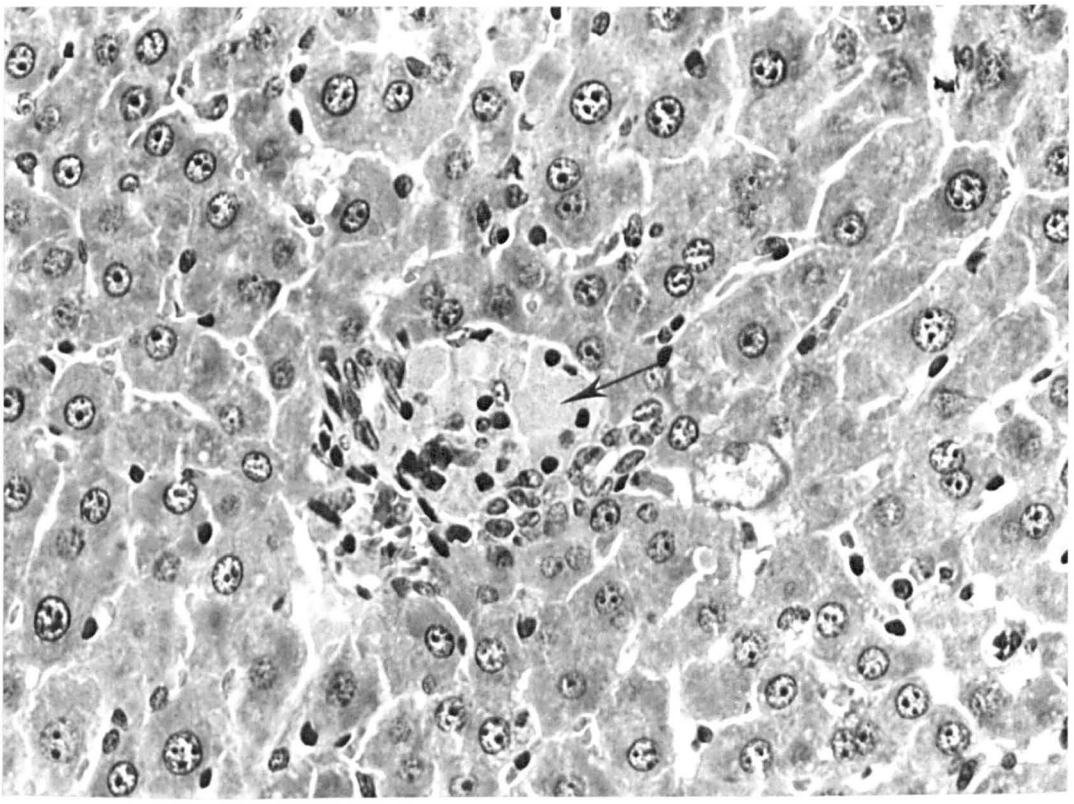


Figure 1.12. Week 16. Copper loaded rat liver. Persistence of hyaline remnants in the portal tracts (➡). Note recovery of hepatic parenchyma and the absence of inflammatory changes. H.E. x440.

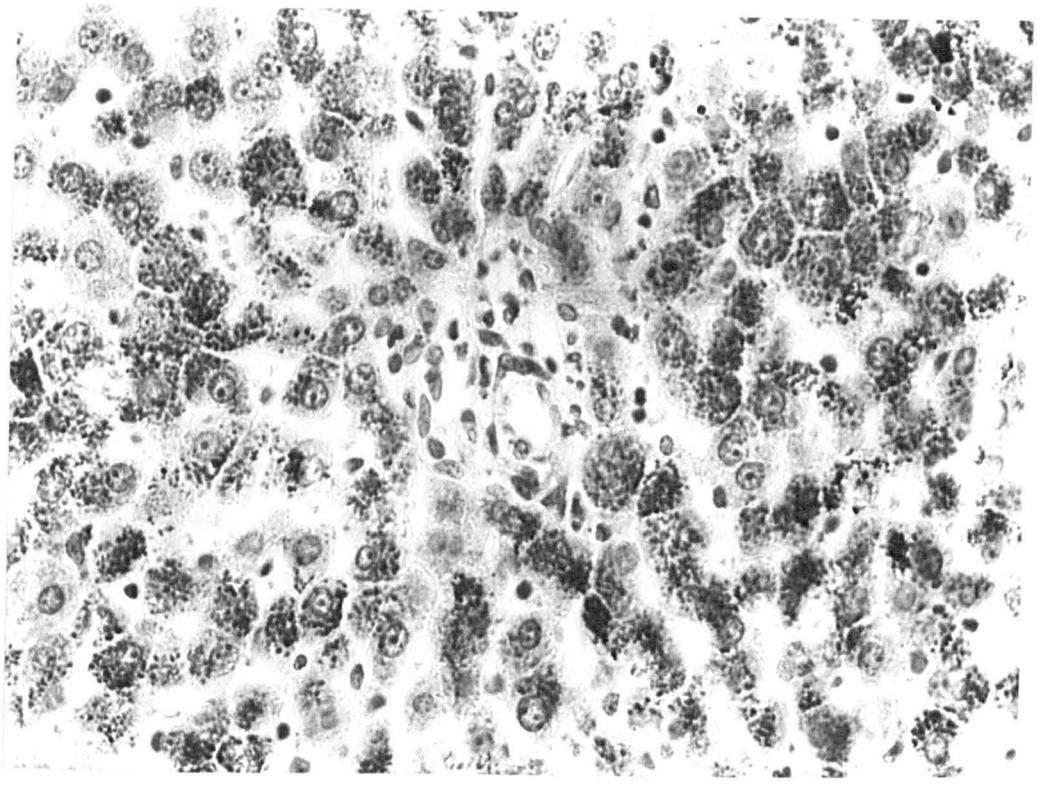


Figure 1.13. Week 5. Copper-loaded rat liver. (Cu concentration 2,920 μg/g). Presence of numerous copper positive granules in the periportal, midzonal hepatocytes. Rhodanine and haematoxylin. x435.

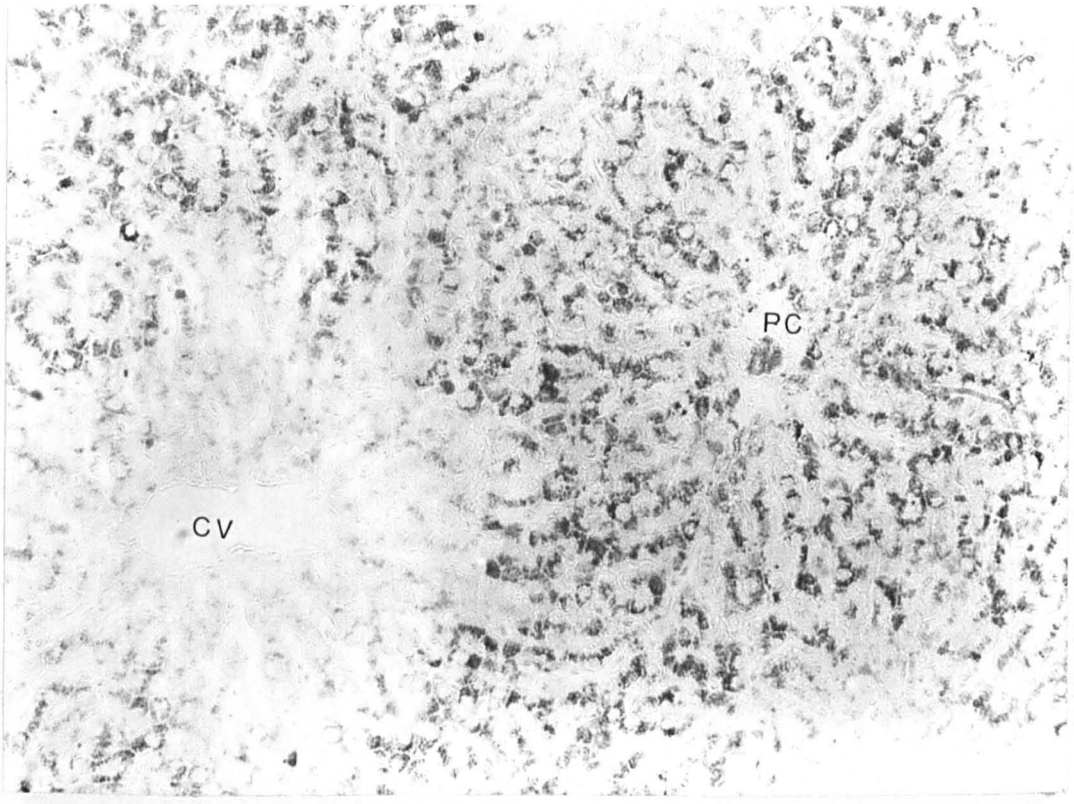


Figure 1.14. Week 5. Copper-loaded rat liver. Heavy deposition of copper in the periportal and midzones in contrast to the central zones. CV= central vein, PC= portal canal. Rubeanic acid and metanil yellow. x175.

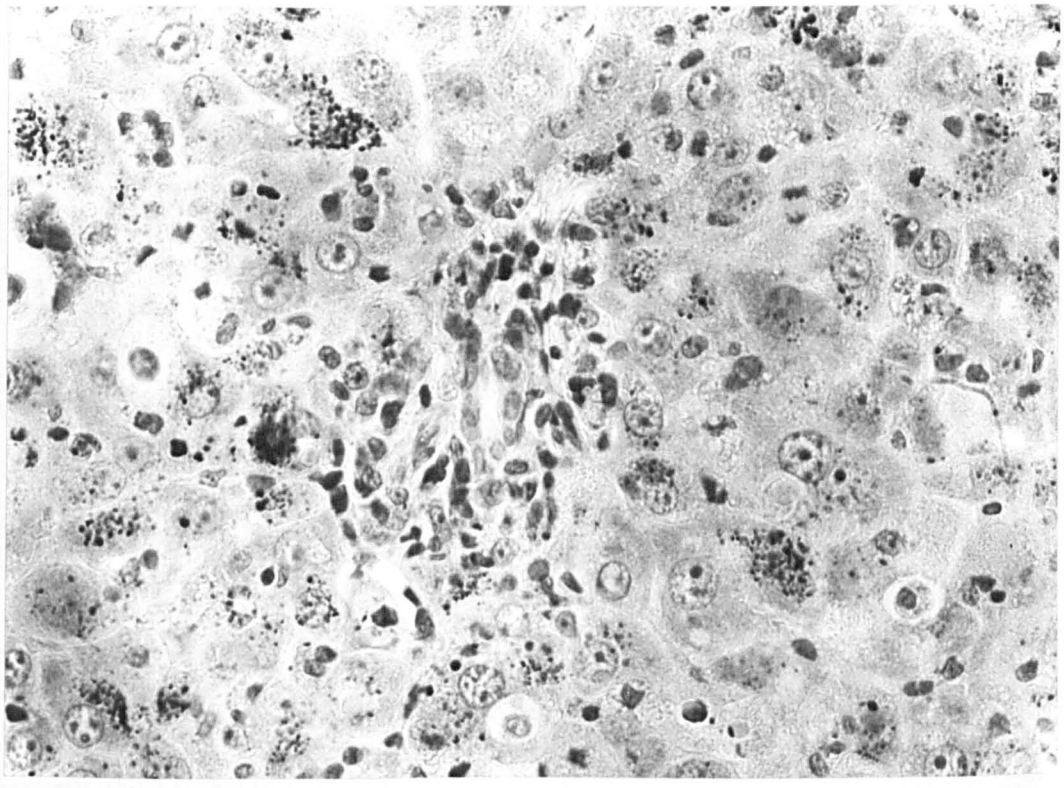


Figure 1.15. Week 5. Copper-loaded rat liver. (Cu concentration 3,151 µg/g). There is loss of overall copper staining intensity compared with figure 1.13. Rhodanine and haematoxylin. x435

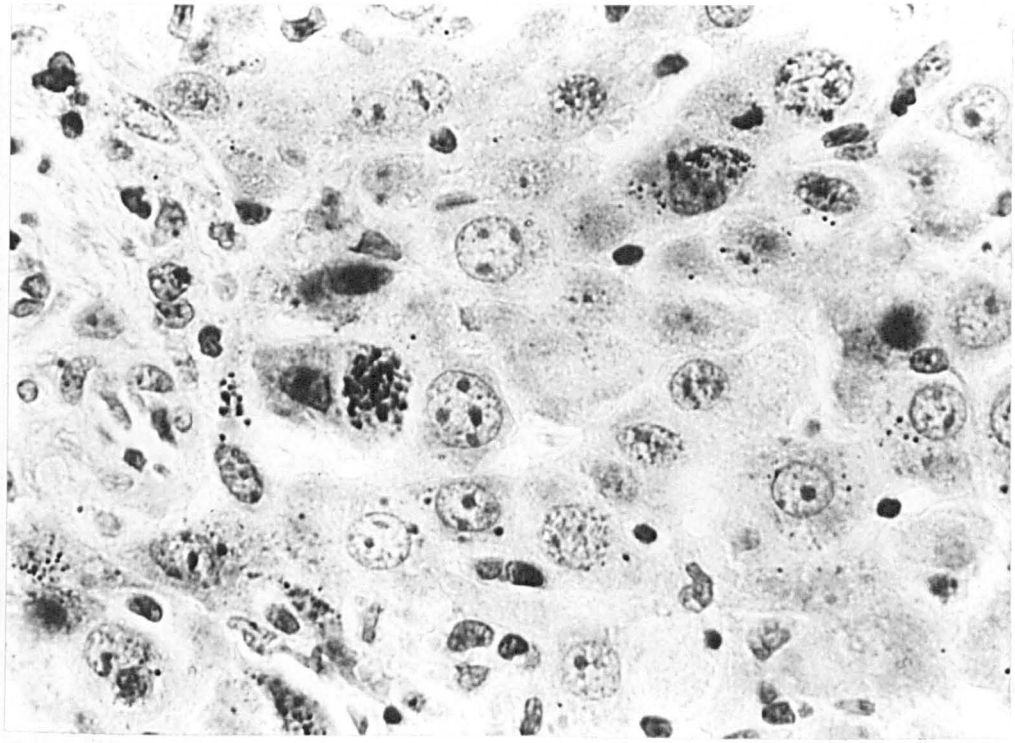


Figure 1.16. Week 6. Copper-loaded rat liver. Note the variability of copper positive granules within individual hepatocytes. Rhodanine and haematoxylin.
x 710.

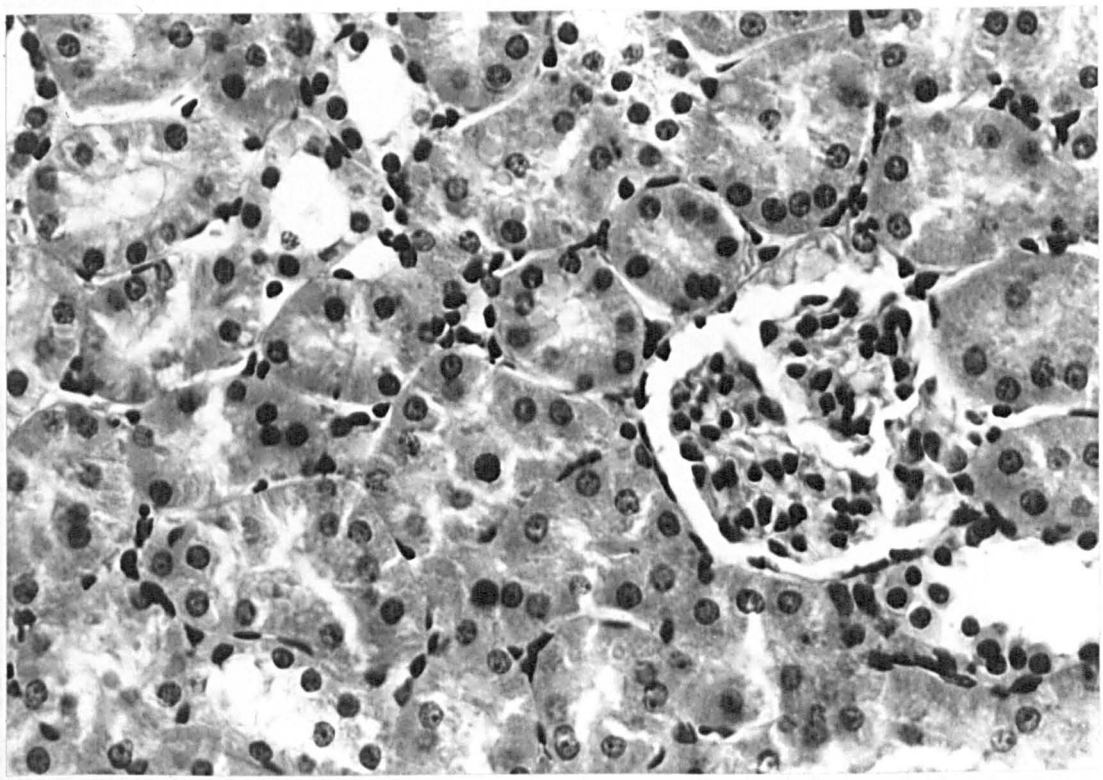


Figure 1.17. Week 3. Copper-loaded rat kidney. Variation in cytoplasmic and nuclear size of the PCT cells. H.E. x450.

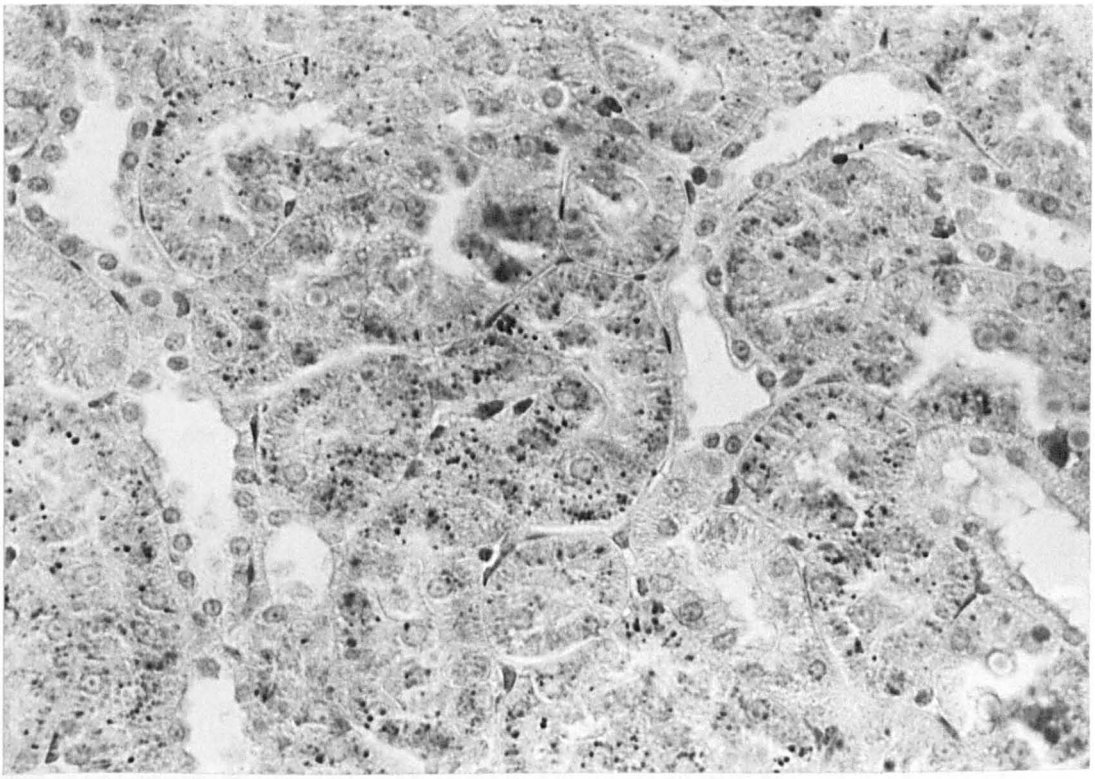


Figure 1.18. Week 4. Copper-loaded rat kidney. Abundant copper positive granules in the PCT. Rubeanic acid and neutral red. x450.

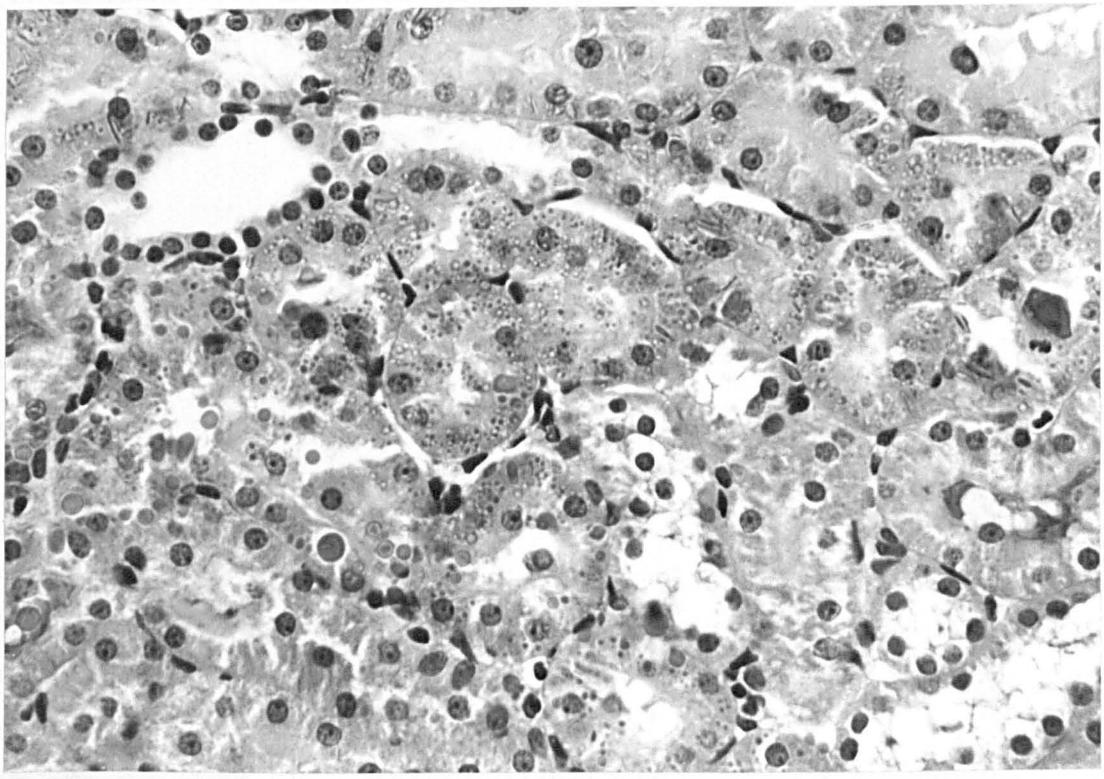


Figure 1.19. Week 5. Copper-loaded rat kidney. Eosinophilic droplets and granules are prominent in the cytoplasm and lumen of PCT. H.E. x 450.

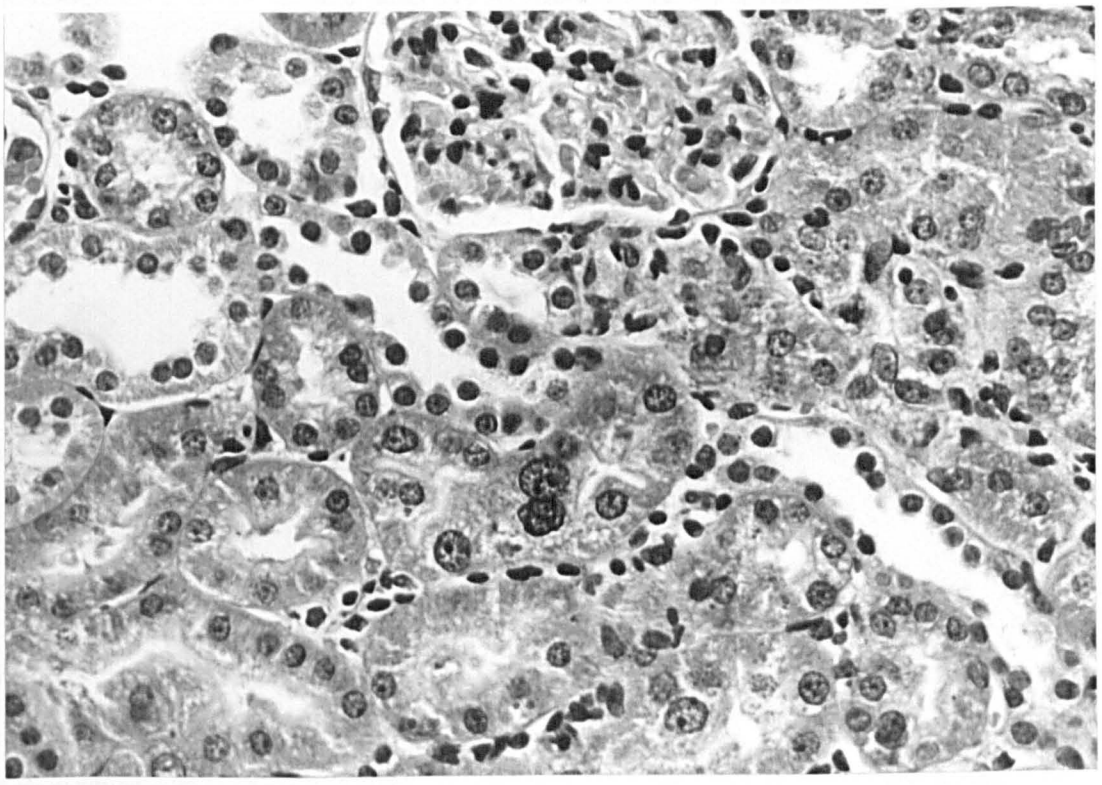


Figure 1.20. Week 6. Copper-loaded rat kidney. Marked differences in nuclear size of PCT cells, denoting regenerative activity. H.E. x 450.

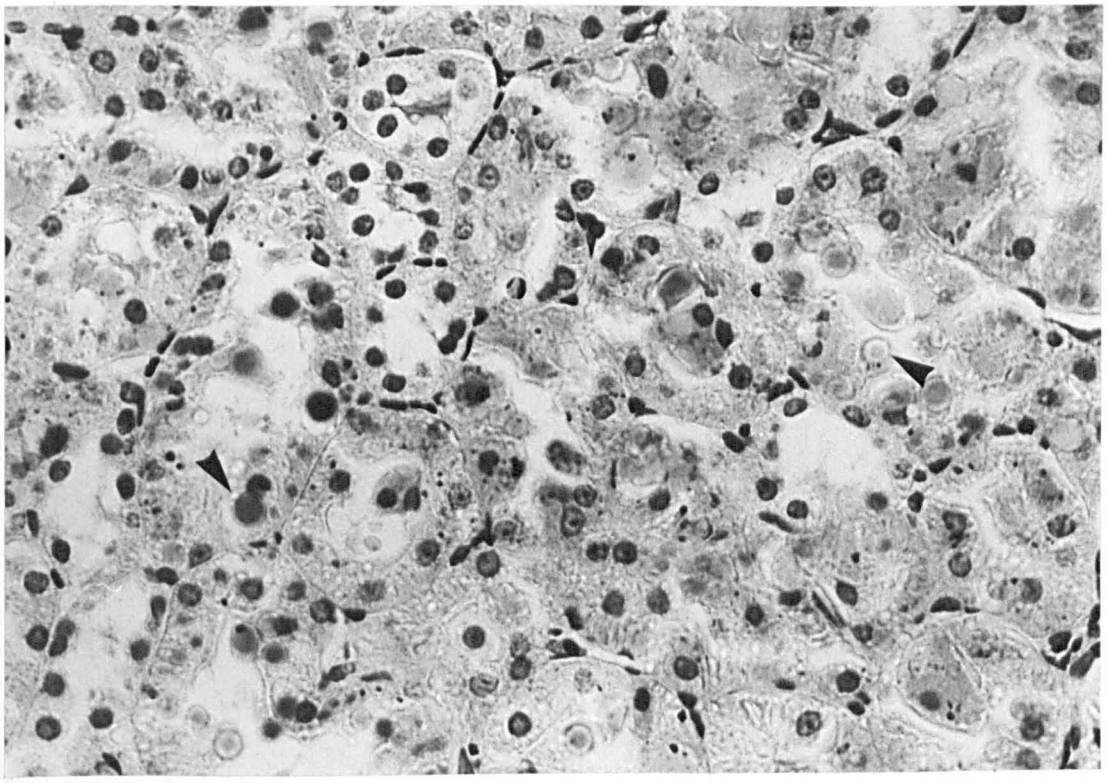


Figure 1.21. Week 6. Copper-loaded rat kidney. Variation in the intensity of copper staining of the droplets (►). Rhodanine and haematoxylin. x450.

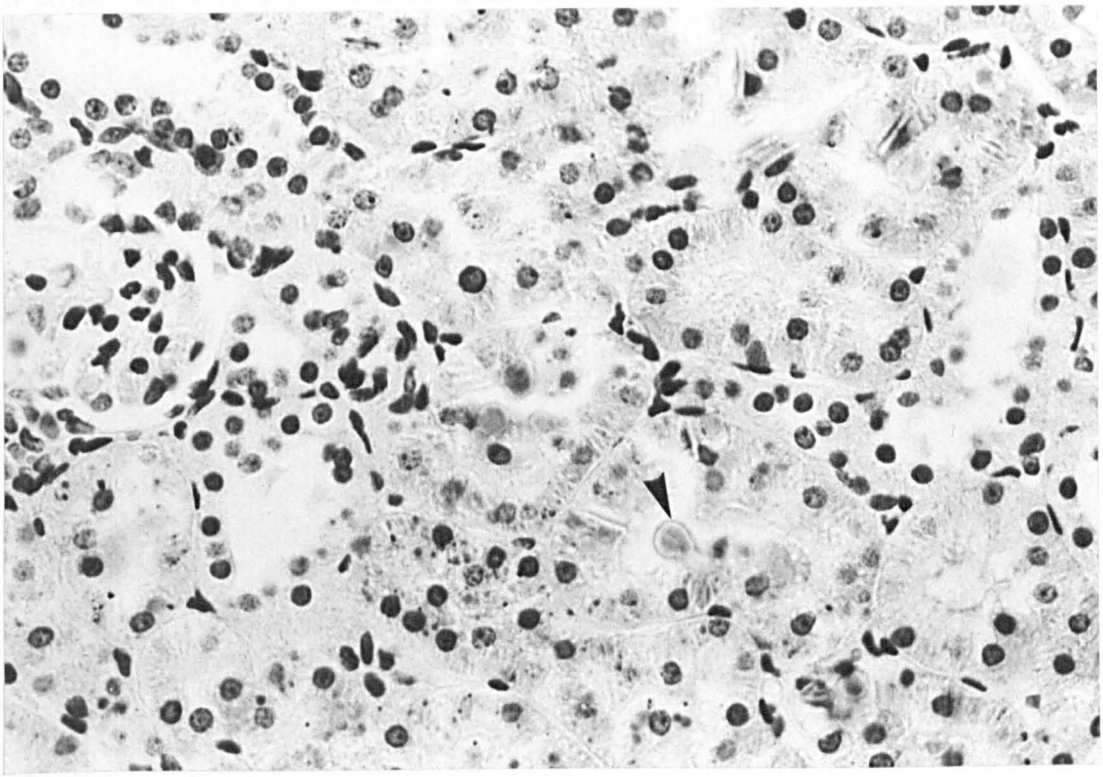


Figure 1.22. Week 16. Copper-loaded rat kidney. Extrusion of copper positive material into the lumen. Rhodanine and haematoxylin. x450.

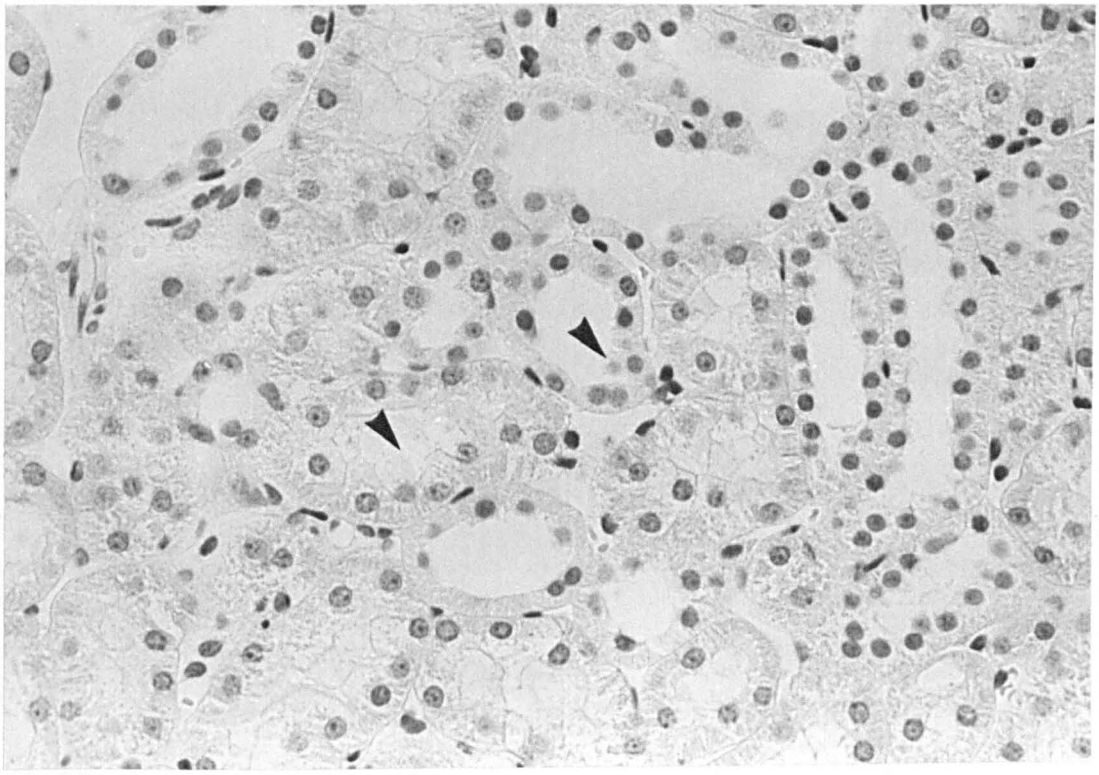


Figure 1.23. Control rat kidney. Copper negative droplets in the PCT cells. Rhodanine and haematoxylin. x450.

4.-DISCUSSION.

The findings from this study confirm the earlier reports of a depressed growth rate in rats fed high copper diets apparently unrelated to diminished food intake (Boyden et al., 1938; Haywood, 1979). Indeed in this particular study overall food intake actually increased in the copper fed groups which poses possible reasons for this particular effects of copper.

It is reasonable to suppose that an overall protein loss from the liver and kidney occurs in copper-loading, accompanied by an increased energy requirement (Haywood, 1979). Copper is usually stored in the hepatocytes in protein-bound form (Underwood, 1977), of which copper-metallothionein is probably the principal component (Mehra and Bremner, 1983). Excess copper is excreted in the bile conjugated to compounds of various molecular weights, including immunoreactive metallothionein and its breakdown products (Sato and Bremner, 1984). Renal excretion of excess copper has also been demonstrated in the rat (Haywood et al., 1985b) characterised at least in part as metallothionein (Bremner et al., 1986).

Alternatively, despite increased intakes of food, absorption may be interfered with in the copper-loaded state, or an altered mucosa may contribute to a protein losing enteropathy (Roger Batt, personal communication). Acute toxicity with copper and other metals is known to damage the gastrointestinal tract (Beliles, 1975, but little is known of the effect of chronic

toxicity.

The severe and erratic interruption in growth rate up to 5 weeks paralleled the cumulative hepatic uptake of copper and the accompanying damage. Subsequently body weight steadied and from 8 weeks onward a steady weight gain took place which reflected the overall recovery of the animals. However, body weights never reached the control weights, presumably because a continuing drain on protein resources would not allow this.

Liver and kidney copper concentrations showed progressive accumulation of the metal to a maximun of 2900 ±57 µg/g at 5 weeks and was maintained to 8 weeks (2960 ± 134 µg/g), thereafter falling to 1930 ± 91 µg/g at 16 weeks. This followed the pattern of loading and subsequent unloading established in earlier work (Haywood, 1985, Haywood et al., 1985a, 1985b). Zinc concentration had no deviation from the normal range indicating that influx of copper into the liver and kidney had not interference with this metal. Similarly, in copper poisoned sheep, copper did not have apparent effect on the zinc levels of livers and kidneys (Wilhelmsen, 1979).

The histopathological changes reflected the damaging period of copper accumulation, followed by recovery. By 16 weeks, despite a still considerable copper burden, the histological appearance of the livers of copper supplemented and control animals scarcely differed.

Histochemical identification of copper in this study has shown that copper-loaded rats store the metal predominantly in the

periportal and midzones (outer zones) of the liver lobule. This is paralleled by pathological changes in these outer zones, unequivocally associated with the progressive accumulation of copper. Subsequently there is a reduction in stainable copper followed by a decline in liver copper concentrations accompanied by recovery. This differential distribution might be attributed to the physiological specialisation which exists within lobules, whereby the outer zones alone have the oxidative capacity to assimilate, store and excrete macromolecules such as iron (Rappaport, 1979). It follows that should there be an excess of metal, either through increased input (absorption) or decreased output (cholestasis) it will normally be stored within the parenchymal cells of the outer zones. This is seen in the swan in which increased exposure to the metal (and therefore absorption) has occurred in contaminated waters (Molnar, 1983). A periportal accumulation of copper is apparent in human neonates, and the neonates of many other species before the biliary secretory route matures (Goldfischer and Berstein, 1969). A similar distribution of copper occurs in cholestatic diseases such as primary biliary cirrhosis in man (Goldfischer et al., 1980). Periportal copper accumulation also occurs in Skye terriers liver disease (Rutgers et al., 1987) and in chronic active hepatitis particularly in Doberman Pinschers which is probably also associated with disruption of biliary excretion (Crawford et al., 1985).

The periportal accumulation of copper observed in the rat contrasts strikingly with the centrizonal retention reported in

cases of familial copper-storage diseases in dogs (Twedt et al., 1979; Ludwig et al., 1980; Erikkson,1983; Thornburg et al., 1986; Haywood and Fuentealba, 1987) and chronic copper poisoning in sheep (Ishmael et al., 1971; Gooneratne et al., 1979; Kumaratilake, 1984) which differences may reflect the abnormal copper metabolism inherent in these animals. It has been suggested that defective biliary excretion of copper is responsible for abnormal copper retention in sheep (Underwood, 1977) and in the Bedlington Terrier (Su et al., 1982a; Owen and Mc Call, 1983). However, the centrizonal accumulation recorded in these species does not support this hypothesis. More likely it indicates a metabolic defect in the handling of copper peculiar to the hepatocytes of this inner zone.

The concept of metabolic zonation has developed from an increasing recognition of the microheterogeneity of liver function whereby hepatocytes in periportal (afferent) and perivenous (efferent) zones differ in enzymic and subcellular structures (Thurman et al., 1986). It is thought that many toxic lesions with a specific zonal pattern may be explained by this functional subcompartmentation.

Pathological changes in the copper-loaded sheep occur initially within the central zone and consist of focal degeneration and necrosis associated with aggregates of polymorphonuclear neutrophilic leukocytes and accumulation of bile pigment in the canaliculi. Later, in the posthaemolytic phase, fibroblast proliferation in the portal areas and increase in bile

ducts and lymphocytic infiltration occur (Gopinath and Howell, 1975; Ishmael et al., 1971).

In Bedlington Terriers there are four grades of abnormality in the liver as the disease progresses: pigment granules without any further hepatic tissue changes, mild focal hepatitis, a chronic active hepatitis and finally cirrhosis (Twedt et al., 1979; Ludwig et al., 1980; Kelly et al., 1984). As with the sheep(Ishmael et al., 1971; Gooneratne et al., 1979;Kumaratilake, 1984) these changes occur initially in the central zone (Twedt et al., 1979; Ludwig et al., 1980; Thornburg et al., 1986).

The early pathological changes consisting of focal degeneration and necrosis that occur as result of copper accumulation in the rat, although having a different microanatomical localisation, are similar to those described in sheep and dogs and indicate the possibility of a similar lesion at the cellular level.

Hyaline bodies present in the periportal zone in this study have previously been observed in copper-overload in rats (Haywood, 1985) and may be similar to the Mallory's hyaline present in primary biliary cirrhosis, long standing biliary obstruction, Wilson's disease and Indian childhood cirrhosis (MacSween et al., 1979). Mallory bodies have not been observed in copper toxicosis in Bedlington Terriers (Ludwig et al., 1980).

The cytotoxic effect of copper has not been fully clarified but there is evidence to suggest that nuclear

destabilization rather than lysosomal disruption may be the primary event (Haywood et al., 1985a). Nuclear changes observed in this study may reflect these events. Karyomegaly has also been recorded in sheep (Ishmael et al., 1971; Gopinath and Howell, 1975; King and Bremner, 1979; Kumaratilake,1984; Seaman, 1985). In Wilson's disease diffuse degenerative changes occur throughout the lobule consisting of vacuolar and fatty change, without reliable copper staining (Goldfischer and Sternlieb, 1968; Goldfischer et al., 1980). It would seem that the primary metabolic defect occurs at a different locus from that in Bedlington Terriers and sheep.

The distinctive patterns of copper distribution that occur in copper-associated diseases indicate different pathogenetic mechanisms and suggest that copper accumulation is not always the result of a simple overload associated with increased intake or reduced biliary excretion but may be more related to aberrations in intracellular events which have a pathoanatomical basis.

In the present study no differences were found between the sensitivity of rhodanine and rubeanic acid staining in the detection of copper. In canine copper storage diseases rubeanic acid has been found to be superior to rhodanine staining for detecting threshold levels of copper in livers and both methods have shown a good correlation with copper content measured by atomic absorption spectrophotometry (Ludwig et al., 1980; Johnson et al., 1984). However, orcein appeared to be of little values in this respect, similarly orcein has not been reliable in the

demonstration of copper in Bedlington terriers copper toxicosis (Johnson et al., 1984; Thornburg et al, 1985). This contrasted with other studies in which orcein has been used more successfully in the copper-loaded rat (Haywood, 1985) and in Bedlington terriers (Herrtage et al., 1987b), and may reflect variations in the quality of different batches of stain.

The findings from this study confirm previous reports (Haywood, 1985; Haywood and Loughran, 1985) that copper stains are unreliable for detecting copper-overload in rats, and in Wilson's disease (Goldfischer et al., 1980), contrasting with canine copper toxicosis where histochemical methods have invariably shown a good correlation with analysis by absorption spectrophotometry (Twedt et al., 1979; Ludwig et al., 1980; Johnson et al., 1984).

The differences with respect to staining in copper storage disease may be concerned with the intracellular localisation and binding of copper and probably indicate a real difference in the pathogenesis of the human and canine forms.

Intralysosomal copper metallothionein (Lys-Cu-MT) has been demonstrated in considerable quantities in Bedlington Terrier toxicosis (Johnson et al., 1981), whereas in Wilson's disease lysosomal bound copper is not marked in the early stages of the disease despite high levels of the metal, although a diffuse distribution of copper has been identified (Goldfischer and Sternlieb, 1968; Goldfischer et al., 1980).

The discussion so far has concerned itself solely with demonstrable cytoplasmic copper but it is interesting to note

that routine histochemical staining has not demonstrated copper in the nucleus of copper-loaded rats (Haywood, 1980; Haywood et al., 1985a), sheep (Ishmael et al., 1971; Gopinath and Howell, 1975; Olsen 1979), or man (Irons et al., 1977), despite considerable evidence of nuclear sequestration of the metal (Panemangalore et al., 1983). It is clear that not all intracellular copper is in a stainable form and that only particular bound forms take up copper stains.

It can be concluded that although copper stains can be useful in defined circumstances, their value as a diagnostic aid unsupported by copper analysis is limited.

Copper storage diseases and experimental administration of high levels of copper had been reported to produce kidney damage in Wilson's disease in man (Bearn et al., 1957; Wolff, 1964), mice (Vogel, 1960), and sheep (Todd, 1969; Ishmael et al., 1971; Ishmael et al., 1972; Gopinath et al., 1974; Gooneratne et al., 1986). Renal changes induced by copper toxicity are largely confined to the proximal convoluted tubules and consist of a variable degree of degeneration and necrosis of the epithelium. However, studies on copper fed rats have shown that accumulated copper is apparently excreted from the proximal convoluted tubules into the lumen and that temporary degenerative changes are followed by recovery and tolerance to copper (Haywood, 1980; Haywood, 1985).

In the present study, despite high renal copper concentrations, damage was minimal. The sequences of events in the

kidney followed a similar pattern to those observed in the liver. First copper accumulated with little effect, by week 6 copper concentration reached their peak and desquamation and cellular necrosis of the proximal convoluted tubules were present; this was followed by regeneration and recovery in the following weeks, associated with a decline in total kidney copper concentrations.

Eosinophilic droplets were observed in the proximal convoluted tubule epithelium, in this study, after 3 weeks of copper overload and were more numerous after 5 weeks; most of these droplets stained positive for copper with rubeanic acid and rhodanine, but some did not take the stain. Similar findings have been reported in copper-loaded rats (Haywood, 1979), and in the sheep kidney in chronic copper poisoning (Gopinath et al., 1974).

Hyaline droplets have been reported in normal mature rat kidney, and cellular fractionation and immunocytochemistry techniques have demonstrated that spontaneous hyaline droplets consist exclusively of alpha $_{2u}$ globulin (Alden, 1986). Several chemicals increase the accumulation of alpha $_{2u}$globulin in the proximal convoluted epithelium of the male rat as the primary acute toxicologic effect, but the spectrum of injury is different from the effects of nephrotoxins such as heavy metals (Alden, 1986). In the present study, hyaline droplets did not stain for copper in control animals suggesting that there are different populations of droplets, some of which are the direct response to copper-overload. The nature of these droplets will be discussed in detail in Chapter 3.

CHAPTER II.

CELLULAR MECHANISMS OF TOXICITY AND TOLERANCE IN THE COPPER-LOADED RAT. II. ULTRASTRUCTURAL CHANGES IN THE LIVER.

1.- INTRODUCTION.

Copper toxicosis, associated with increased hepatic copper, occurs in Wilson's disease, a familial disorder in man, (Underwood, 1977) and has been a well recognised problem in sheep for many years (Soli, 1980). More recently inherited copper storage diseases have been reported in Bedlington (Hardy et al., 1975) and West Highland White terriers (Thornburg et al., 1986).

Pathological changes described in the liver in copper toxicosis in man and sheep include degeneration and necrosis of parenchymal cells (Schaffner et al., 1962; King and Bremner, 1979; Gooneratne et al., 1980).

Fine structural changes have been reported in the liver in Wilson's disease (Schaffner et al., 1962; Goldfischer, 1963; Sternlieb, 1968; Goldfischer and Sternlieb, 1968), in copper-poisoned sheep (Gooneratne et al., 1980; Kumaratilake 1984), and in copper-loaded rats (McNary, 1963; Barka et al., 1964; Lindquist, 1968). All these studies have concentrated attention on the increased numbers of pericanalicular lysosomes, but have tended to overlook changes in other organelles, particularly the nucleus.

There are differing hypotheses to explain the mechanism of copper toxicity: copper sequestered in lysosomes may induce lipid peroxidation of lysosomal membranes with release of acid hydrolases into the cell (Lindquist, 1968; Gooneratne et al., 1980), alternatively other evidence suggests that nuclear

destabilisation rather than lysosomal disruption may be the initiating event (Haywood et al., 1985a). The copper-loaded rat can recover from copper-induced liver damage (Haywood, 1985) and is therefore a useful model in which to study the injurious effects of the metal and the subsequent regeneration that takes place.

The aim of the present study is

a) to describe the ultrastructural changes in the livers of rats during 16 weeks of copper-supplementation.

b) to relate the morphological alterations to the hepatic copper concentrations.

It is hoped thereby to clarify the pathogenesis of copper-induced damage and to increase understanding of the subsequent adaptation that takes place.

2.- MATERIALS AND METHODS.

a.-Animals.

The fixation of liver samples from the previous experiment (Chapter 1) was considered unsatisfactory for ultrastructural evaluation. The study of paraffin-sections of livers stained with haematoxylin-eosin revealed vacuolation of hepatocytes (Fig.2.1.); in sections stained with PAS, the vacuoles contained PAS (+ve) material identified as glycogen (Figs.2.2; 2.3). Ultrastructural examination confirmed the presence of excess glycogen in the hepatocytes which caused serious distortion of the cells and also interfered with fixation (Fig 2.4). Consequently it became necessary to previously fast the rats to remove this complication.

Male 6 weeks-old, Wistar rats of uniform weight were randomly allocated to groups of four, caged and fed a pelleted diet (Labsur Animal Diet, RHM Agriculture South Ltd) with a copper content of 1,500 mg/kg (range 1,300-1,800 mg/kg) (Appendix 1). Tap water and food were always available. Control animals were fed the unsupplemented diet with a copper content of 10 mg/kg.

The animals were regularly observed and weighed at weekly intervals (Appendix 9), and the food was weighed and consumption per group recorded weekly (Appendix 10). Rats on the supplemented diet were killed at intervals of 1,2,3,4,5,6,8,12, and 16 weeks. Five groups of 4 control rats were similarly killed at 1,4,8,12 and 16 weeks

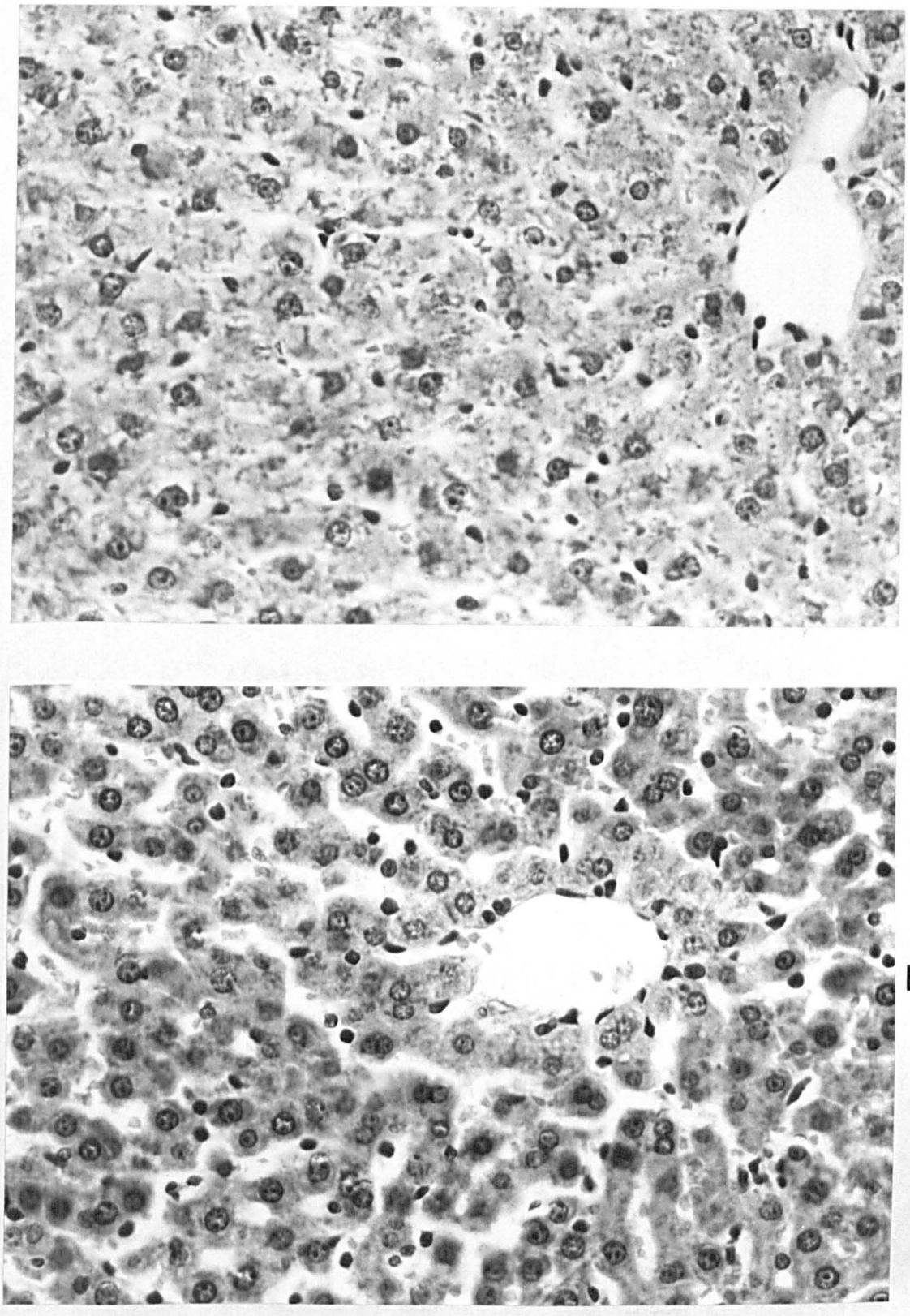


Figure 2.1. A) Liver from a rat with unrestricted food. Note intense cytoplasmic vacuolation compared with B) Liver from a rat starved overnight. H.E. x440.

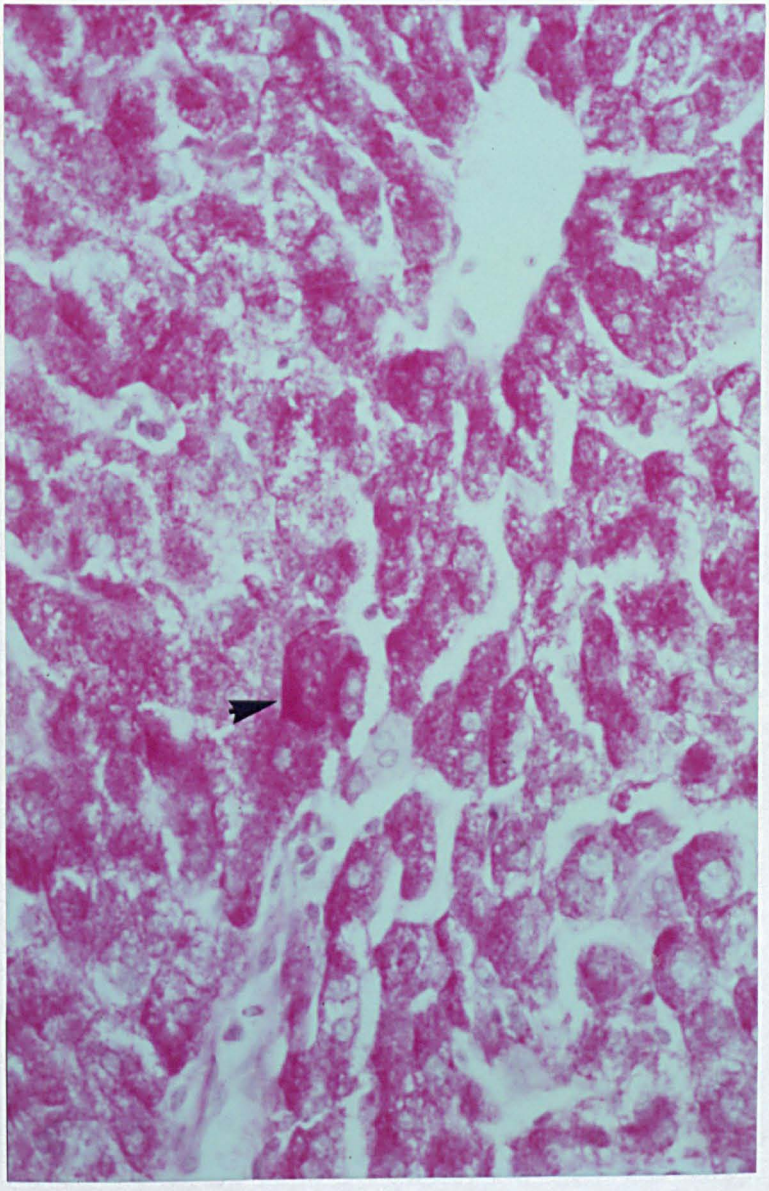


Figure 2.2. Liver from a rat with unrestricted food overnight. PAS positive material (►). x 440

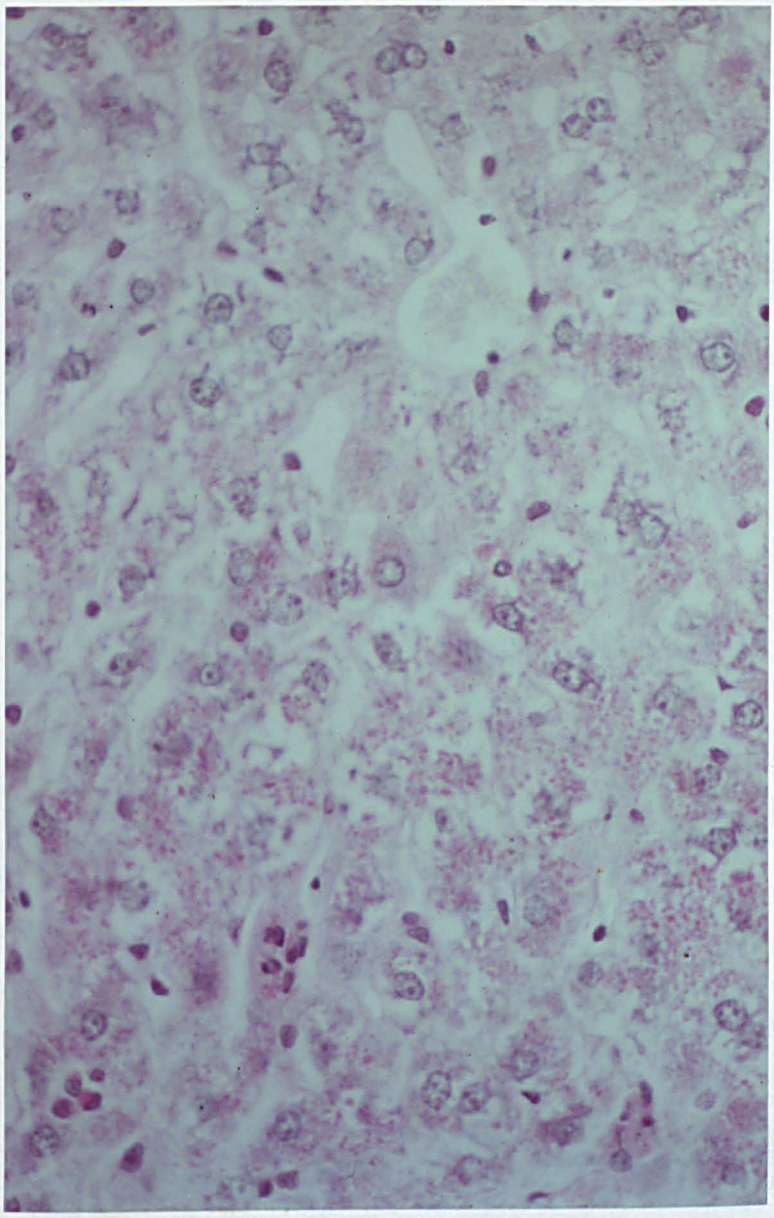


Figure 2.3. Liver from a rat with unrestricted food overnight. PAS + amylase digestion. Absence of PAS positive material. x440.

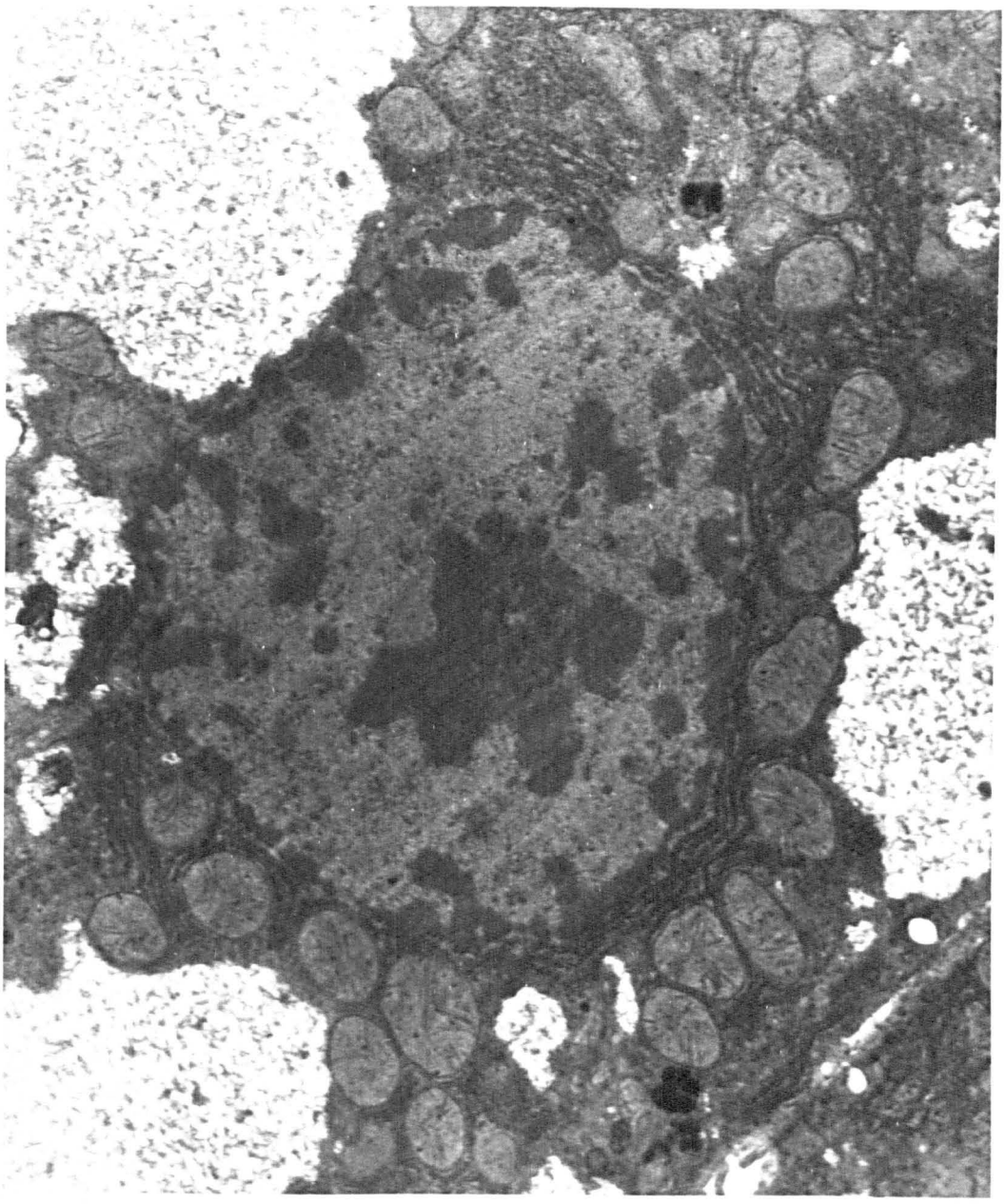


Figure 2.4. Liver from a normal rat without food restriction overnight and fixed with glutaraldehyde in phosphate buffer. The nucleus is irregularly-shaped, the chromatin is clumped in the periphery and abundant glycogen deposit is seen in the cytoplasm. 16,000x.

Groups of four rats on the supplemented diet were fasted for 18 hours previous to being killed when placed in a polythene chamber in which the air was gradually replaced by CO_2. After 20-30 seconds, respiration was depressed or had ceased altogether and the animals appeared unconscious. The rats were removed from the chamber and subjected to cervical dislocation..

The right median lobe of the livers (Haywood, 1981) were removed immediately for ultramicroscopy and copper analysis.

b.-Copper analysis.

Liver copper concentrations were determined by atomic absorption spectrophotometry as described in chapter 1.

c.-Transmission Electron Microscopy.

Fixation

Thin slices (2-3mm) of liver were immersed immediately after removal in chilled 4 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M phosphate buffer (pH7.4), (Karnovsky, 1965) (Appendix 11), further cut into small cubes (approximately 1.0 mm^3) and kept in this fixative overnight at 4 ^oC. The tissues were rinsed twice in buffer for 30 min, post-fixed for 1 hour in cold (4 ^oC) 1 % unbuffered Os O_4.

Resin embedding:

The post fixed blocks were rinsed in distilled water, immersed in 2% uranyl acetate in 0.69% maleic acid for 1.5 hours

and then dehydrated through a graded series of ethanol, cleared in acetone (Appendix 12) and finally embedded in TAAB resin (Appendix 13). Subsequently 4 blocks were embedded in fresh resin, polymerised at 60 ° C for 24 hours and trimmed. Tissues from the rat with a copper concentration closest to the group mean were selected for E.M. examination, however, in some circumstances blocks from all rats in a particular group were examined. Semi-thin sections (0.5-0.3μm) were cut using glass knives on a Reichert-Jung ultramicrotome (Reichert Ltd. Austria), stained with alkaline toluidine blue (Appendix 14) to select a periportal area for ultrathin sectioning. The blocks were retrimmed before cutting ultrathin (silver-grey) sections. These sections were collected on plain 200 mesh copper grids (TAAB Laboratories, Reading, U.K.), contrast stained for 5 minutes with lead citrate (Reynolds, 1963a), rinsed in destilled water, left to dry and examined at 50KV in a Hitachi electron microscope.

d.- **Photography.**

EM sections were photographed using Ilford EM film (Ilford Ltd., Mobberley Cheshire, U.K.) and printed on Ilfospeed Multigrade II photographic paper.

3.- RESULTS.

Hepatic copper concentrations are recorded in Table 2.1. There was an initial rapid accumulation of the metal up to 4 weeks when maximum concentrations were achieved. This was followed by falling levels so that by 16 weeks liver copper concentrations (2,271 ± 93 µg/g) was significantly less ($p < 0.05$) than at 4 weeks (2,840 ± 46 µg/g). When compared with experiment 1 (Fig.2.5) copper accumulation occurred more rapidly, associated with an earlier increased food intake (Appendix 10).

The fine structure of liver (Fig.2.6) in the control group conformed in general to previous descriptions of normal rat liver (Bruni and Porter, 1965; Rhodin, 1974).

Copper supplemented groups.

Week 1: Liver copper concentration was 679 ± 40 µg/g.
Parenchymal cells varied in size and electron density so that light and dark cells were distinguished (Fig.2.7). The rarefaction of the light cells was associated with vacuolation and focal dilatation of the smooth endoplasmic reticulum (SER) and lucency of the intervening cytosol (Fig.2.8).

Pericanalicular regions of the hepatocytes contained single-membrane bound electron-dense bodies identified as lysosomes (Fig.2.9). Bile canalicular changes consisted in swelling of the microvilli (Fig.2.10).

Week 2: Liver copper concentration was 1346 ± 72 µg/g.

The vacuolation of the SER persisted. There was an increase in the number of lysosomes dispersed throughout the cytoplasm. In some hepatocytes there was condensation of nuclear chromatin (Fig.2.11).

Week 3: Liver copper concentration had risen to 2697 ± 57 µg/g.

The nuclear changes were more pronounced: condensation of chromatin was found in the nuclei of most parenchymal cells and many nuclei were misshapen with crenated nuclear membranes (Figs.2.12a and b). The mitochondria appeared swollen. Irregularly-shaped lysosomes were numerous and dispersed throughout the cytoplasm; they were also noted in Kupffer cells (Fig.2.13). Ovoid masses of condensed cytoplasm (apoptotic bodies) were present in parenchymal cells (Fig.2.14a and b) and the accumulation of cellular debris was observed within sinusoids (Figs.2.15).

At this time inflammatory cells were seen in some sections.

Week 4: Liver copper concentration was maximal at 2840 ± 46 µg/g.

Inflammatory cells were numerous; nuclear changes had progressed to include dissolution of nuclear membranes and karyolysis as shown by the presence of numerous "ghost-like" forms

(Fig.2.16). There was fragmentation of the rough endoplasmic reticulum (RER), disruption of the parallel arrays of membranes and disaggregation of ribosomes (Fig.2.17). Lysosomes were very numerous, and heterogeneous with regard to size, shape and electron-density (Fig.2.18). They were widely distributed throughout the cytoplasm of the parenchymal cells; membrane integrity was always maintained.

Week 5 and 6: Liver copper concentration has fallen to 2358 ± 106 µg/g in week 5 and 2389 ± 91 µg/g in week 6.

Inflammatory cells were numerous; nuclear changes in recovered cells were minimal. A large number of laminar concentric osmiophilic membranes were seen during this period (Fig.2.19), some included cytoplasmic organelles (mitochondria, SER). Intact lysosomes were still numerous and noticeably heterogeneous with regard to electron density (Fig.2.20). Mitochondria and SER swelling persisted. The bile canalicular microvilli showed pronounced vesiculation and frequently loss of microvilli (Fig.2.21) and the space of Disse appeared dilated (Fig.2.22). Apoptotic bodies were numerous both in the sinusoids and within Kupffer cells (Fig.2.23 a and b).

Week 8: Liver copper concentration was 2429 ± 156 ug/g.

This period was characterised by hypertrophy of parallel arrays of the RER (Fig.2.24), reduced numbers of lysosomes, apoptotic bodies and inflammatory cells.

Week 12: Liver copper concentration was 2540±147 μg/g.

Hypertrophy of the RER persisted. There was an accumulation of membrane-bound cytoplasmic floccular material (Mallory body-like structures) within the hepatocytes.

Week 16: Liver copper concentration was 2271 ±93 μg/g.

The hepatocytes were nearly normal in appearance (Fig. 2.25) and no abnormal changes were detected in the bile canaliculi or nuclei. There was abundant floccular material in the cytoplasm of hepatocytes and Kupffer cells (Fig. 2.26). In some hepatocytes there was residual swelling and electron lucency of the cytosol (Fig. 2.27). Lysosomes were still present but were considerably reduced in numbers.

Table 2.1. Hepatic copper concentrations in copper-loaded and control rats.

Time (weeks)	Copper concentration μg/g) *
1	679 ± 40
2	1,346 ± 72
3	2,697 ± 57
4	2,840 ± 46
5	2,358 ±106
6	2,389 ± 91
8	2,429 ±156
12	2,540 ±147
16	2,271 ± 93
Control **	22 ± 1

* mean ± SE of the mean (n=4)

** n= 20

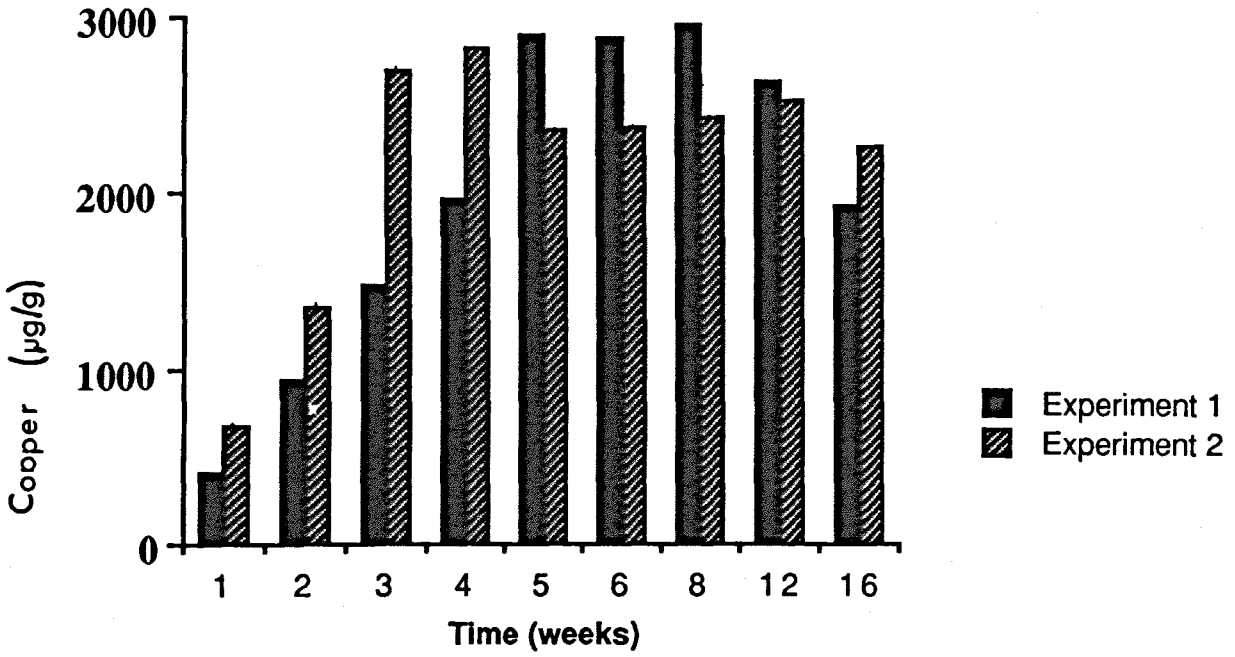


Figure 2.5. Liver copper concentrations in copper-supplemented rats for 16 weeks; experiment 2 compared with experiment 1. Mean and standard error of the mean. N= 4.

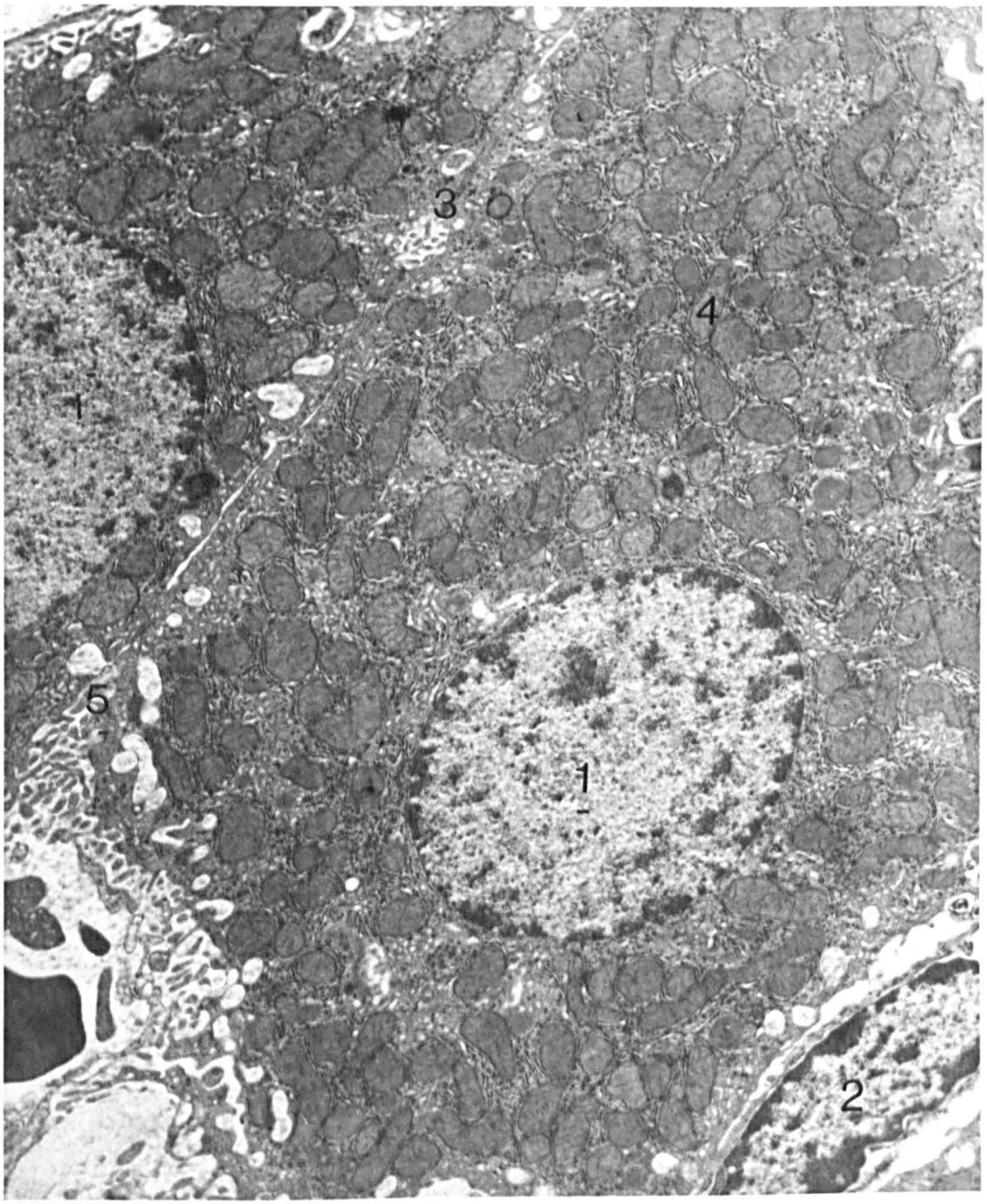


Figure 2.6. Liver from a control rat. Karnovsky fixative. 10,000x.
1. Nuclei of hepatic cells
2. Nucleus of sinusoidal endothelial (Kupffer) cell
3. Longitudinally sectioned bile canaliculus
4. Mitochondria
5. Perisinusoidal space of Disse

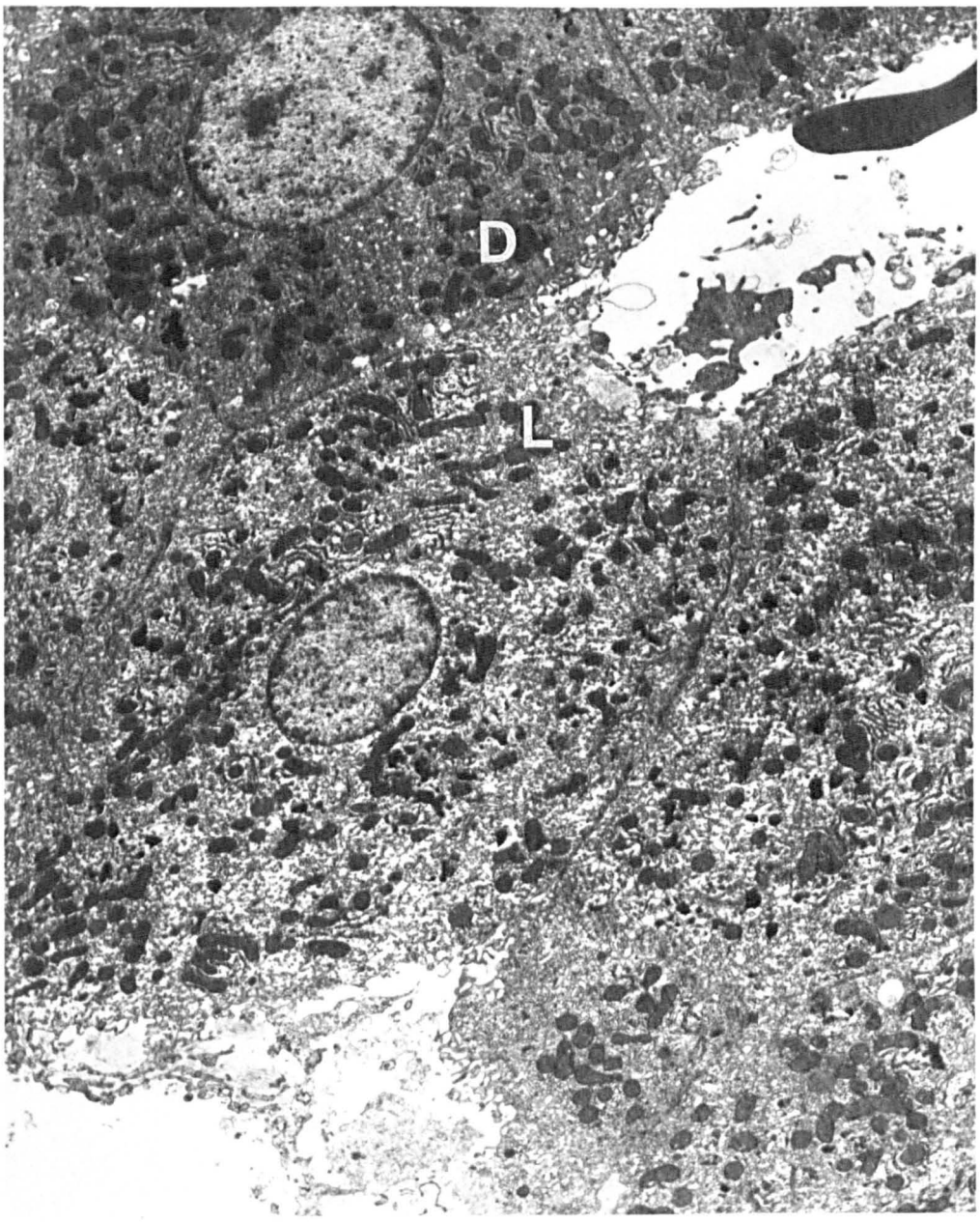


Figure 2.7. Week 1. Copper-loaded rat liver. Presence of light (L) and dark (D) parenchymal cells. x 6,000.

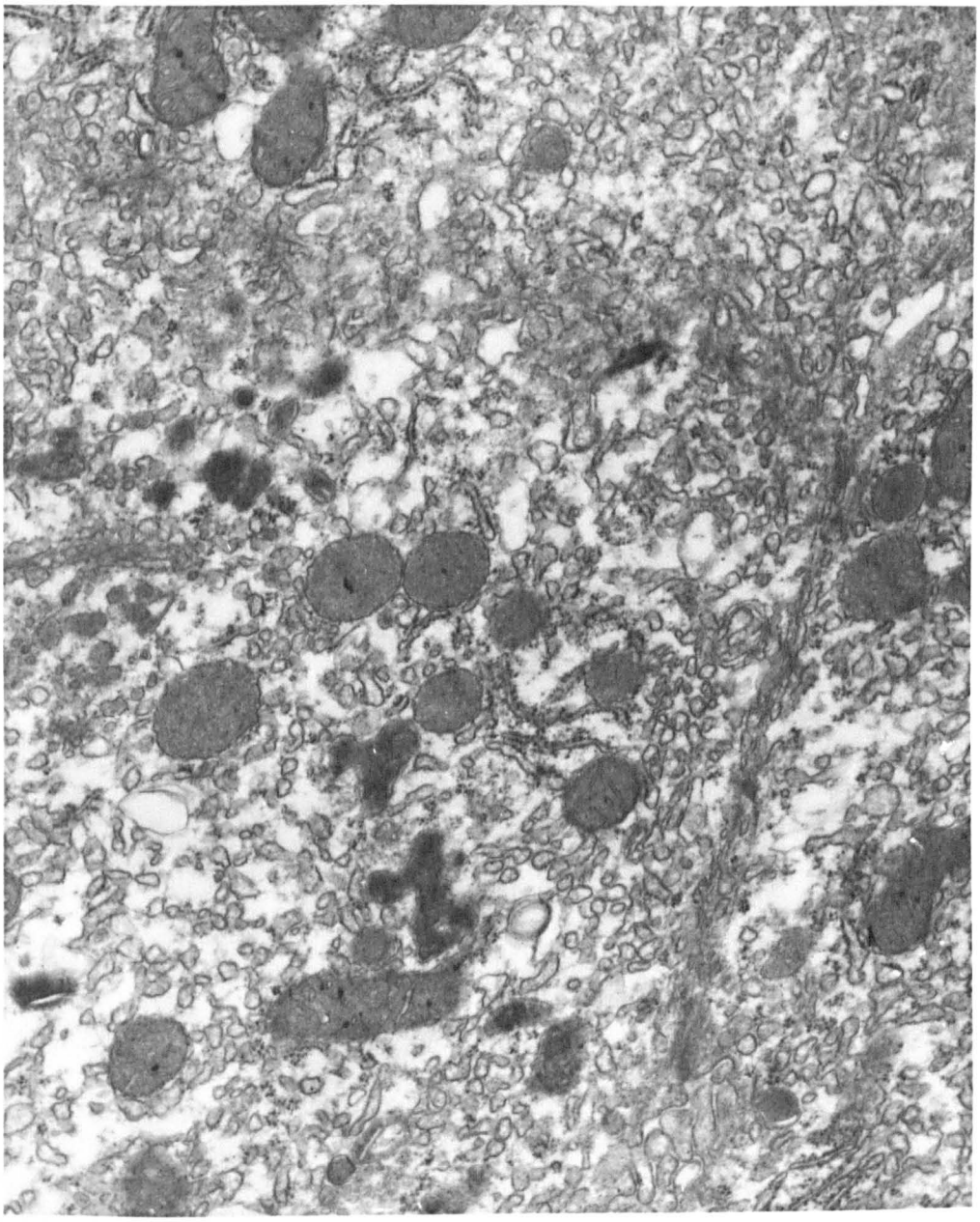


Figure 2.8. Week 1. Copper-loaded rat liver. Swollen smooth endoplasmic reticulum within a light hepatocyte. x 30,000.

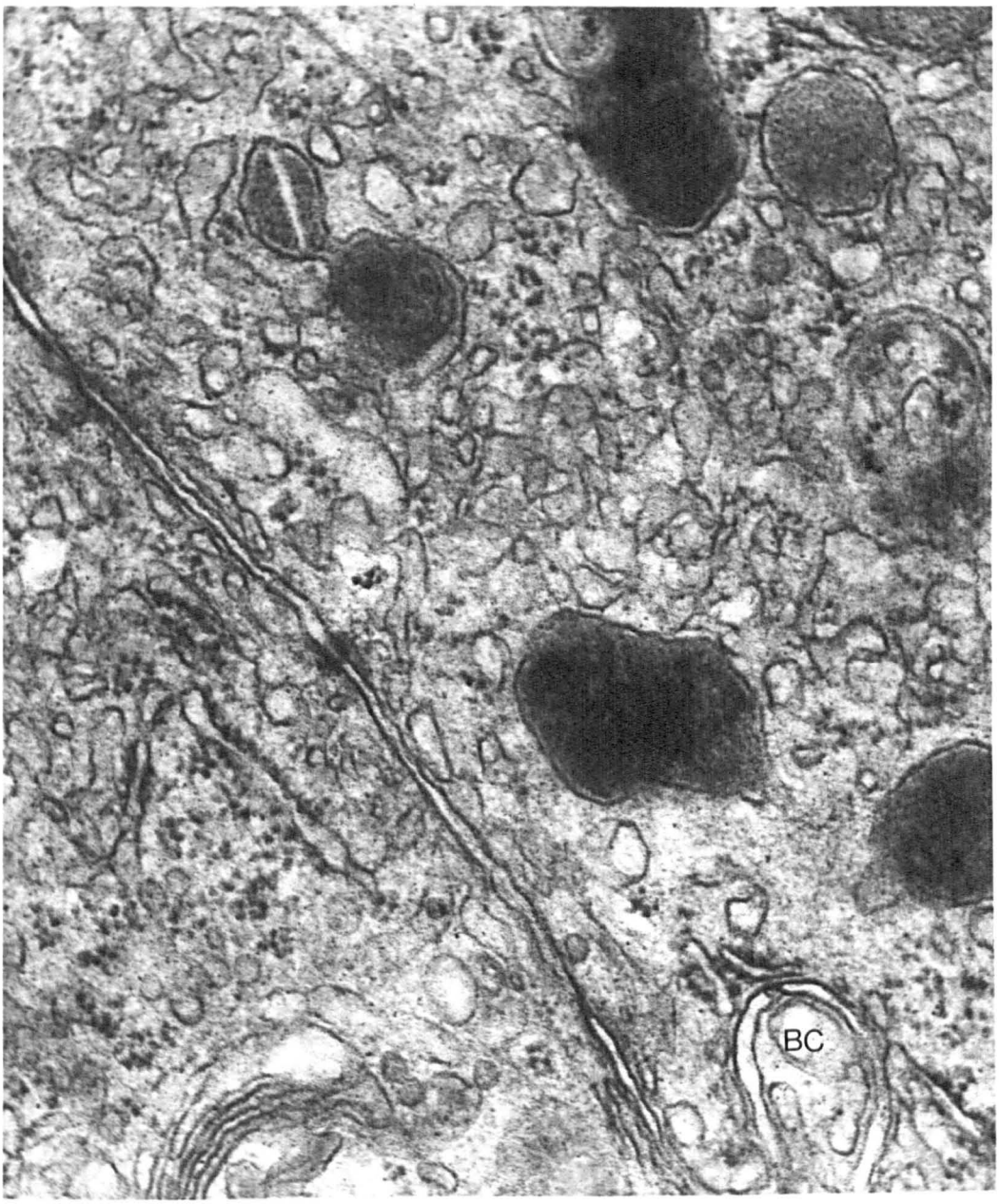


Figure 2.9. Week 1. Copper-loaded rat liver. Pericanalicular electron dense membrane-bound lysosomes. x 70,000.
B.C = Bile canaliculus

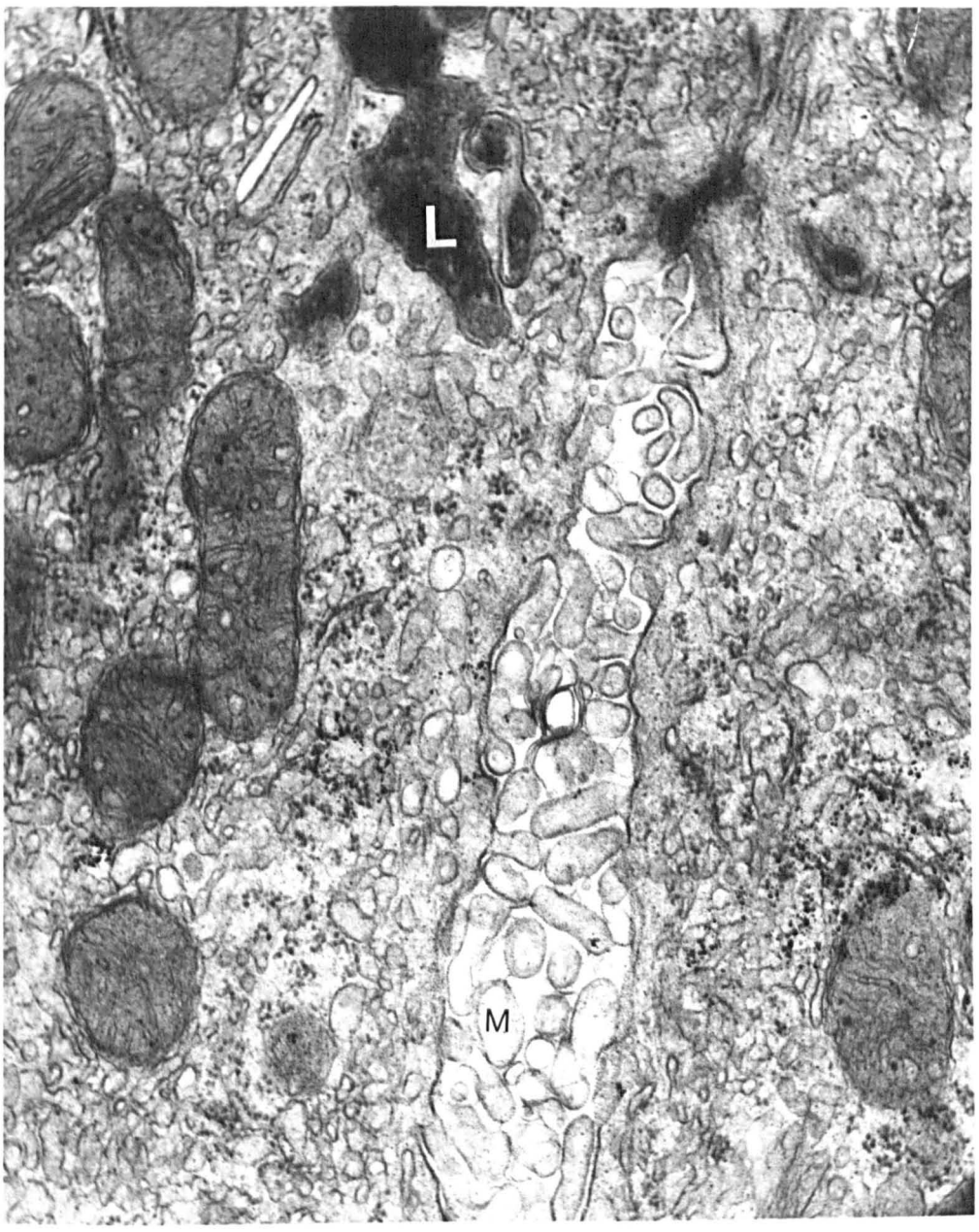


Figure 2.10. Week1. Copper-loaded rat liver. Swelling of canalicular microvilli (M) and presence of pericanalicular lysosomes (L). x 20,000.

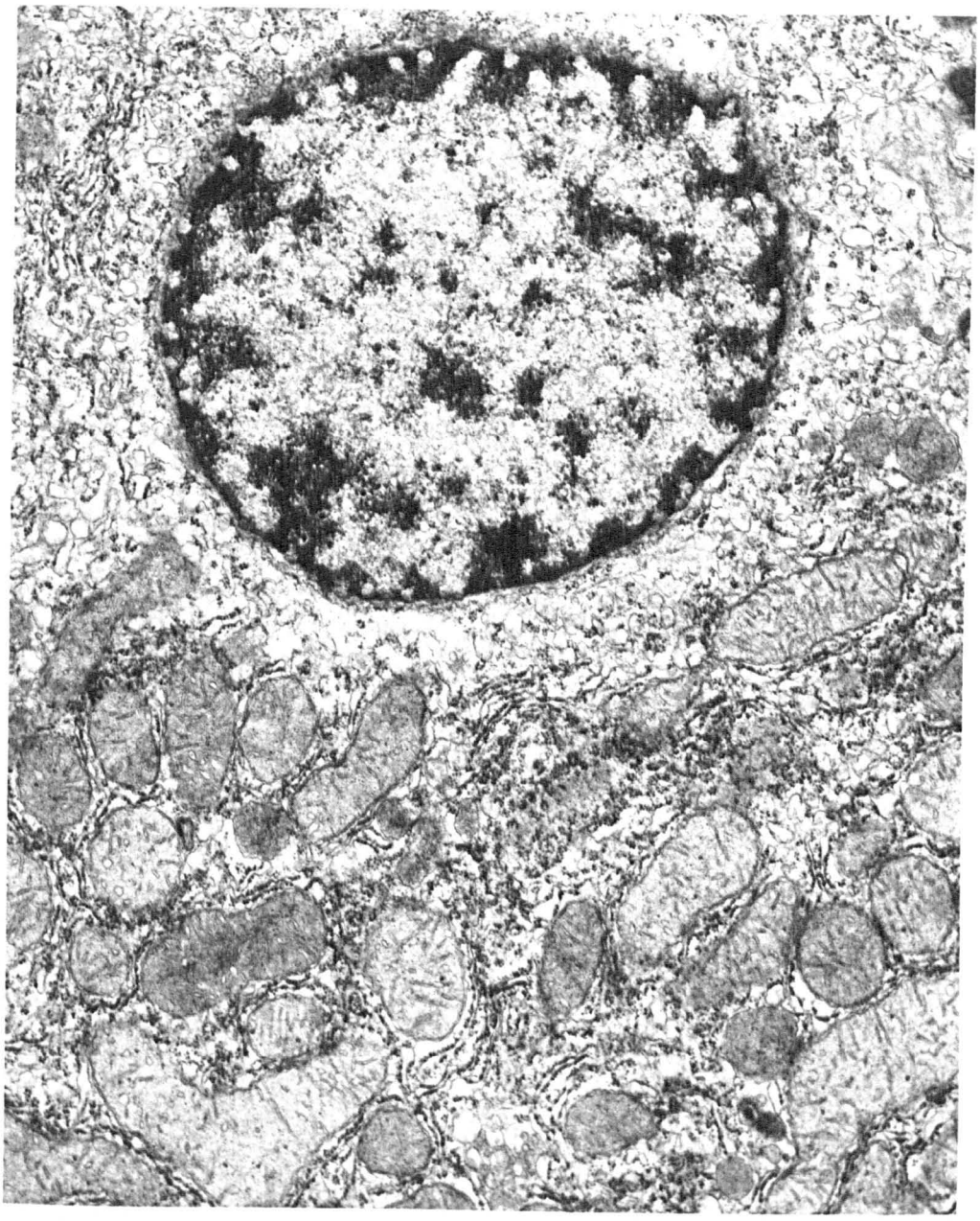


Figure 2.11. Week 2. Copper-loaded rat liver. Hepatocyte nucleus with margination of nuclear chromatin. x 20,000.

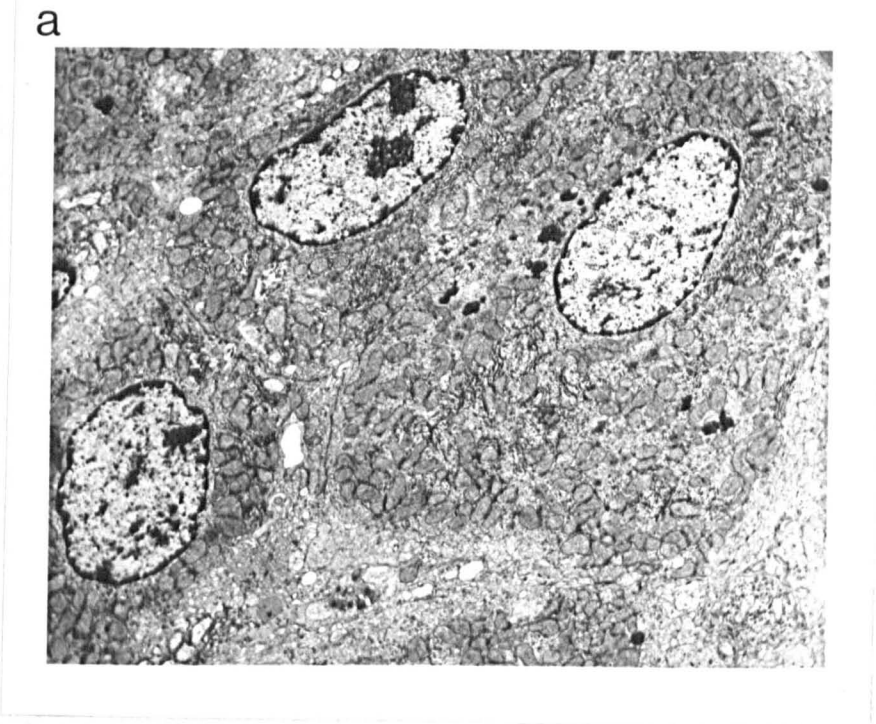


Figure 2.12a. Week 3. Copper-loaded rat liver. Condensation of chromatin and crenation of nuclear membranes. x 4,200.

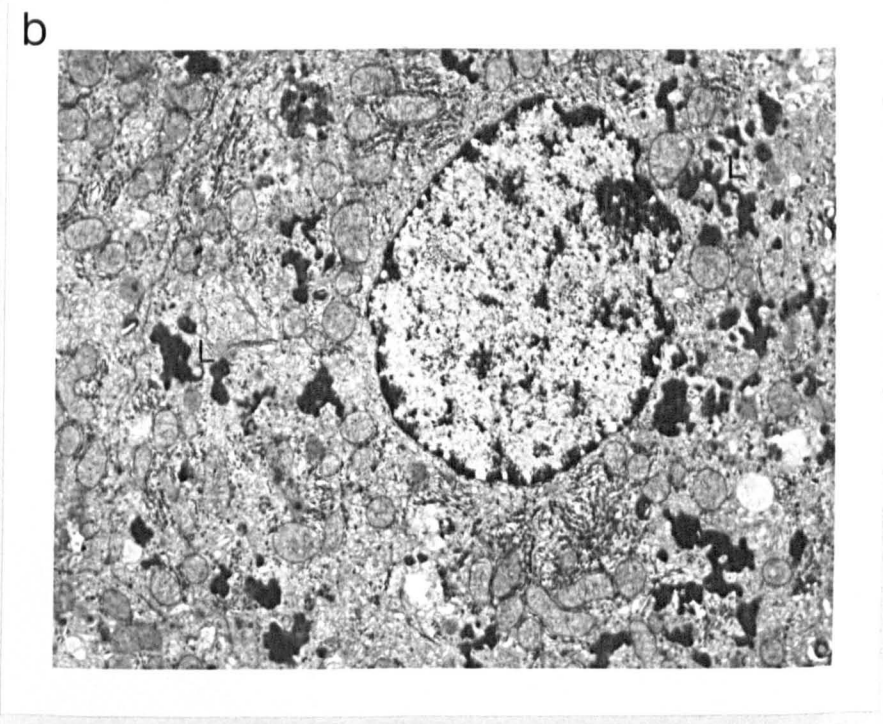


Figure 2.12b. Week 3. Copper-loaded rat liver. Crenated nuclear membranes and dispersed distribution of lysosomes (L). Also note swollen mitochondria. x 7,200.

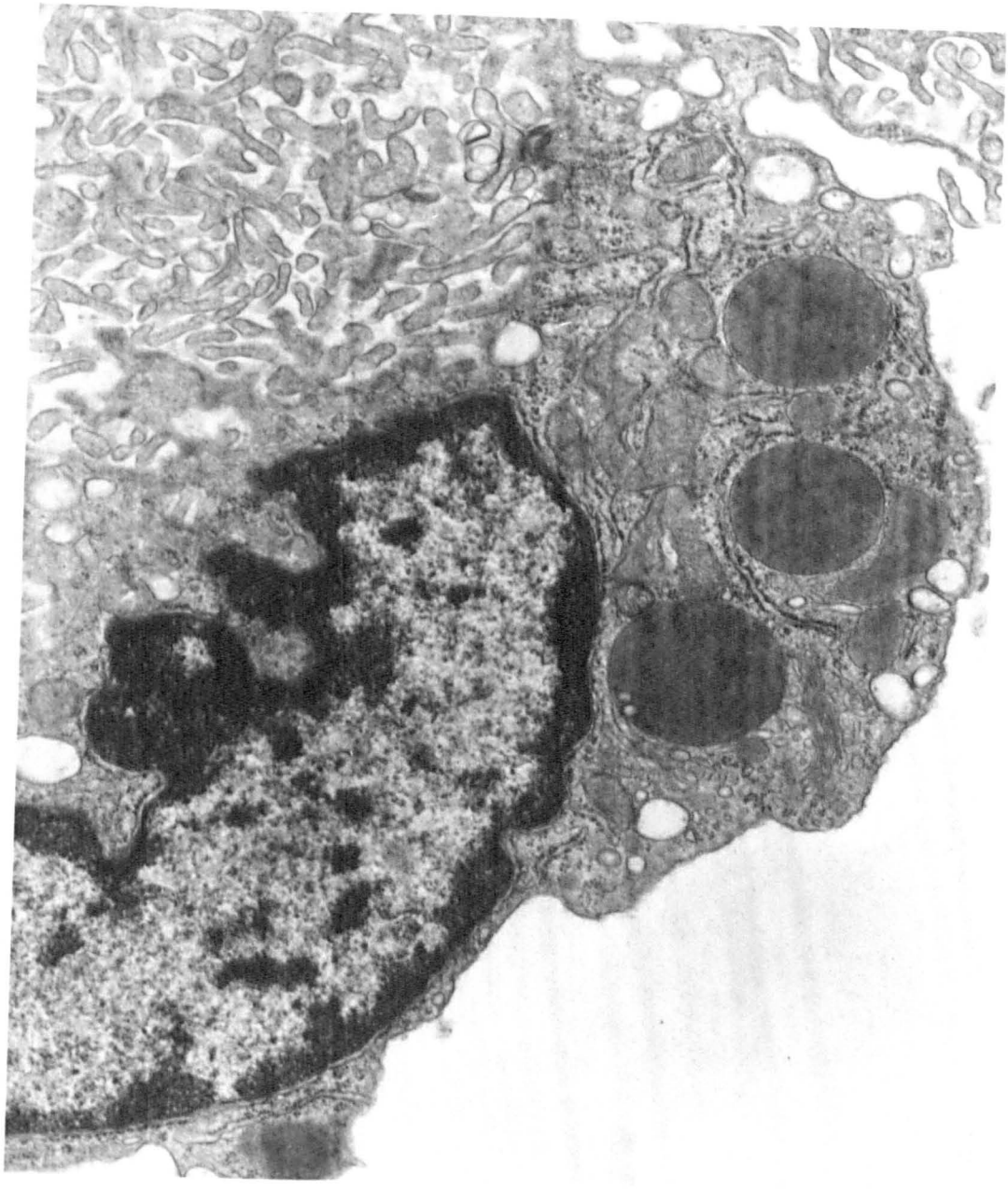


Figure 2.13. Week 3. Copper-loaded rat liver. Presence of electron-dense lysosomes in a Kupffer cell. x 30,000.

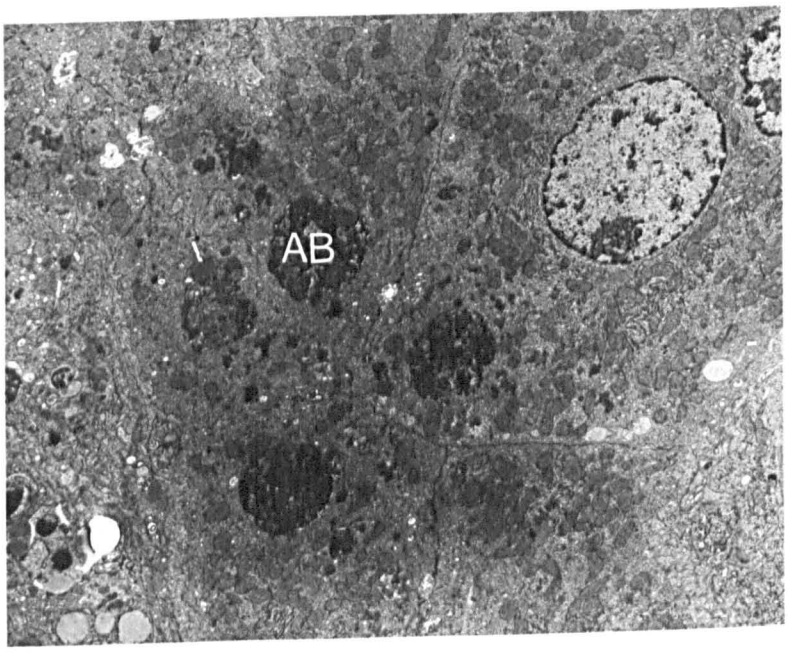


Figure 2.14a. Week 3. Copper-loaded rat liver. Ovoid masses of condensed cytoplasm (apoptotic bodies) (AB) are present in the cytoplasm of hepatic parenchymal cells. x 3,600.

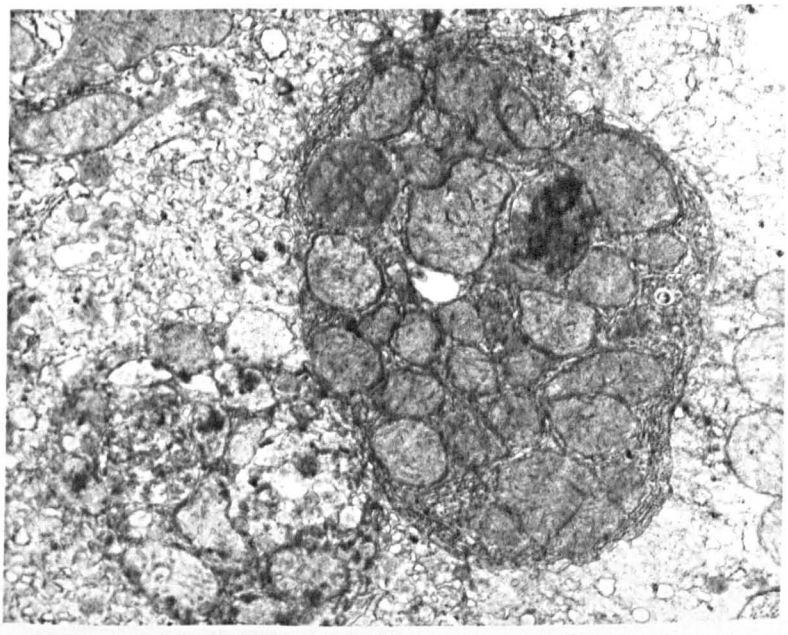


Figure 2.14b. Week 3. Copper-loaded rat liver. Higher magnification of apoptotic bodies demonstrate the inclusion of well-preserved organelles. x 14,400.

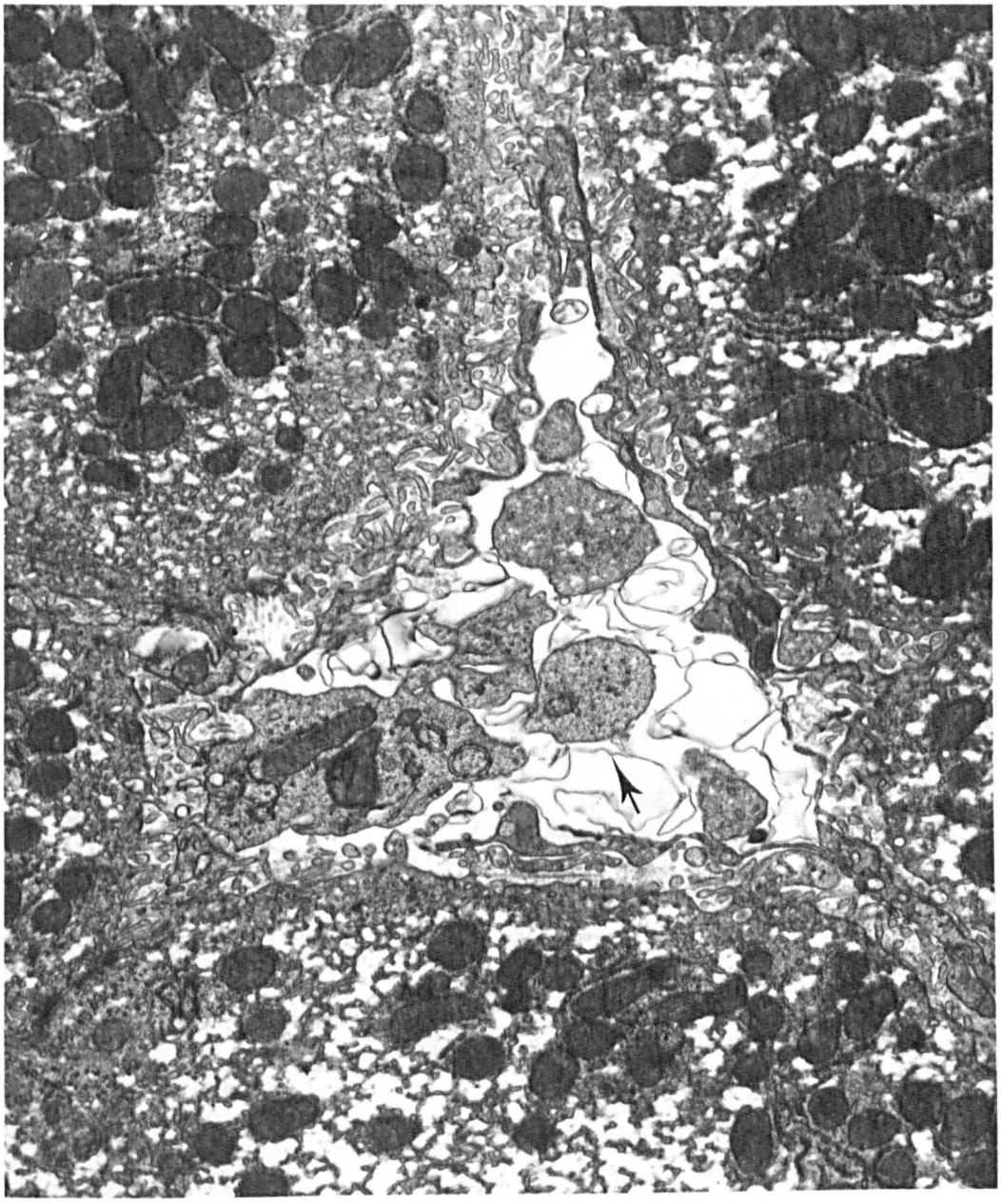


Figure 2.15. Week 3. Copper-loaded rat liver. Cellular debris within sinusoids (→). x 12,000.

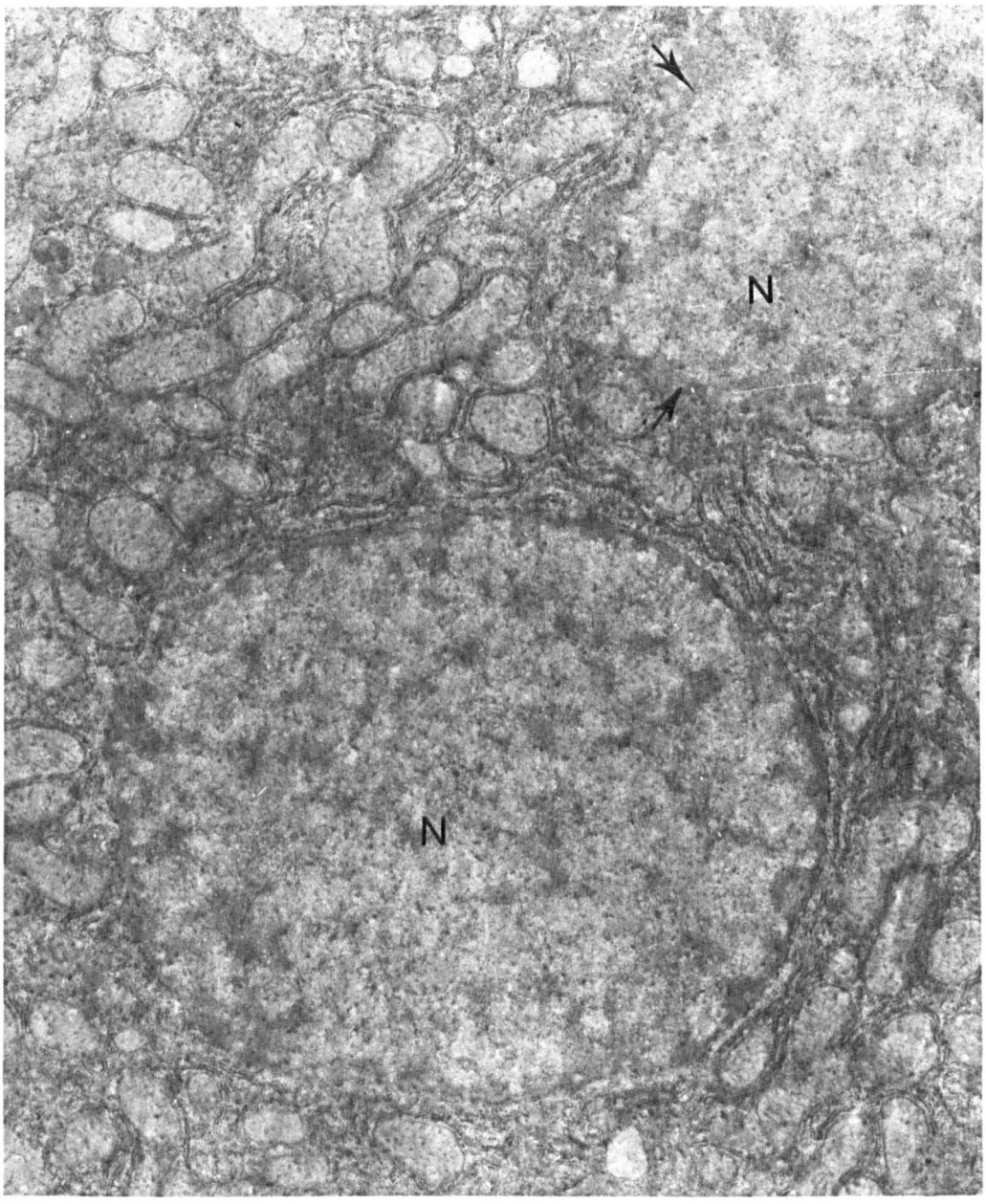


Figure 2.16. Week 4. Copper-loaded rat liver.
Karyolysis of hepatic cell nuclei (N) and loss of distinct nuclear membrane (►). x16,000.

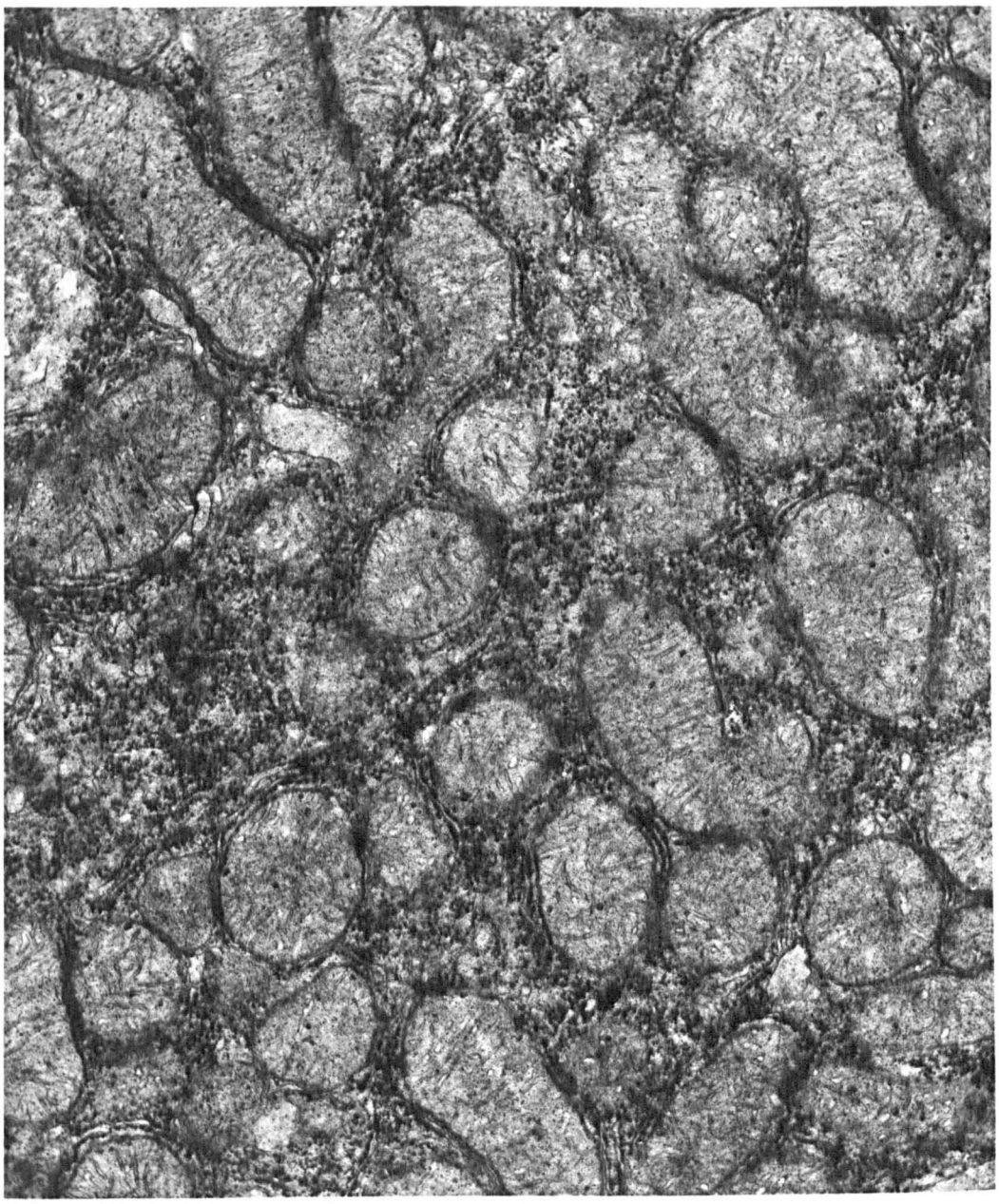


Figure 2.17. Week 4. Copper-loaded rat liver. Liver from a rat copper-loaded during 4 weeks. Fragmentation of RER and disaggregation of ribosomes. x 30,000.

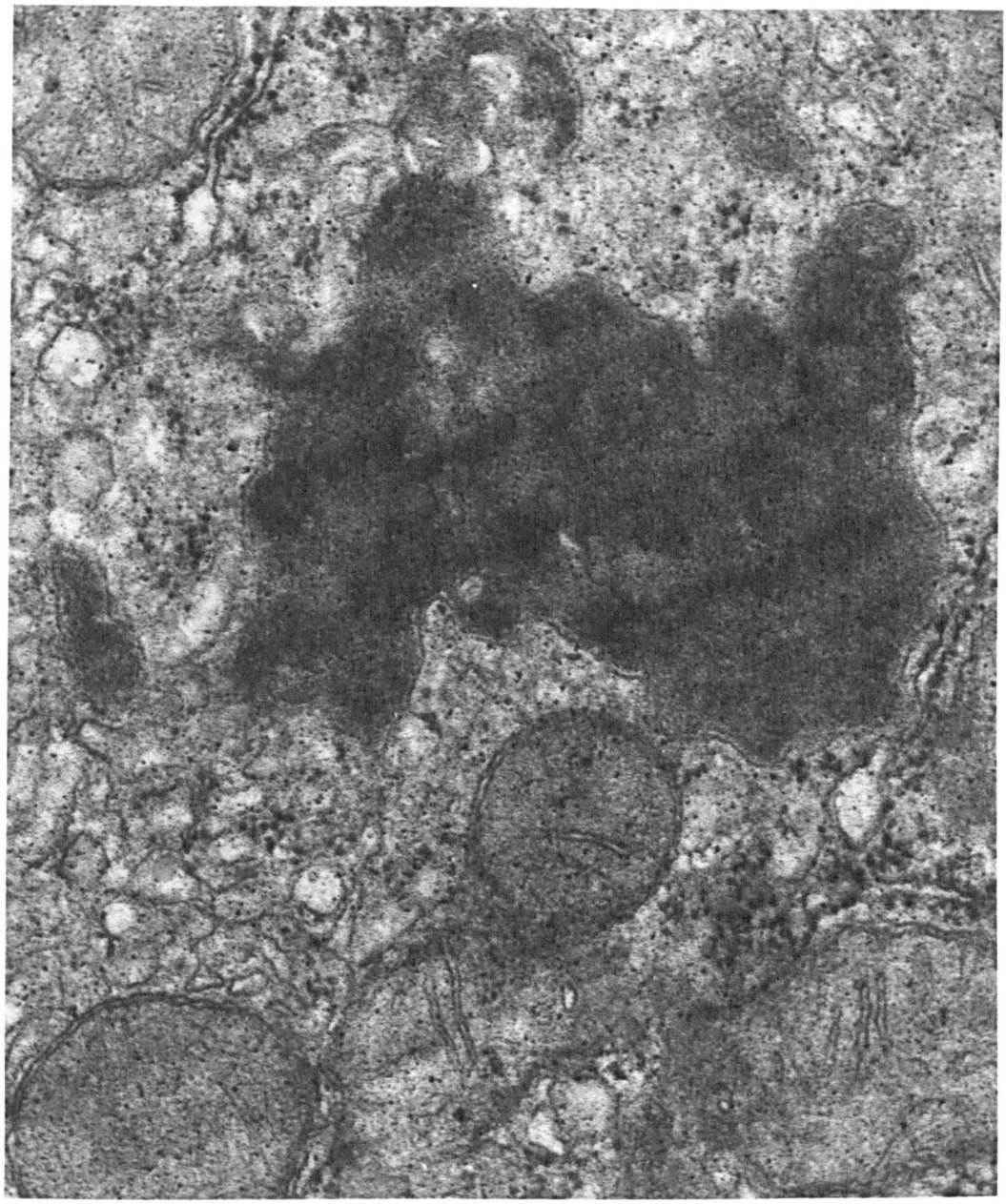


Figure 2.18. Week 4. Copper-loaded rat liver. Large irregularly-shaped lysosome; note the intact limiting membrane. x 75,000.

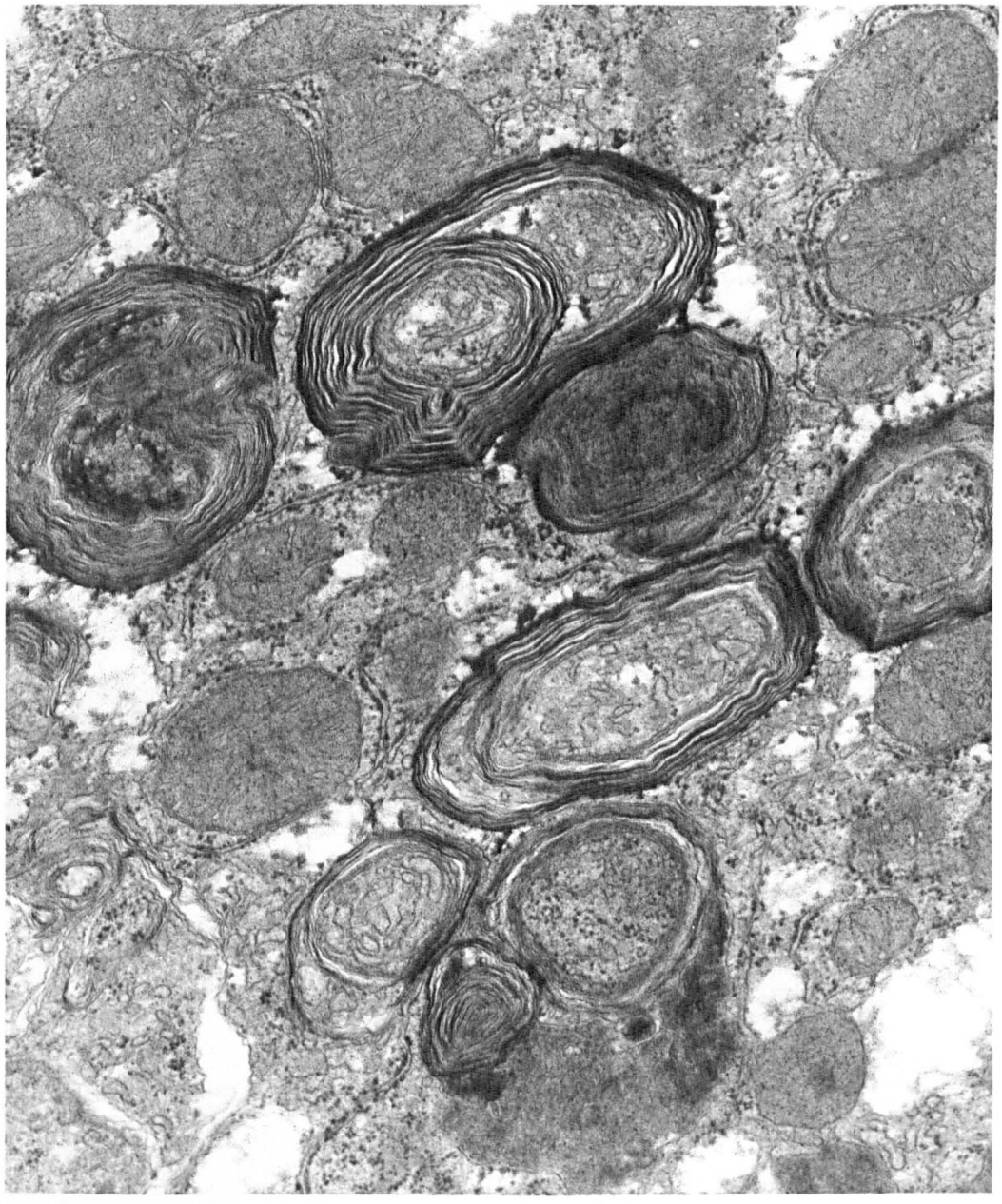


Figure 2.19. Week 5. Copper-loaded rat liver. Osmiophilic concentric laminar membranes surrounding cytoplasmic organelles. x 34,000.

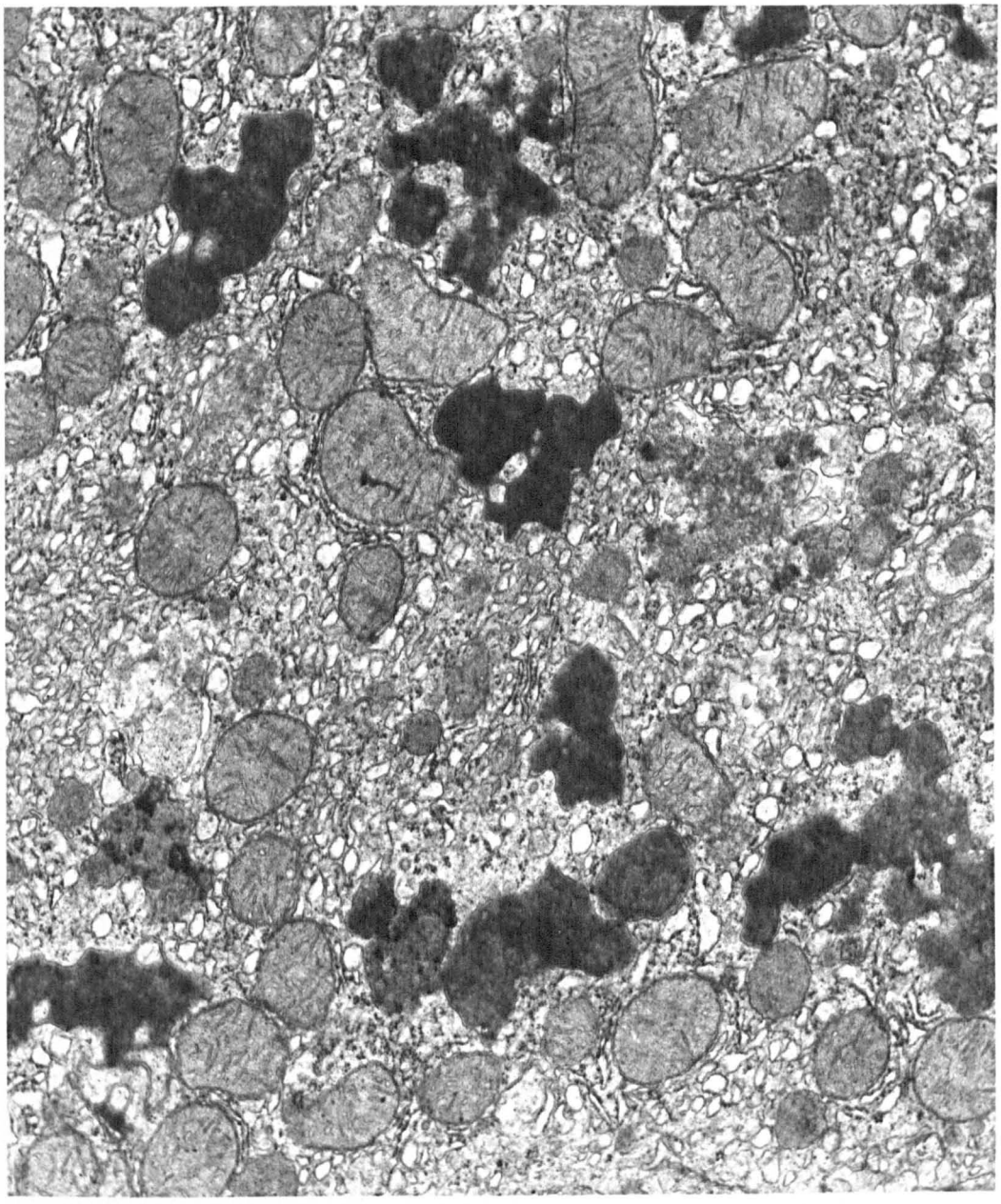


Figure 2.20. Week 5. Copper-loaded rat liver. Irregularly shaped lysosomes with variable electron density, bounded by a distinct membranes. Note also swollen mitochondria and vacuolation of SER. x 20,000.

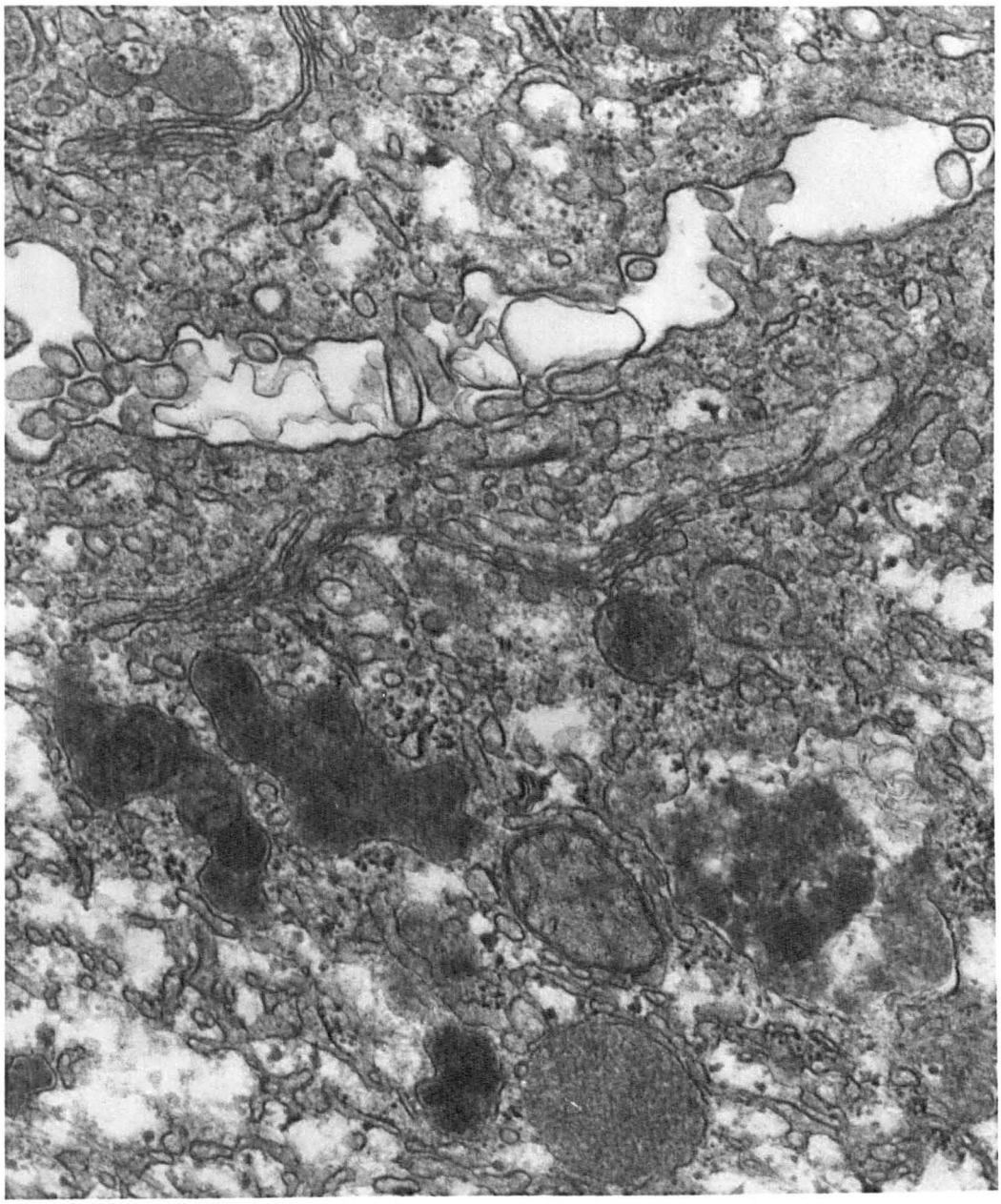


Figure 2.21. Week 6. Copper-loaded rat liver. Vesiculation of bile canaliculi. x 40,000.

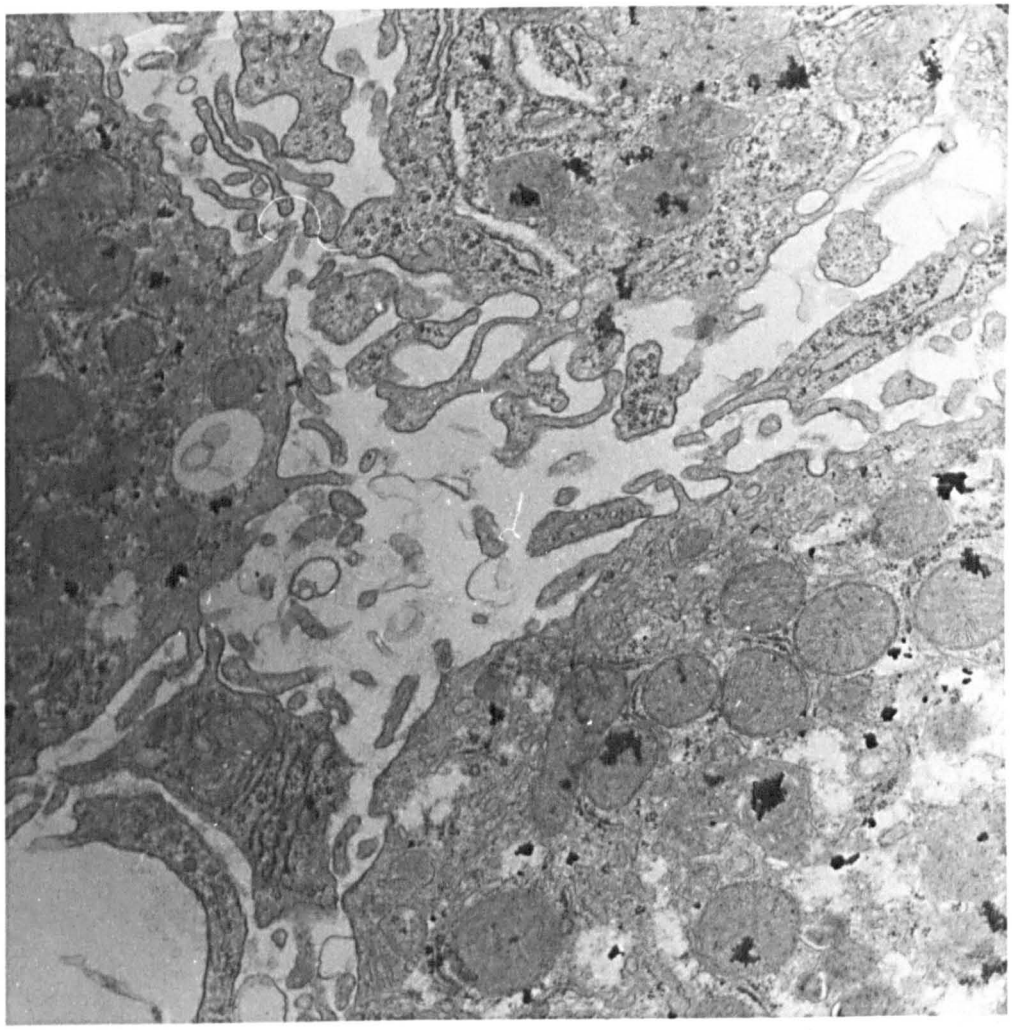
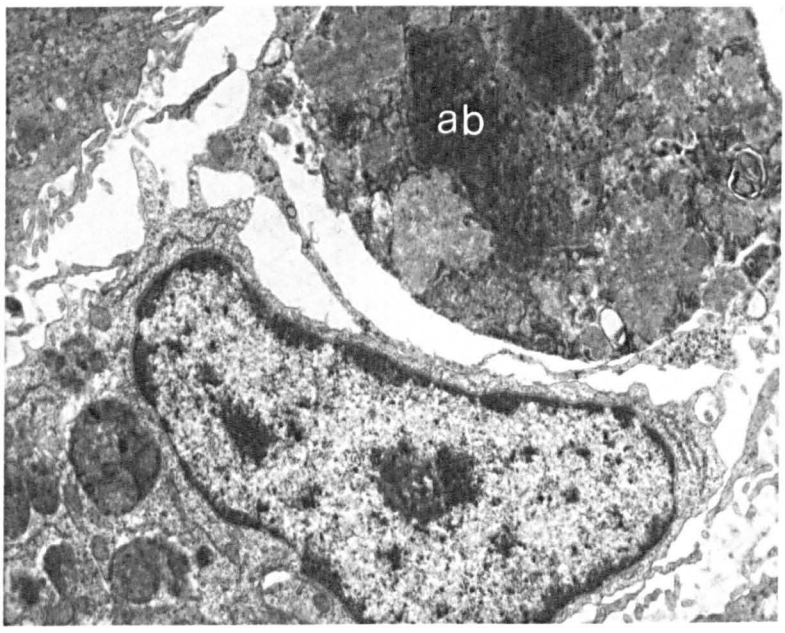
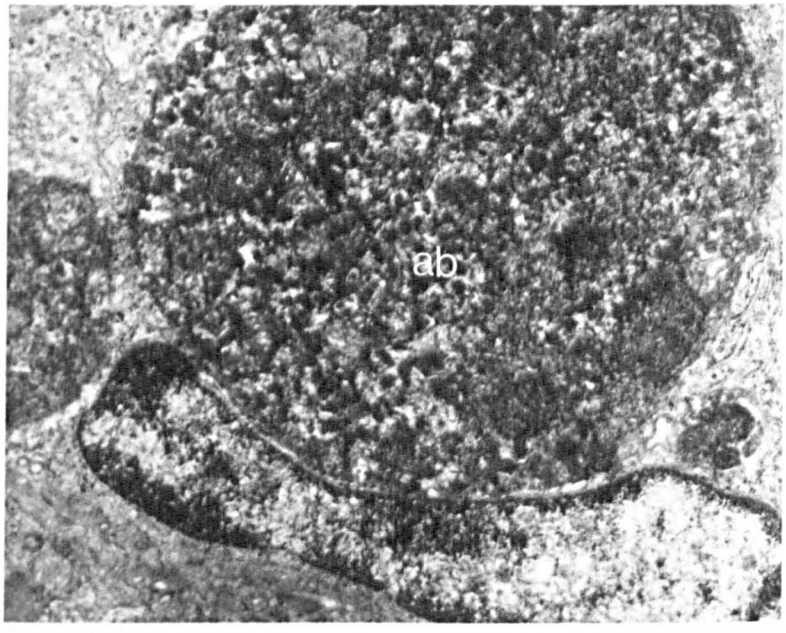


Figure 2.22. Week 6. Copper-loaded rat liver. Dilatation of space of Disse. x 15,000.



A



B

Figure 2.23. Week 6. Copper-loaded rat liver. Presence of Apoptotic bodies (ab) in A) the sinusoids (x 9,600) and (B) Kupffer cells (x 14,400).

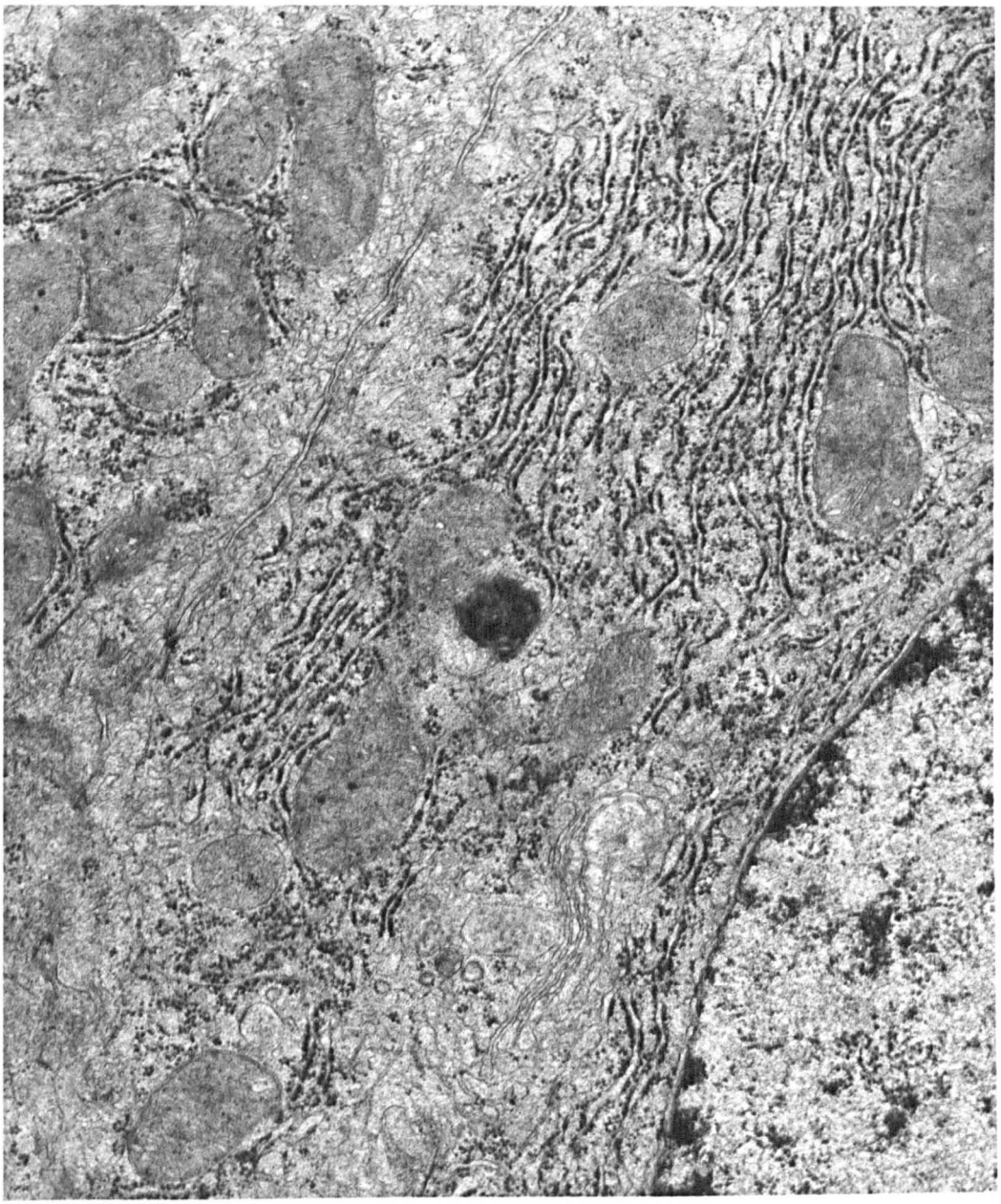


Figure 2.24. Week 8. Copper-loaded rat liver. Hypertrophy of parallel arrays of the RER. x 30,000.

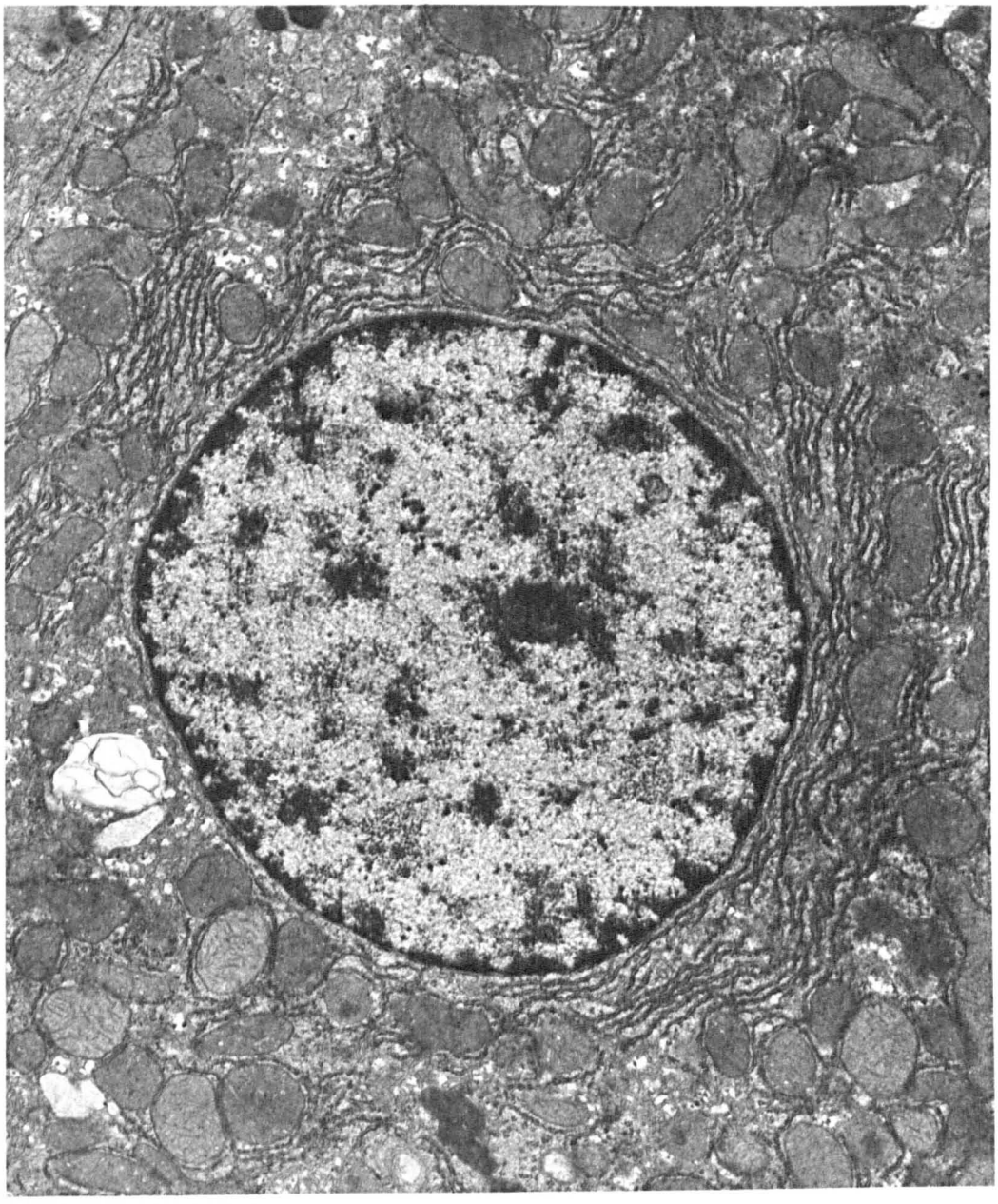


Figure 2.25. Week 16. Copper loaded rat. Recovered hepatocyte. x 16,000.

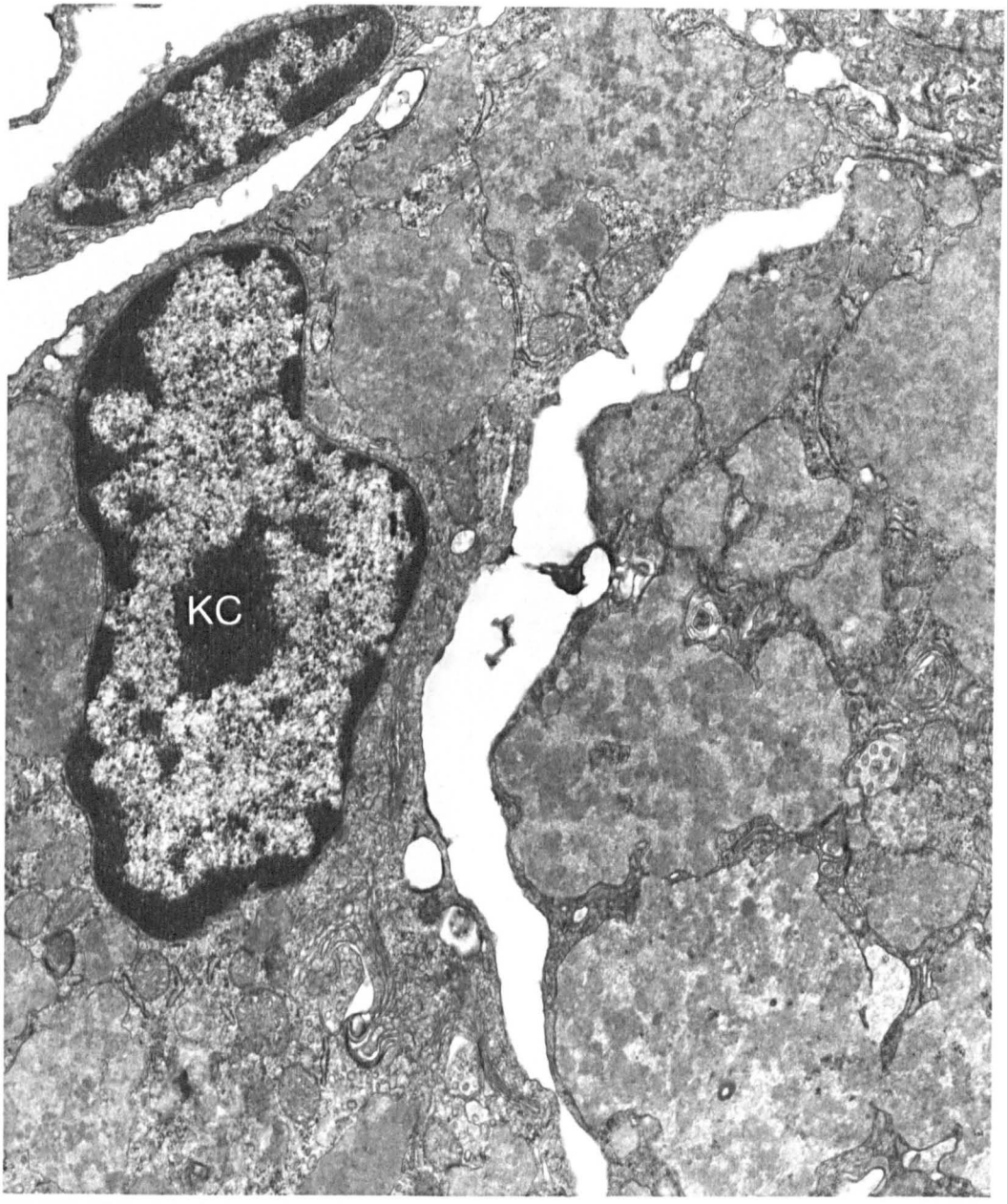


Figure 2.26. Week 16. Copper-loaded rat liver. Abundant floccular material in the cytoplasm of hepatocytes and Kupffer cells (KC). x 16,000.

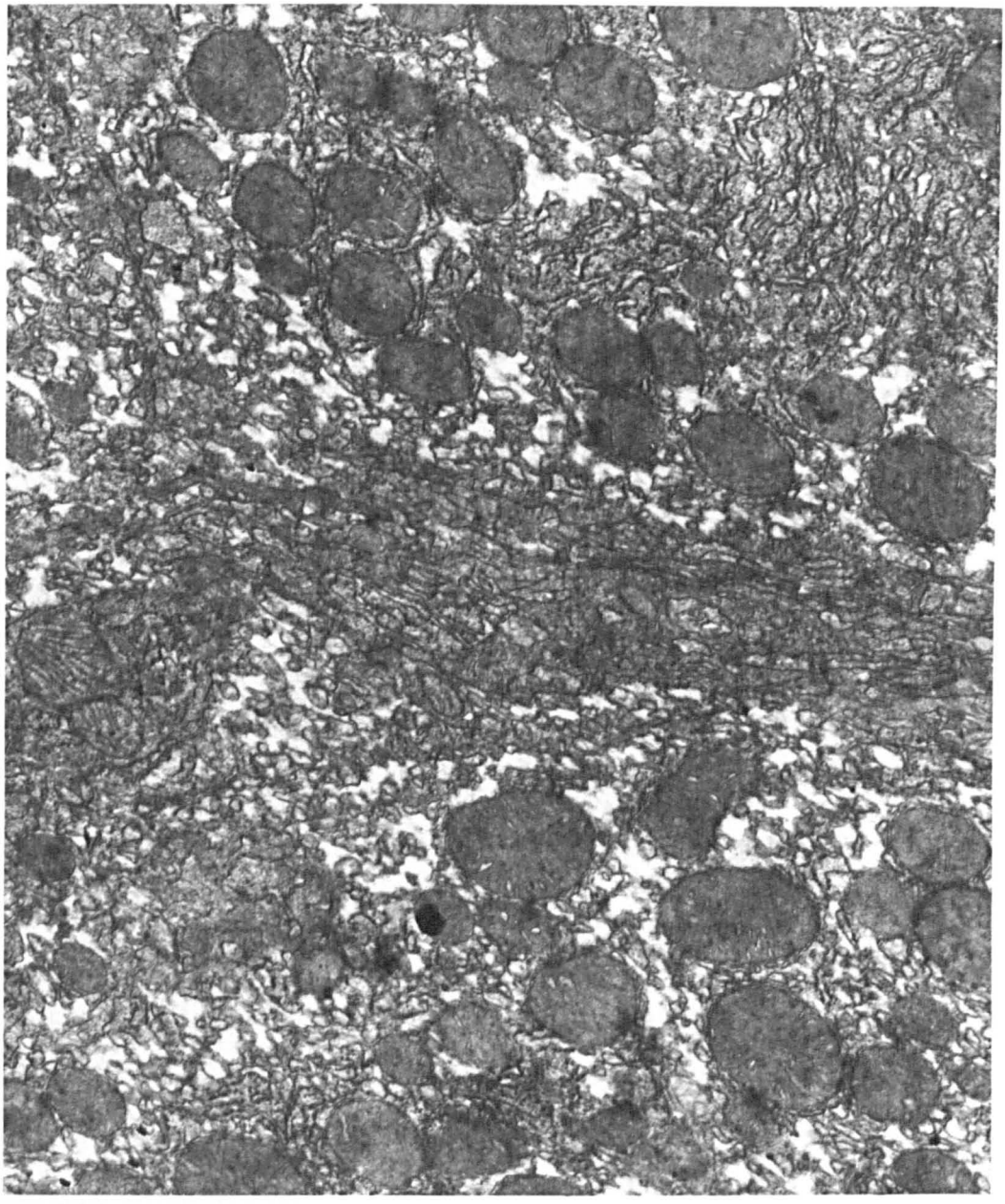


Figure 2.27. Week 16. Copper-loaded rat liver. Residual swelling and electron lucency of the cytosol. x 20,000.

4.-DISCUSSION.

In this study copper accumulated rapidly in the liver for the first four weeks, and then had fallen to lower concentrations at 16 weeks as previously described in chapter 1. This was characteristic of the adaptation that takes place in the rat.

Ultrastructural examination showed an increasing cellular dissarray up to week 4, culminating in nuclear lysis; this was followed by regeneration of surviving cells as adaptation took place.

Variations in parenchymal cell electron density, identified here as primarily due to hypertrophy and dilatation of the smooth endoplasmic reticulum (SER), have been described previously in copper intoxication in the rat (Barka et al., 1964), copper toxicosis in the sheep (King and Bremner, 1979) and in abnormal copper storage in Teleost fish (Bunton et al., 1987). This is apparently a non-specific change, since an increase in the number of light cells in the liver has been commonly observed following a variety of insults such as: -restricted food intake (Herdson et al., 1964), liver circulatory arrest (Hubner and Bernhard, 1961), common bile duct ligation (Steiner et al., 1962), portal vein occlusion in the dog (Gansler et al., 1962) and following the administration of ethionine (Herman et al., 1962) and carbon tetrachloride (Smuckler et al., 1962).

Many toxic compounds have been found to induce the proliferation of SER in parenchymal cells (Shinozuka, 1971;

Desnoyers and Chang, 1975), including cadmium intoxication (Stowe et al., 1972; Hoffmann et al., 1975) and acute lead intoxication (Hoffmann et al., 1972; 1974). Although the mechanism remains to be established (Miyai, 1979), SER hypertrophy is believed to represent a detoxification response of the liver (Desnoyers and Chang, 1975); enzymes of the SER are thought to be responsible for the detoxification of endogenous and exogenous lipid soluble compounds (Trump et al., 1978). Hypertrophy of the smooth endoplasmic reticulum is regarded as an adaptation (tolerance) by which an animal develops an enhanced ability to handle potentially lethal doses of drugs (Ghadially, 1975). Alternatively hypertrophy of SER may be indicative of cell injury, since dilatation and vesiculation of SER cisternae with loss of intramitochondrial dense granules and mitochondrial swelling are among the main features of cloudy swelling and hydropic degeneration associated with increased water flux into cells.

Mitochondrial changes similar to those described here in the parenchymal cells have been described in a variety of conditions such as hypoxia (Trump et al., 1965), ischaemia (Buffa et al., 1970), Reye's syndrome (Partin et al., 1971), Wilson's disease (Sternlieb, 1968); and copper (Verity et al., 1967; Gooneratne et al., 1980), lead (Hoffmann et al., 1972), and cadmium intoxication (Hoffmann et al., 1972)

All these changes described previously were observed in this study during the early stages of copper overloading, and were accompanied by fragmentation and degranulation of the rough

endoplasmic reticulum (RER), a common change observed in the livers of experimental animals subjected to various noxious influences (Miyai, 1979), including copper (Barka et al., 1964; King and Bremner, 1979 and cadmium (Stowe et al., 1972; Hoffmann et al., 1975).

Starvation causes disarray of the parallel arrangement of cisternae of the RER which is restored upon refeeding (Smuckler and Arcasoy, 1969). Varying degrees of disruption of RER have been reported in rat liver after administration of carbon tetrachloride (Smuckler et al., 1962; Reynolds, 1963b), phosphorus (Jezequel, 1958), cysteine (Emmelot et al., 1962), also following partial hepatectomy, portocaval shunt (Fisher and Fisher, 1963), in dietary necrotic liver degeneration (Svoboda and Higgins, 1963), and occlusion of liver circulation (Hubner and Bernhard, 1961). Additionally, degranulation of SER has been observed after ligation of the common bile duct (Steiner et al., 1962), in human liver in biliary atresia (Steiner and Carruthers, 1961) and in hepatocytes in Wilson's disease (Schaffner et al., 1962). Disruption of RER is considered a useful morphological indicator of depressed or arrested protein synthesis (Smuckler et al., 1962; Hoffmann et al., 1975).

Osmiophilic membranes in the liver cell cytoplasm have been observed after administration of hexachlorophene in sheep (Reid and Hall, 1975), carbon tetrachloride (Shinozuka, 1971) and in the liver of rats treated with acetyl salicylic acid or malonic acid (Reide, 1973). Membranous whorl formation has previously been

described in copper intoxication in the rat (Barka et al., 1964), and during the pre-haemolytic phase in sheep (Gooneratne et al., 1980) and presumably reflects membrane destabilisation in cell injury. The formation of concentric whorls has been interpreted as an adaptative phenomenon in view of their appearance during recovery from injuries (Steiner et al., 1964) and in regenerative nodules in cirrhotic livers (Stenger, 1966). Hwang et al., (1974) found that the formation of membrane whorls is associated with an imbalance in protein: phospholipid synthesis in favour of the latter.

In the present study changes in the canalicular microvilli were detected as early as the first week of copper-overload and persisted until week 6. This change was also observed in copper intoxication in rats (Barka et al., 1964), in the pre-haemolytic and haemolytic phase in chronic copper-poisoned sheep (King and Bremner, 1979; Gooneratne et al., 1980) and in rat liver following repeated vespine envenomation, in which it was interpreted as evidence of increased bile secretion (Barr-Nea et al., 1985). However, dilatation of bile canaliculi and absence of microvilli have been also observed in intrahepatic cholestasis caused by cytochalasin B in the rat (Phillips et al. 1975). Bleb formation and dilatation of bile canaliculi has also been observed in benign recurrent cholestasis in man (Raymond et al., 1987).

Kerr et al. (1972) introduced the term apoptosis to describe a mechanism of controlled cell deletion involving the formation of cytoplasmic fragments or apoptotic bodies and the

phagocytosis and degradation of these bodies by other cells. Apoptotic bodies containing portions of well preserved cytoplasm and also degraded and necrotic portions of cytoplasmic material confined within the cell by a limiting membrane have been observed in the liver after exposure to methylmercury (Desnoyers and Chang, 1975) and in other systems after various toxic conditions (Chang and Hartmann, 1972; Chang and Yamaguchi, 1974). It has been demonstrated that lysosomal enzymes are important in the degradation of phagocytosed bodies and the latter are rapidly reduced to electron-dense lysosomal residual bodies (Kerr et al., 1972).

Eosinophilic hyaline cytoplasmic bodies (Mallory bodies) occur in a variety of chronic liver disorders in man including copper associated conditions such as Primary biliary cirrhosis (Monroe et al., 1973), Indian childhood cirrhosis (Nayak and Roy, 1976), and Wilson's disease (Levi et al., 1967; Sternlieb, 1972). They have also been observed in disorders unrelated to copper such as alcoholic (Flax and Tinsdale, 1964) and non-alcoholic cirrhosis (Gerber et al., 1973), infantile cirrhosis (Smetana et al., 1961), post hepatic cirrhosis (Baggenstone and Stanffer, 1952), hepatocellular carcinoma (Norkin and Campagne-Pinto, 1967) and amyloidosis (Thiery and Caroli, 1962). Mallory bodies have also been described in the lung in alveolar epithelial cells associated with asbestosis (Kuhn and Kuo, 1973), radiation pneumonitis, diffuse interstitial fibrosis, and organizing bacterial pneumonia (Warnock et al., 1980), as well as in scar adenocarcinoma of the

lung (Michel et al., 1982).

There have been various interpretations about the nature of these non-membrane bound hyaline bodies (Biava 1964; Smuckler 1968; Yokoo et al., 1972). They have been thought to be megamitochondria and their degenerate remains (Porta et al.,1965), alternatively areas of focal cytoplasmic degeneration involving organelles other than mitochondria (Flax and Tinsdale, 1964), or compacted masses of non-membrane bound cytoplasmic filaments (Ma, 1972). This hypothesis has gained further support (French, 1981a, 1981b; Denk et al., 1981) and it is now accepted that some types of Mallory bodies represent a pathologic expression of intermediate filaments (Lane and Anderton, 1982). The liver cell, like all other cell types, has a cytoskeleton composed chiefly of actin microfilaments located at the cell borders, (French and Davies,1975) and intermediate filaments and microtubules located throughout the cytoplasm (Irie et al., 1982; French et al., 1982).

According to Yokoo et al. (1972) and Nayak and Roy (1976) three morphological variants of Mallory bodies may be recognized. The first type consists of bundles of fibrils in parallel arrays which measure 9-12 nm. The second type is composed of a meshwork of randomly orientated fibrils which measure 11.5- 20.0 nm and the third of a granular or almost amorphous substance containing only scattered remains of fibrils.

The cytoplasmic inclusions observed in the present study are similar in some aspects to the Type III-Mallory body, however due to the absence of fibrils and the presence of membrane they

were denominated Mallory body-like structures.

The status and diversity of the lysosomes is very interesting and will be discussed further in chapter 4. The prominence of the lysosomes at any one time reflects the overall copper content of the liver, supporting earlier findings that lysosomes accumulate the metal (Haywood et al., 1985a). The presence of copper in lysosomes has been further confirmed by x-ray emission analysis (Chapter 4). However, contrary to the situation in sheep (Gooneratne et al., 1980) these were the only organelles in the copper-loaded rat liver parenchymal cells from which degenerative changes, as evidenced by membrane disapearance or rupture, were conspicuously absent. This supports the view that lysosomes help to protect the cell interior from the cytotoxic effects of copper (Sternlieb, 1980; Helman et al., 1985).

In contrast to the preceeding observations severe and irreversible changes occurred only in parenchymal cell nuclei and abnormalities of this organelle were seen as early as 2 weeks of copper-overload.

Margination of chromatin is an early nuclear change in irreversible injury leading to cell death (Ghadially, 1975). In the present study chromatin condensation was followed by karyolysis, and the latter appearance coincided with the highest liver copper concentration.

Nuclear changes have been described in the pre-haemolytic phase of chronic copper toxicosis in sheep prior to the rupture of lysosomal membranes (King and Bremner, 1979; Gooneratne et al.,

1980; Kumaratilake, 1984), but their significance is unclear. It is not unusual to overlook the ultrastructural changes in the nucleus of injured cells (Ghadially, 1975). Early studies in ultrastructure concentrated on the cytoplasmic structures rather than the nucleus which lead to the comment that "in electron microscopical cytology the interphase nucleus of the somatic cell has been the neglected orphan compared with cytoplasmic organelles" (Bernhard and Granboulan, 1963). In copper toxicosis the main attention has been given to the presence and role of electron dense lysosomes (Schaffner et al., 1962; Goldfischer, 1963; Mc Nary, 1963; Barka et al., 1964; Goldfischer and Sternlieb, 1968; Lindquist, 1967 ;1968; Gooneratne et al., 1980).

In the present study nuclear changes were detected at an early stage and in contrast with other reports in the sheep (Gooneratne et al., 1980) lysosomal membranes were at all times intact. This observation supports the hypothesis of nuclear destabilisation being the primary event in copper-induced cell damage (Haywood et al., 1985a) .

It is now possible to construct a probable sequence of events in the liver of the copper-loaded rat. Copper accumulates and is sequestered initially within lysosomes with only mild perturbation of cytoplasmic organelles associated with the detoxification/complexing and excretion of copper. Excess copper does not appear to damage lysosomes but is responsible for irreversible nuclear damage leading to lysis. The anucleate cytoplasmic remains (apoptotic bodies) are deleted from the liver

mainly into the sinusoids. Subsequently adaptation occurs; this is associated with regeneration, falling hepatic copper levels and restoration of cellular equilibrium.

It is reasonable to suppose that the ability to recover and adapt is absent or deficient in copper-sensitive species and studies on this aspect of cellular homeostasis must be regarded as equally contributory to the understanding of the copper toxicoses as the primary damage.

CHAPTER III

CELLULAR MECHANISMS OF TOXICITY AND TOLERANCE IN THE COPPER-LOADED RAT. III ULTRASTRUCTURAL CHANGES IN THE KIDNEY CORTEX.

1.- INTRODUCTION.

The renal tubules and interstitium are particularly susceptible to injury by a variety of exogenous and endogenous toxins. This susceptibility is determined in large part by normal physiological functions of the kidney, such as abundant blood flow and the high local concentration of various substances, including toxins. The most important exogenous toxins causing tubulo-interstitial disease are drugs and heavy metals. (Wedeen and Batuman, 1982.

The kidney is composed of many different types of cells with varying sensitivities to toxic metals. Proximal tubule cells, owing in part to their reabsorptive functions, are highly sensitive to most nephrotoxic metals or organometallic complexes (Fowler, 1983). There is marked structural, biochemical, histochemical, and functional heterogeneity within the proximal tubule of the kidney (Maunsbach, 1973; Jacobsen and Jorgensen, 1973a). Three sequential segments (S_1, S_2, S_3) occur in the rat proximal tubule, each characterised by a unique cell type. Proximal convoluted tubules (PCT) include the first segment (S_1) and most of the second segment (S_2). A sharp transition to the third segment (S_3) takes place in the upper portions of the proximal straight tubules (PST) (Jacobsen and Jorgensen, 1973b).

Differences have been observed between PCT and PST in their susceptibility to injury (Venkatachalam et al, 1978). Acute

mercuric chloride administration has long been known to preferentially damage the third segment (Gritzka and Trump, 1968; Ganote et al, 1975), whereas acute administration of the cadmium metallothionein complex (CdMT) has been shown to produce toxicity in the first and at higher doses, second segments of the proximal nephron (Nordberg et al, 1975; Cherian et al, 1976; Fowler and Nordberg, 1978; Squibb et al, 1979; Squibb et al, 1982). Administration of other agents such as methyl mercury may exert toxicity in all segments of the proximal tubule depending on dose and experimental protocol (Fowler, 1972a; Fowler et al, 1974; Fowler and Woods, 1977; Carmichael and Fowler, 1979).

Copper as a cause of renal injury was first reported by Wolff (1964) who indicated that copper toxicosis in Wilson's disease is associated with degeneration and necrosis of the PCT epithelium. Since then, although the kidney has not been of primary concern in most investigations, several authors have also reported damage of this organ, in sheep (Ishmael et al, 1971; Gopinath et al, 1974; Howell et al., 1984) and in experimental copper toxicosis in the rat (Wolff, 1960; Haywood et al, 1985b). Ultrastructural studies in chronic copper toxicosis in sheep have confirmed that the renal changes were largely confined to the PCT and consisted of a variable degree of degeneration and necrosis of tubule cells (Gooneratne and Howell, 1983; Gooneratne et al, 1986).

The pathogenesis of copper-induced damage in the kidney remains obscure. Ultrastructural and microanalytical techniques

have demonstrated the presence of copper in lysosomes within the PCT epithelium (Jones et al, 1984). It has been proposed that the rupture of such lysosomes is responsible for the cytotoxicity of copper (Gooneratne et al, 1980); however, kidney fractionation studies have shown that a temporary nuclear accumulation of copper coincides with tubule cell necrosis strongly suggesting that cell death occurs primarily as a result of nuclear disorganization rather than lysosomal damage (Haywood et al, 1985a).

The aim of the present study is:

a) to describe the ultrastructural changes in the PCT of the kidneys of rats during 16 weeks of copper-supplementation.

b) to relate the morphological alterations to the renal copper content.

2.- MATERIALS AND METHODS.

The animals, their feeding and management are described in detail in Chapter 2. Copper-loaded and control rats were killed at the predetermined intervals and their kidneys removed.

METHODS

a.-Copper analysis

Triplicate samples of approximately 0.3 g of kidney cortex were removed with plastic knives. Copper concentrations were determined by atomic absorption spectrophotometry as described in Chapter 1.

Copper concentrations are expressed as the mean and the standard error of the mean (M±SEM), in ug/g of tissue on a dry weight basis.

b.-Transmission Electron Microscopy.

Fixation

Small pieces of kidney cortex were immersed immediately after removal in chilled 4 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M phosphate buffer (pH7.4), (Karnovsky, 1965) (Appendix 11), cut into small cubes (approximately 1.0 mm^3) and kept in this fixative overnight at 4 ^{o}C. The tissues were then

processed for transmision electron microscopy as described in Chapter 2.

c.- **Photography.**

EM sections were photographed using Ilford EM film (Ilford Ltd., Mobberley Cheshire, U.K.) and printed on Ilfospeed Multigrade II photographic paper.

3.- RESULTS.

Renal copper concentrations are recorded in Table 3.1. There was an initial rapid accumulation of the metal up to 4 weeks when maximum concentrations (1,142 ± 88 µg/g) were achieved. This was followed by falling levels so that by 16 weeks kidney copper concentrations (500 ± 30 µg/g) were significantly less ($p < 0.05$) than at 4 weeks. As with the liver, when kidney copper concentrations are compared with experiment 1 (Fig.3.1) copper accumulation occurred more rapidly, associated with an earlier increased food intake (Appendix 10).

The fine structure of the kidney cortex (Fig.3.2) in the control groups conformed in general to previous descriptions of normal male rat kidney cortex (Rhodin,1974).

Copper supplemented groups.

Week 1: Kidney copper concentration was 56 ± 2 µg/g.

The proximal convoluted cells tubules contained large single-membrane bound electron-dense bodies identified as lysosomes (Fig.3.3). Electron-lucent irregularly shaped vacuoles (pinocytotic vesicles) were present in the apical regions. Brush border microvilli were unremarkable (Fig.3.4)

Week 2: Kidney copper concentration was 306 ± 21 µg/g.

There was vacuolation of the smooth endoplasmic reticulum (SER) and mitochondrial swelling. There was an increase in the

number of lysosomes dispersed throughout the PCT cytoplasm. Pinocytotic vesicles were prominent. In some epithelial cells there was condensation of nuclear chromatin (Fig.3.5).

Week 3: Kidney copper concentration was 458 ± 29 µg/g.

The nuclear changes were more pronounced: condensation of chromatin was found in the nuclei of most PCT epithelial cells and many nuclei were misshapen with crenated nuclear membranes (Fig. 3.6). There was dissaggregation of ribosomes and swelling of SER, the mitochondria appeared swollen, some contained flocculent small electron-dense areas (see arrow fig.3.6). In addition to large membrane-bound lysosomes, numerous membrane-bound spherules, some containing fine granular material were seen in the PCT (Figs. 3.6 and 3.7). Pinocytotic vacuoles were still present in the vicinity of the tubule lumen.

Week 4: Kidney copper concentration was maximal at 1142± 88 µg/g.

Nuclear changes had progressed to include karyolysis and PCT cell nuclei were characterised by their irregular shape, intact membranes and mottled appearance caused by spotty fading and loss of granularity and electron density of the nuclear substance (Fig.3.8). Fragmentation of the rough endoplasmic reticulum (RER), disruption of the parallel arrays of membranes and disaggregation of ribosomes persisted at this sampling point. Mitochondrial swelling had progressed to include extensive

disruption of cristae. Lysosomes were very numerous. Brush border microvilli were distended and closely packed together, sometimes coalescing and forming cytoplasmic bridges between adjacent microvilli with lamellar profiles and a labyrinthine network (Fig.3.9). Cellular debris was seen in the tubular lumen (Fig.3.10). Pinocytotic vacuoles and spherules were still present.

Week 5 and 6: Kidney copper concentration had fallen to 1001± 64 µg/g in week 5 and 768± 72µg/g in week 6.

Nuclear changes were still apparent and included condensation of chromatin, misshapen appearance and occasional karyolysis. In addition to membrane-bound large lysosomes (Fig.3.11); numerous elongated, irregularly-shaped, electron-dense crystalline structures, which were also surrounded by a membrane, were seen during this period (Fig.3.12).

There was extrusion of apical cytoplasm including lysosomes and pinocytotic vesicles into the tubule lumina (Figs.3.13 and 3.14). Microvillar changes, similar to those described during the previous weeks, were marked in some tubules (Fig.3.15). Occasional myelin figures were also seen in the microvilli (see arrow fig. 3.13). Pinocytotic vesicles and spherules persisted during this sampling time.

Weeks 8 and 12: Kidney copper concentration was 526 ± 48µg/g and 703 ±50 µg/g.

This period was characterised by reduction in the numbers

of lysosomes and crystalline structures. Cellular debris, including intact lysosomes, was abundant in the tubule lumina (Fig.3.16). There was recovery of organelles and microvilli. Occasional accumulation of myelin figures was seen in the cytoplasm of some PCT cells (Fig.3.17).

Week 16: Kidney copper concentration was 500 ± 30 µg/g.

The proximal convoluted tubules were nearly normal in appearance and no abnormal changes were detected in the cytoplasmic organelles, nuclei or microvilli. Lysosomes were still present but were reduced in numbers and crystalline structures were reduced in size and numbers. Pinocytotic vesicles and spherules were still present and abundant cellular debris was seen in the tubule lumina (Fig.3.18).

Table 3.1. Renal copper concentrations in copper-loaded and control rats.

Time (weeks)	Copper concentration (µg/g)*
1	56 ± 2
2	306 ±21
3	458 ±29
4	1,142±88
5	1,001±64
6	768±72
8	526±48
12	702±50
16	500± 30
Control **	34± 1

* mean ± SE of the mean (n=4)

** n=20

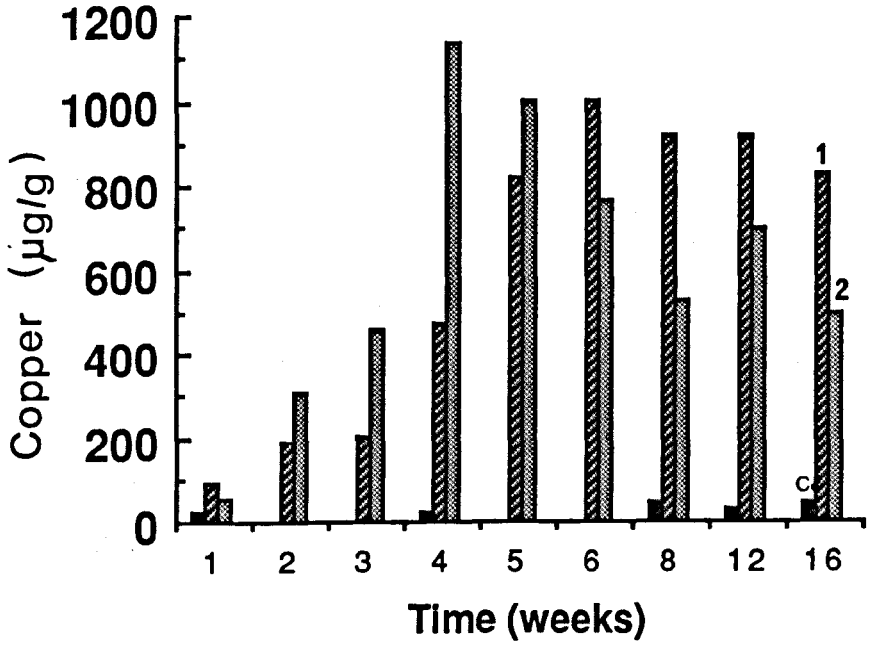


Figure 3.1. Kidney copper concentrations in copper-supplemented rats for 16 weeks; experiment 2 compared with experiment 1.

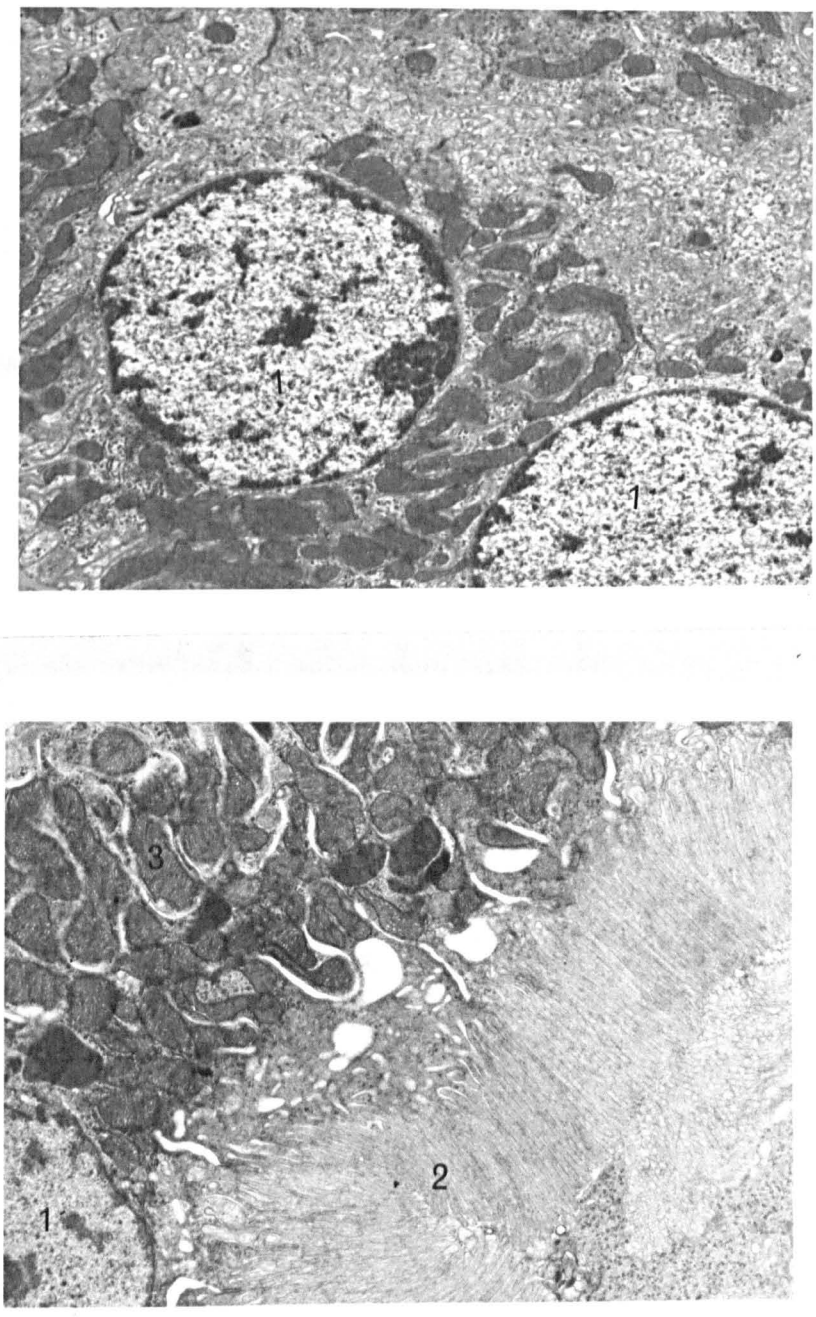


Figure 3.2. Kidney from a control rat. Karnovsky fixative. a)x7,200; b)x8,100.
1. Nuclei
2. Microvilli (Brush border)
3. Mitochondria

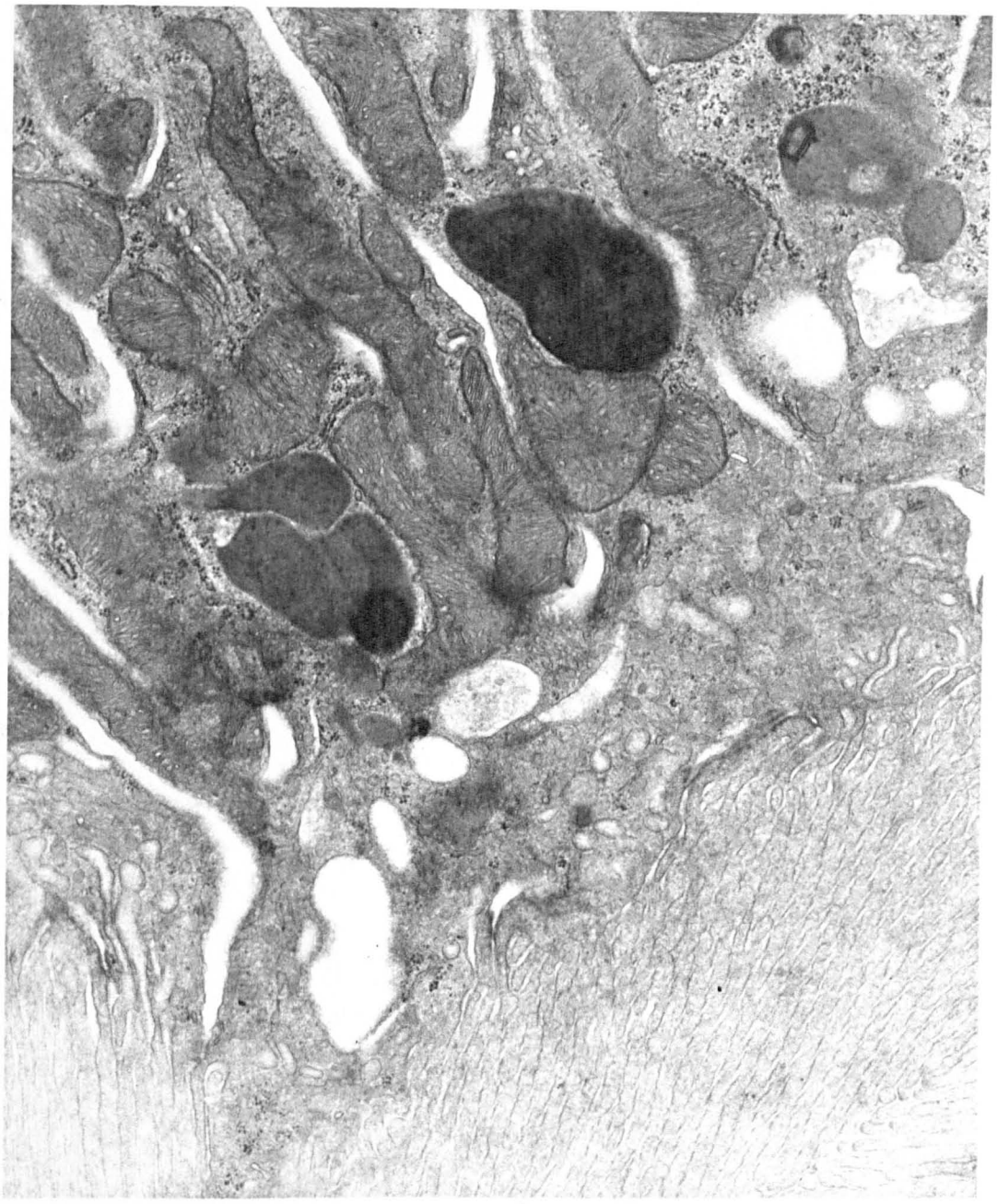


Figure 3.3. Week 1. Copper-loaded rat kidney. Electron-dense lysosomes in the PCT cells. Pinocytotic vesicles (PV) are numerous in the apical region. x 24,000.

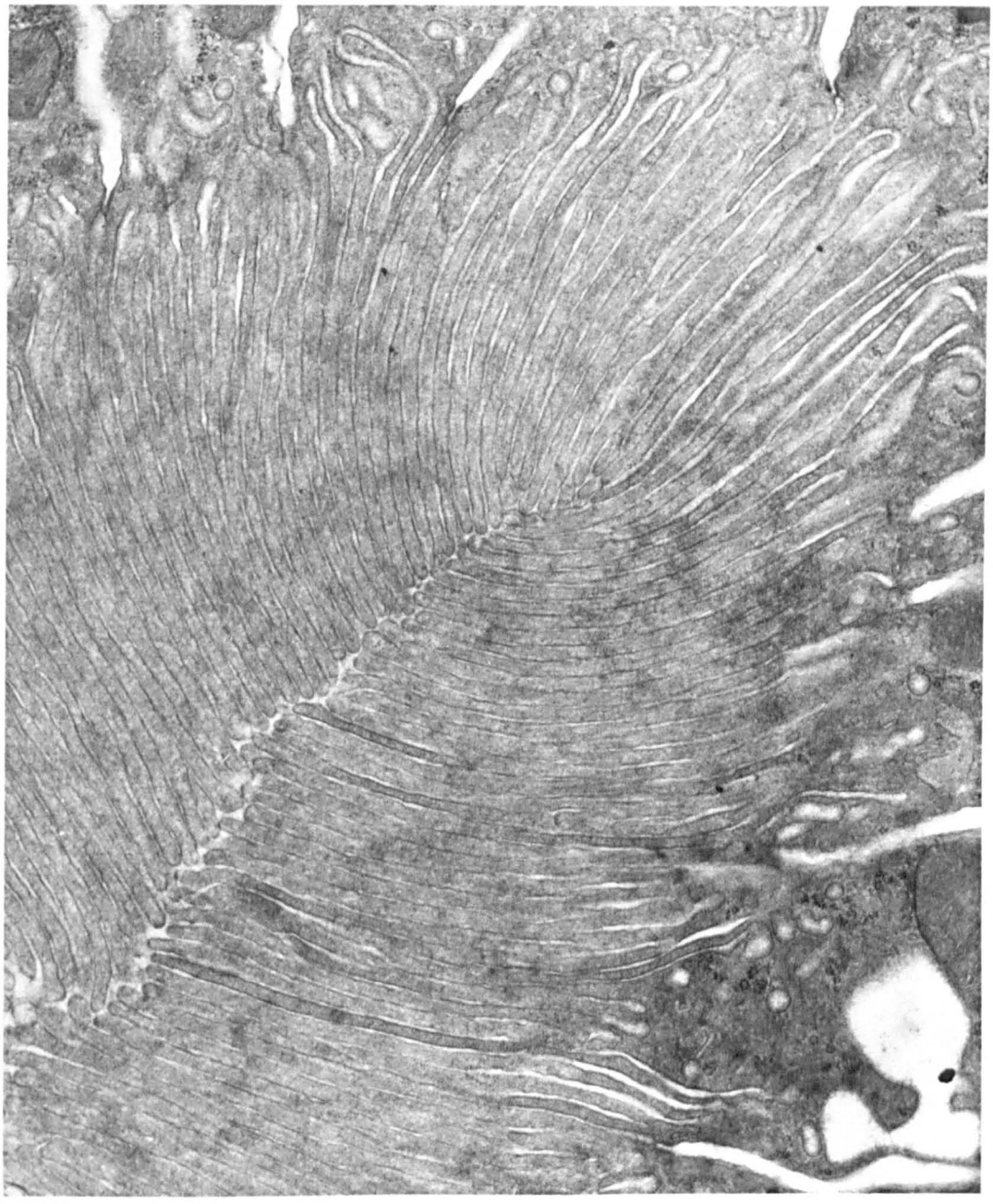


Figure 3.4. Week 1. Copper-loaded rat kidney.
The PCT cells have long uniform intact microvilli. x 24,000.

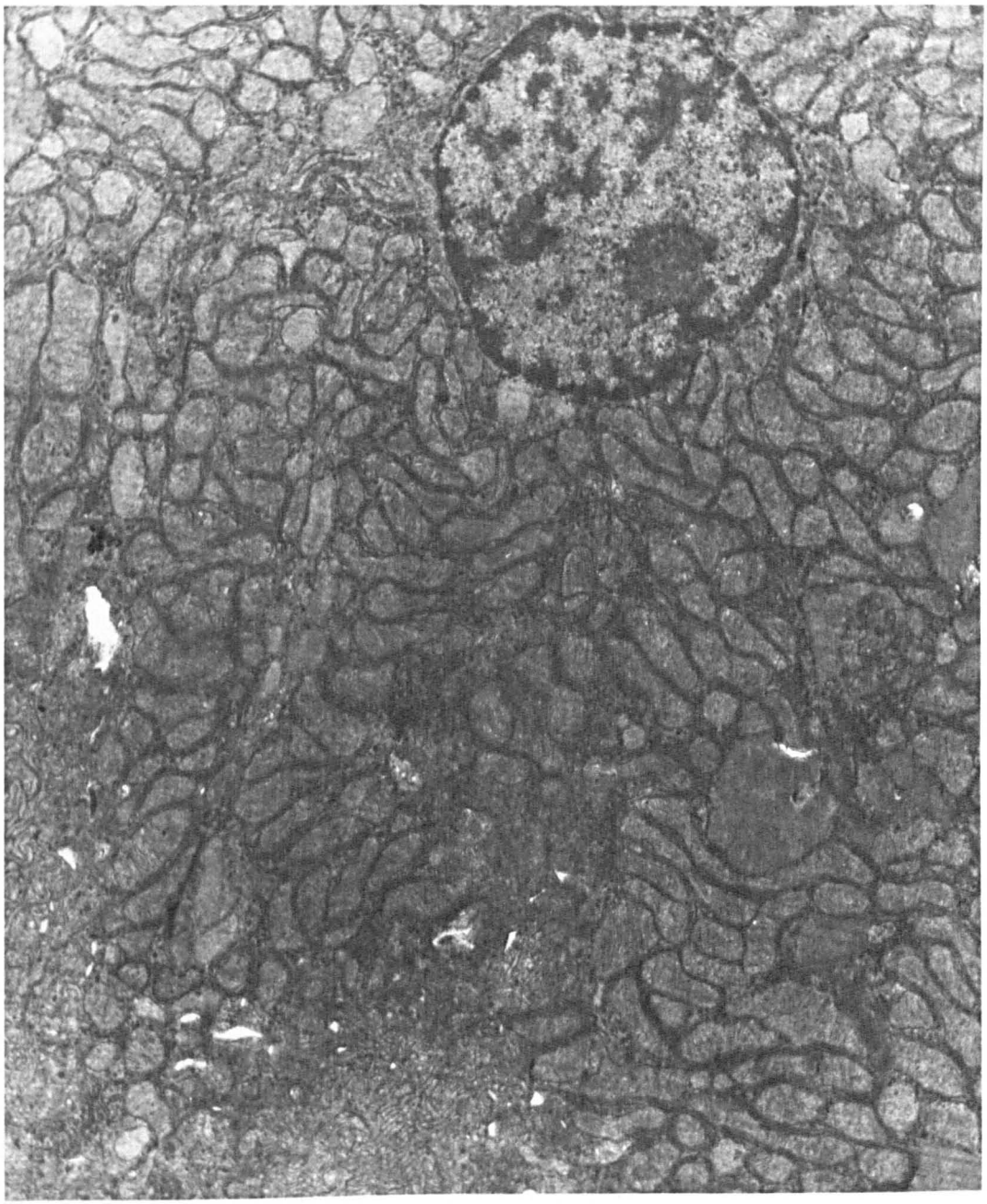


Figure 3.5. Week 2. Copper-loaded rat kidney.
Condensation of nuclear chromatin and swelling of mitochondria.
x12,000.

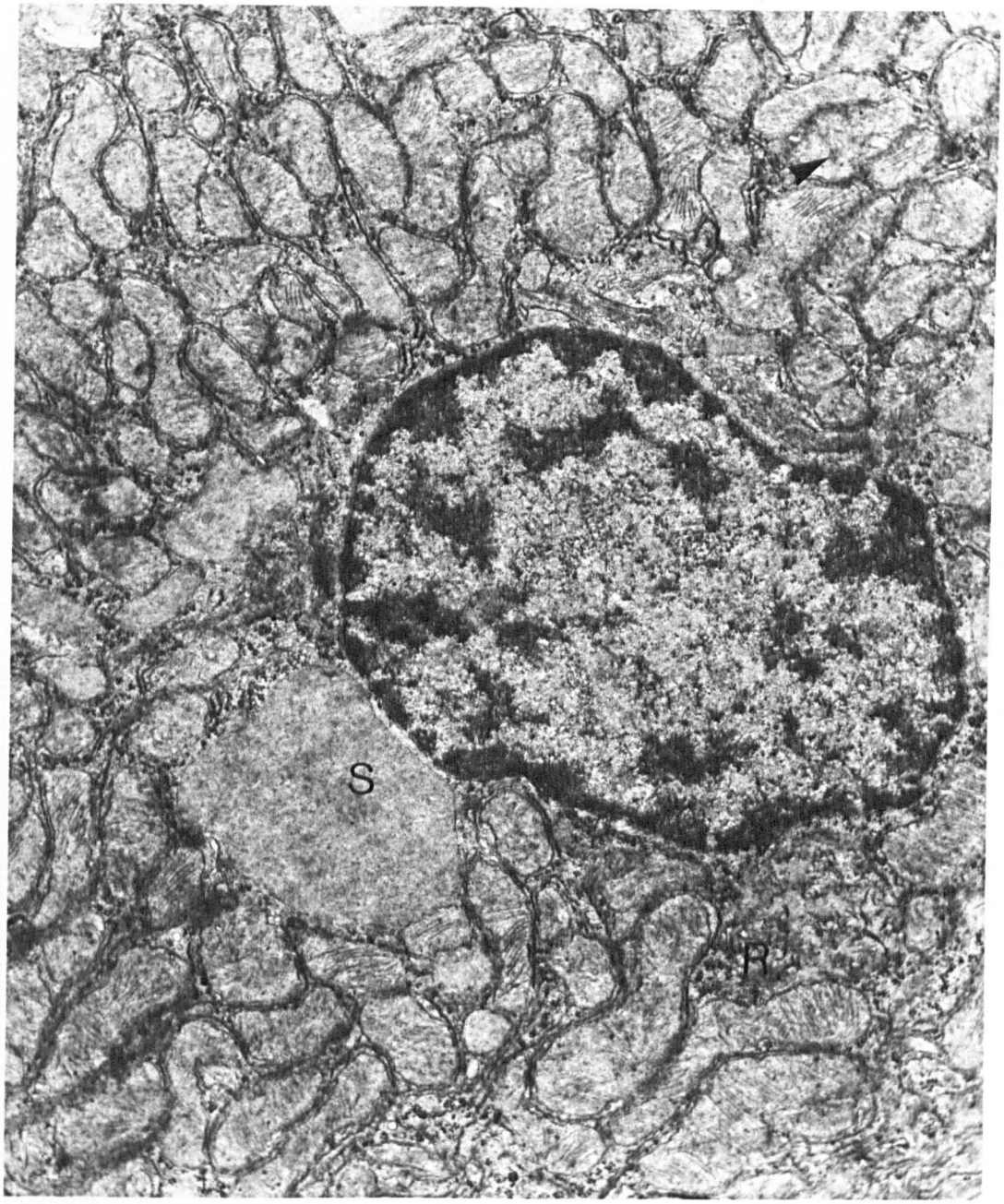


Figure 3.6. Week 3. Copper-loaded rat kidney. Misshapen nucleus with crenated nuclear membrane. Mitochondria are swollen, some contain flocculent areas (►). Also note dissagregation of ribosomes (R) and presence of electron lucent, membrane-bound spherules (S). x20,000.

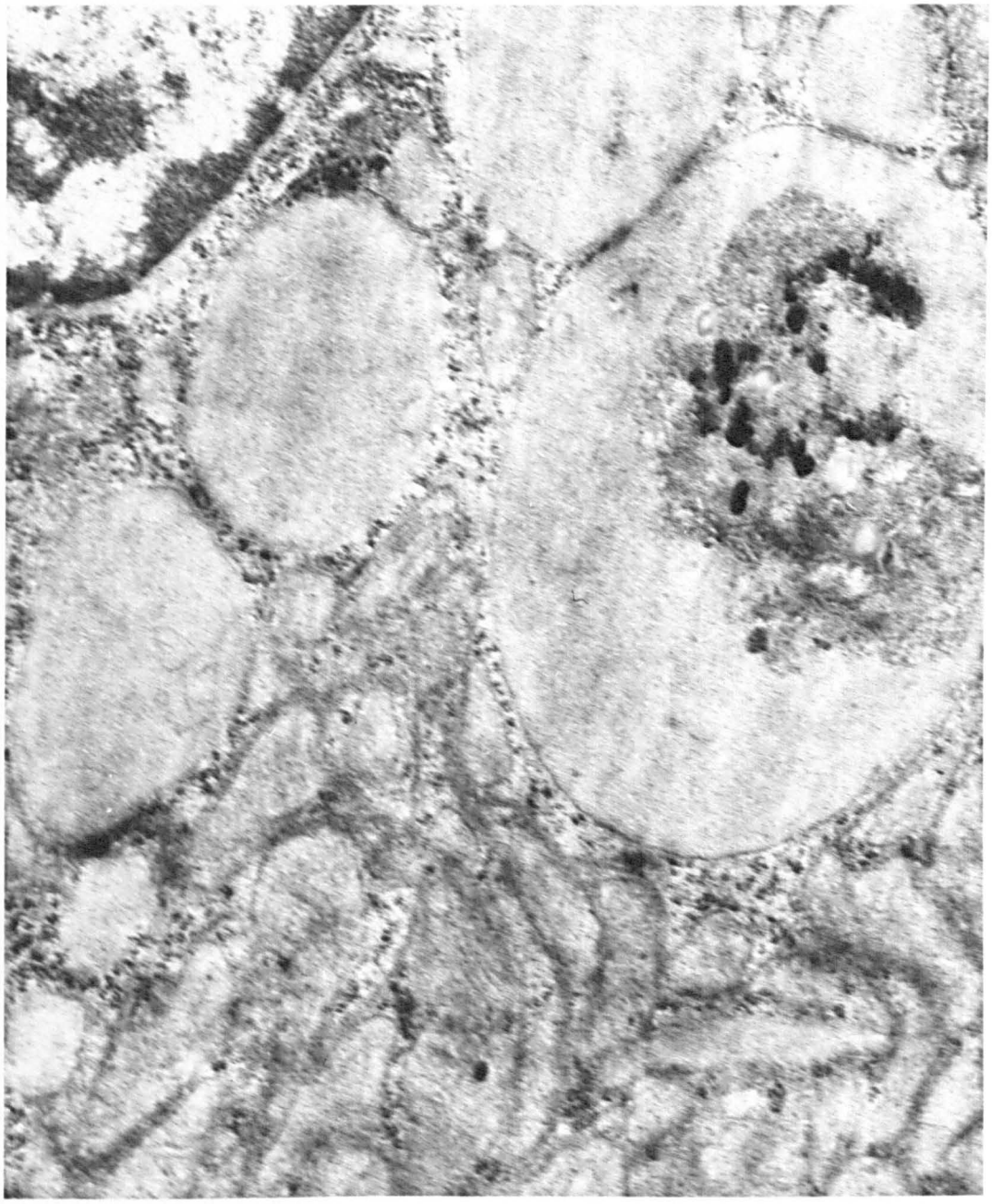


Figure 3.7. Week 3. Copper-loaded rat kidney. Numerous membrane-bound spherules, some containing fine granular material are seen in the PCT cells. x 34,000.

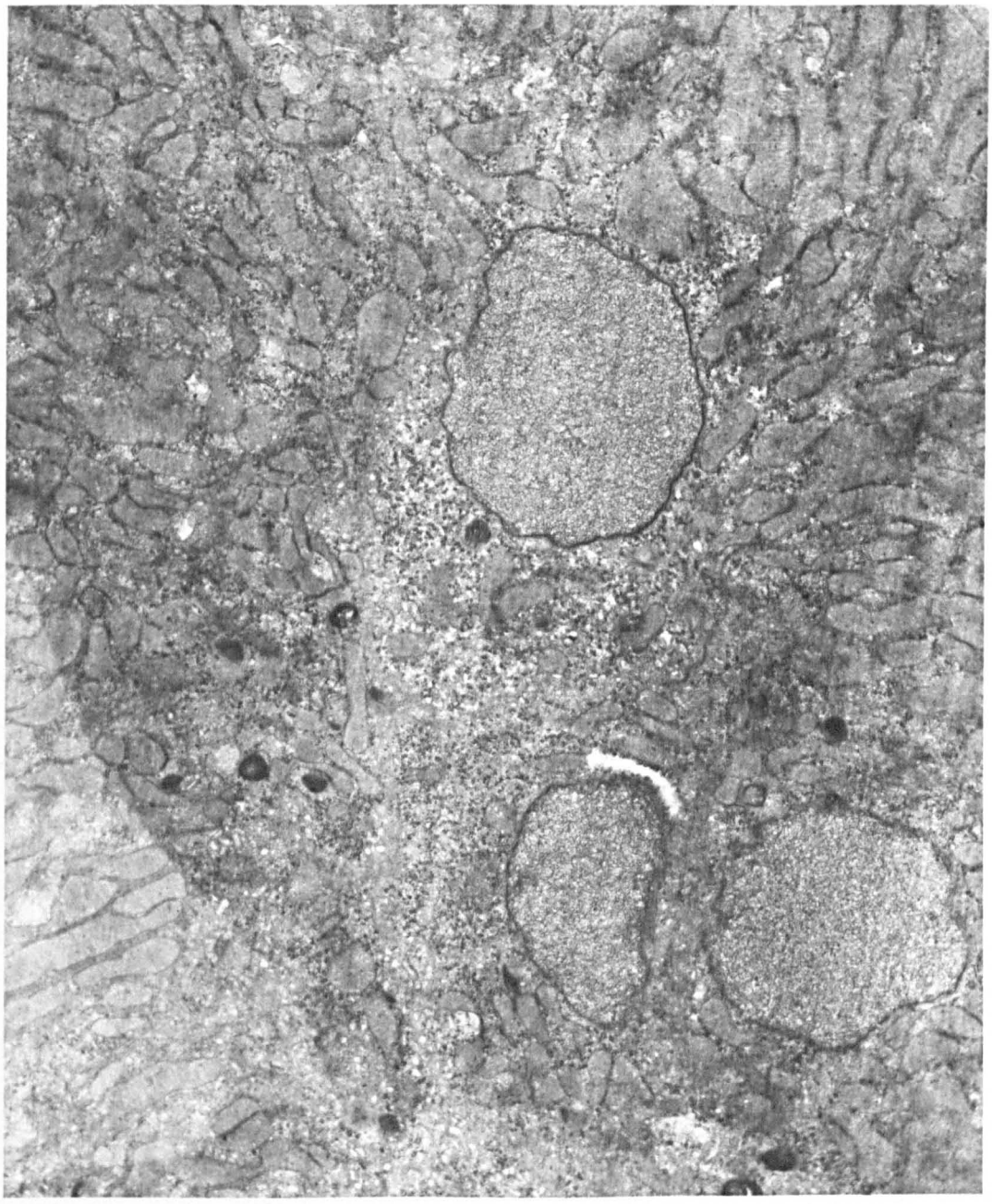


Figure 3.8. Week 4. Copper-loaded rat kidney. Karyolysis of PCT cell nuclei. x10,000.

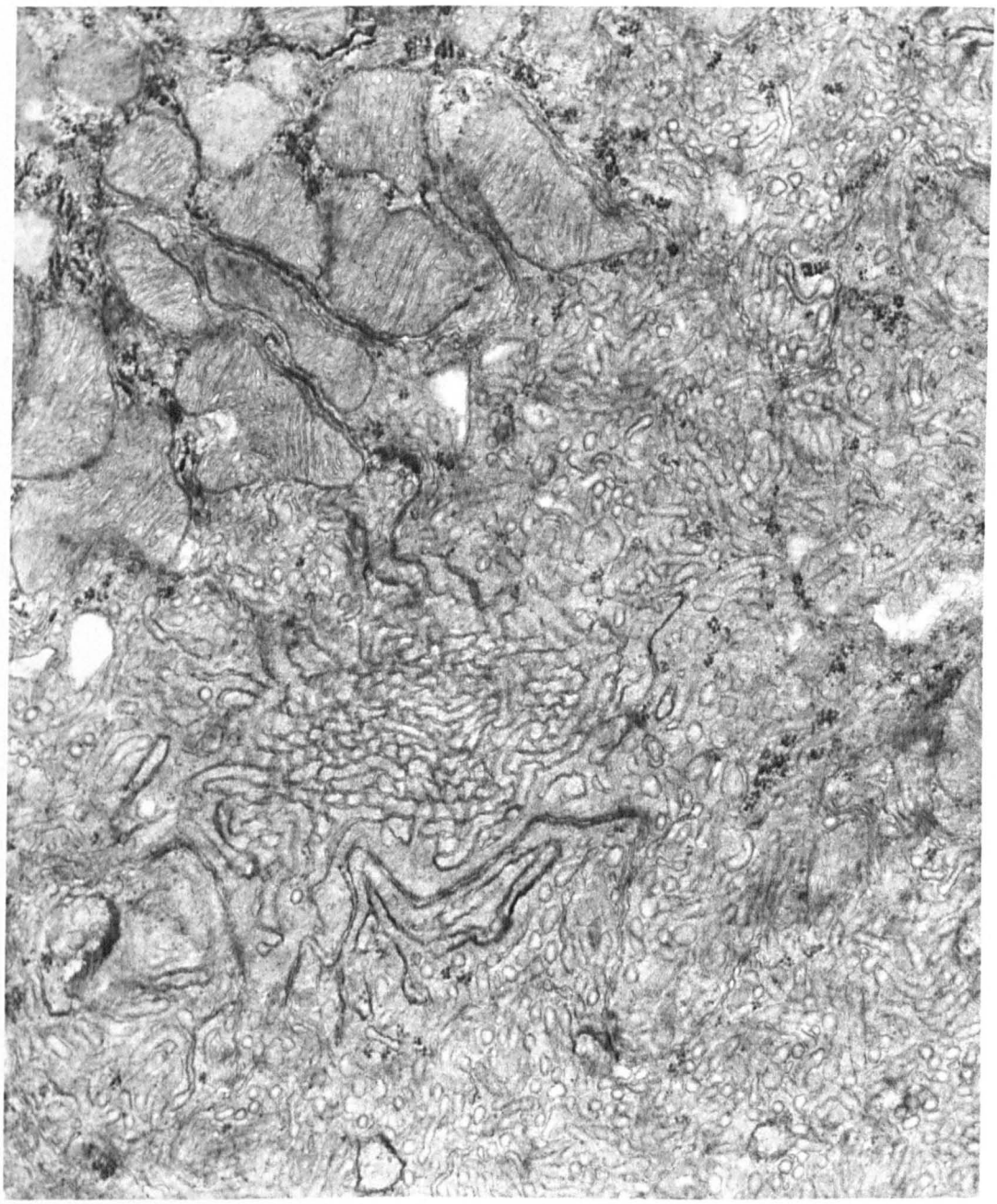


Figure 3.9. Week 4. Copper-loaded rat kidney. Coalescence of brush border microvilli. x30,000.

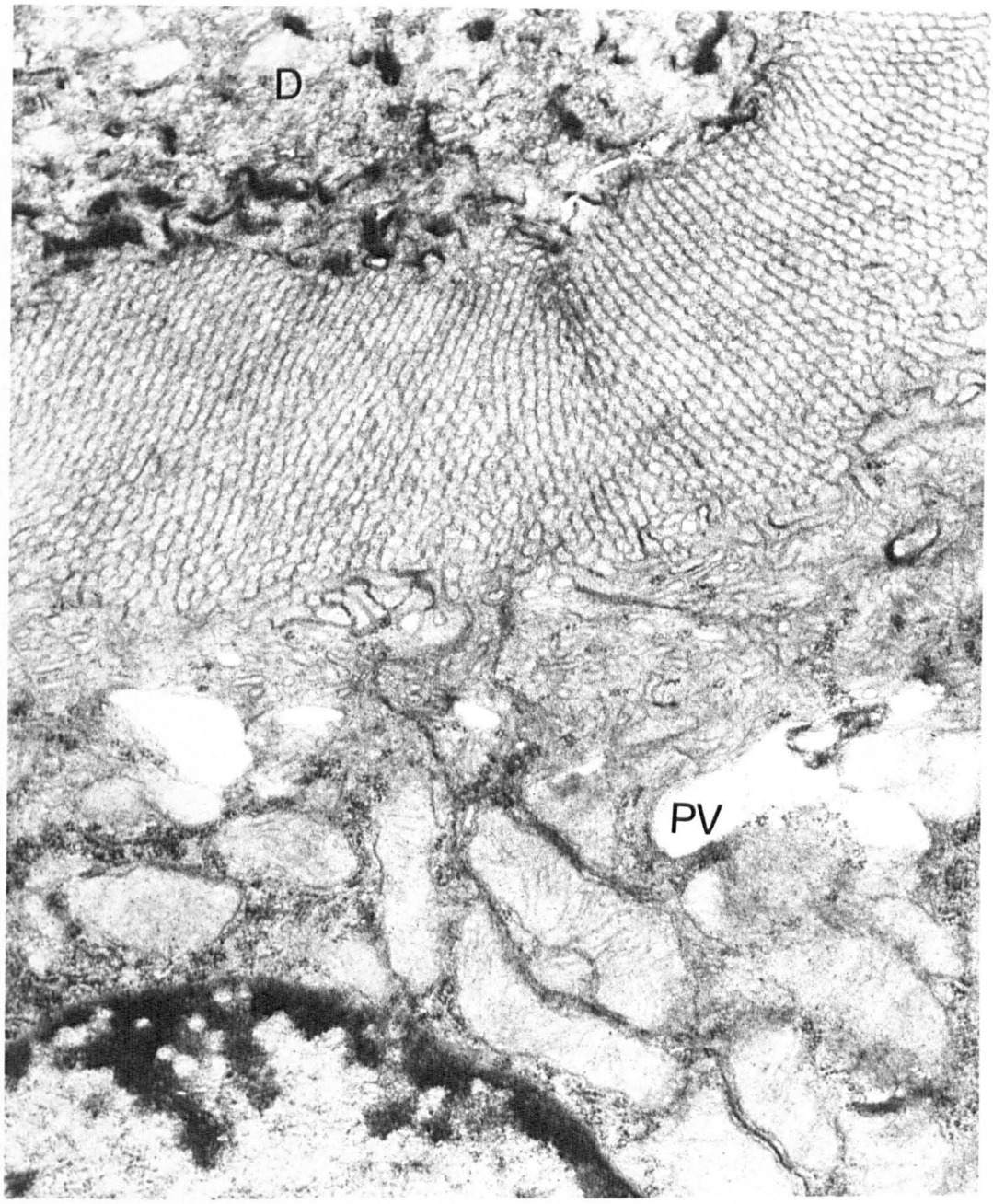


Figure 3.10. Week 4. Copper-loaded rat kidney. Presence of cellular debris in the PCT lumen (D). Also note pinocytic vesicles and swollen mitochondria. x30,000.

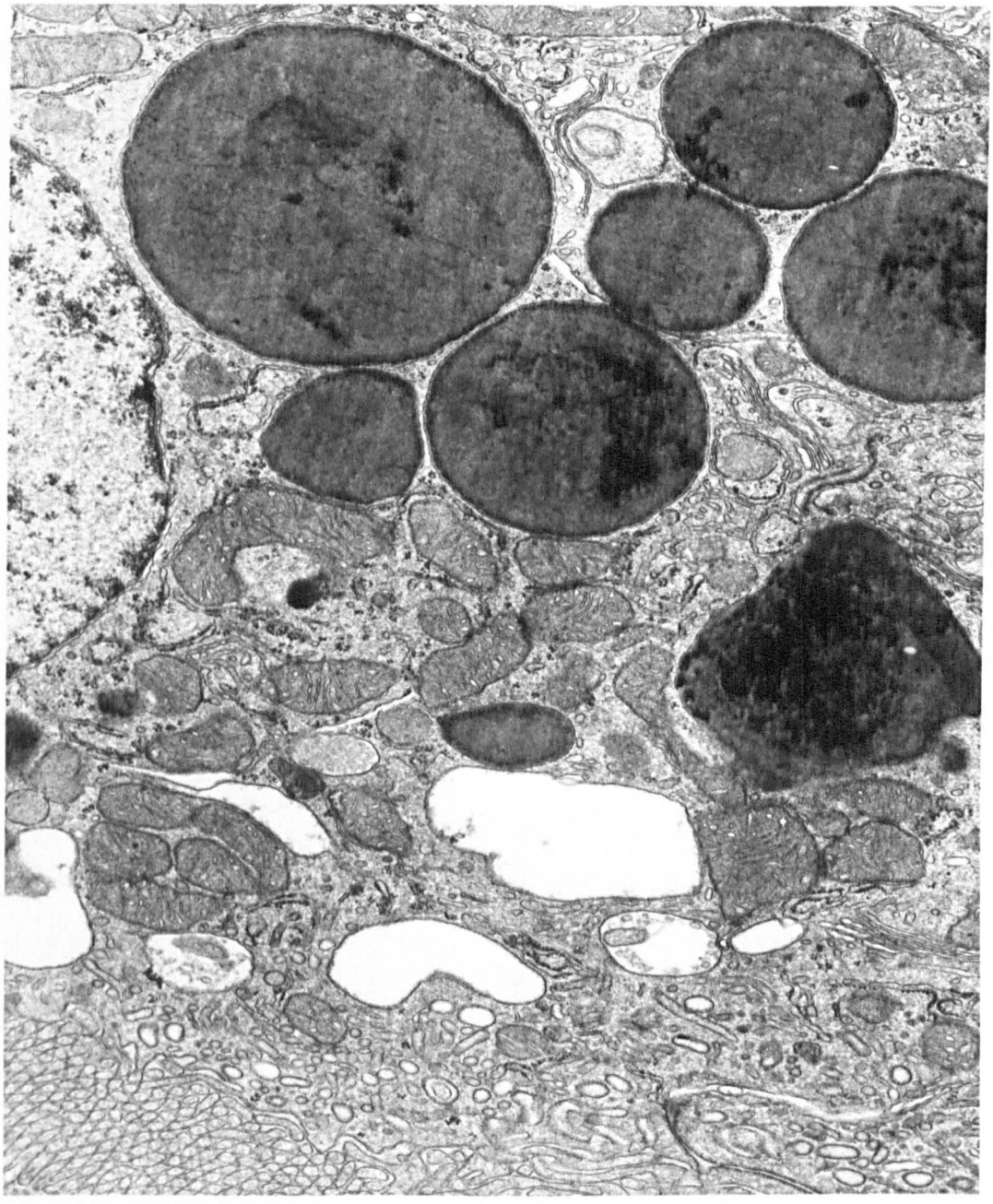


Figure 3.11. Week 5. Copper-loaded rat kidney. Numerous large membrane-bound lysosomes are seen in the PCT cells. x 20,000.

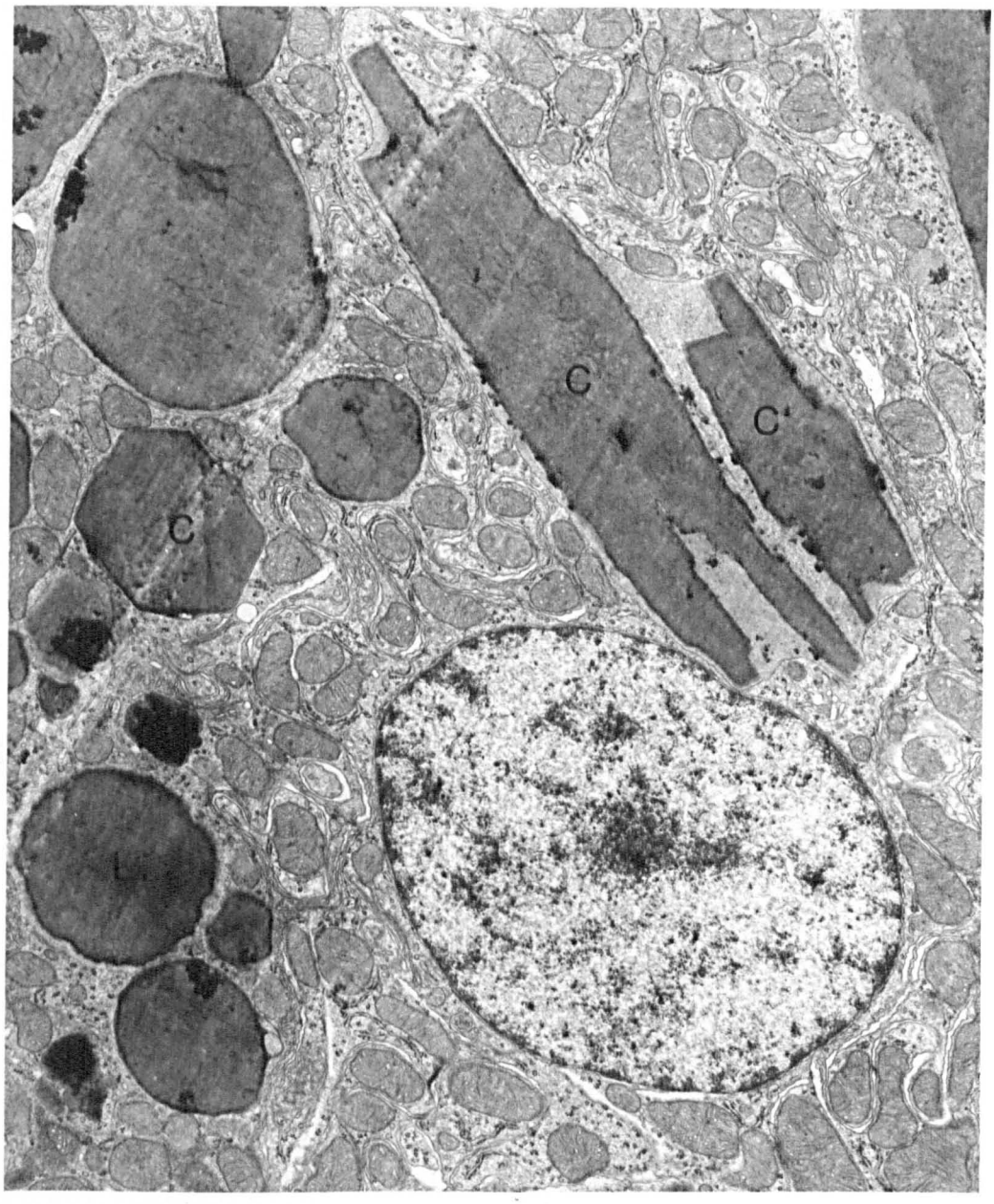


Figure 3.12. Week 6. Copper-loaded rat kidney.
Lysosomes (L) and elongated crystalline, electron-dense structures are surrounded by a distinct membrane (C). x12,000.

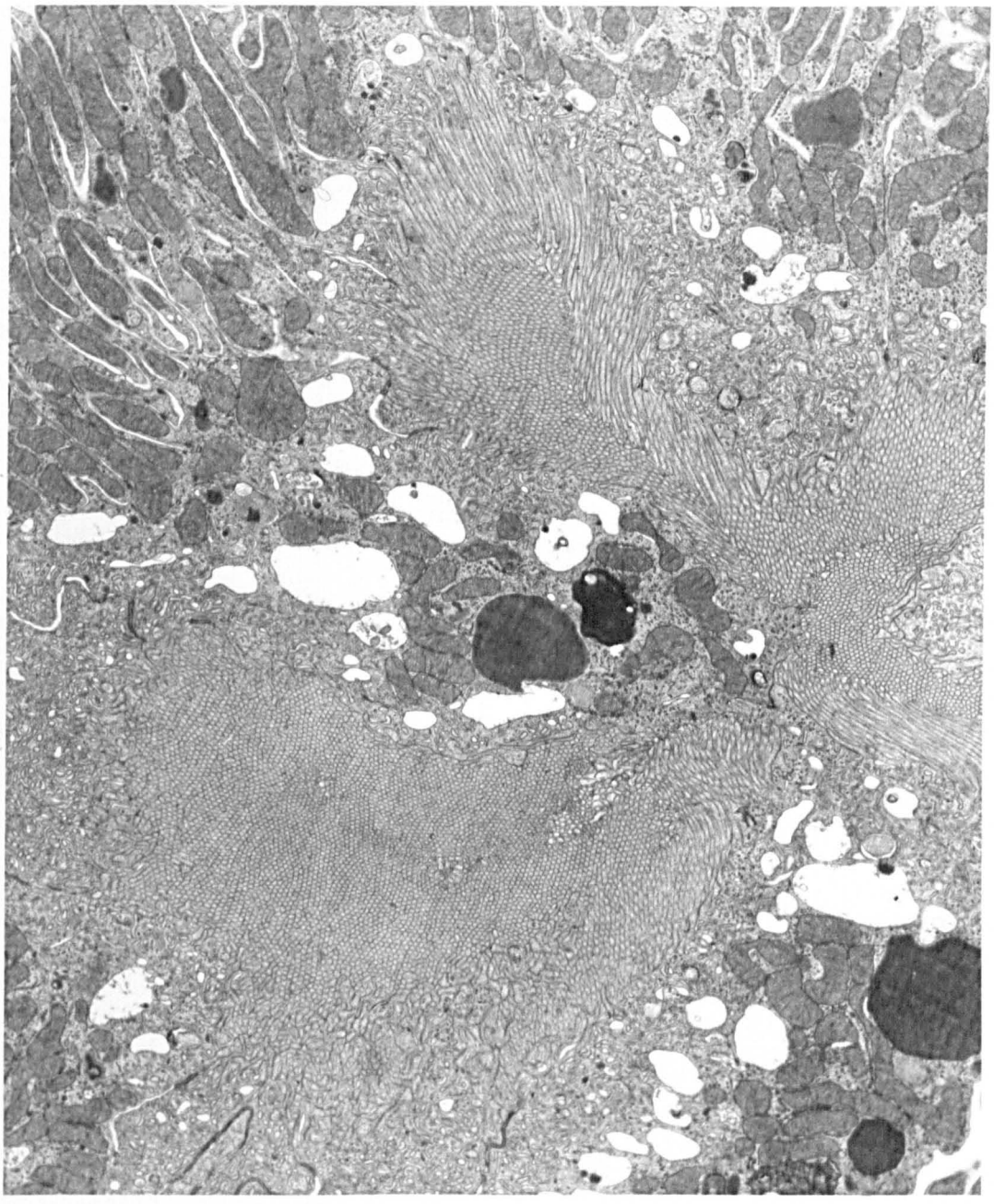


Figure 3.13. Week 5. Copper-loaded rat kidney.
Extrusion of apical cytoplasm which contains pinocytic vesicles and lysosomes. x6,000.

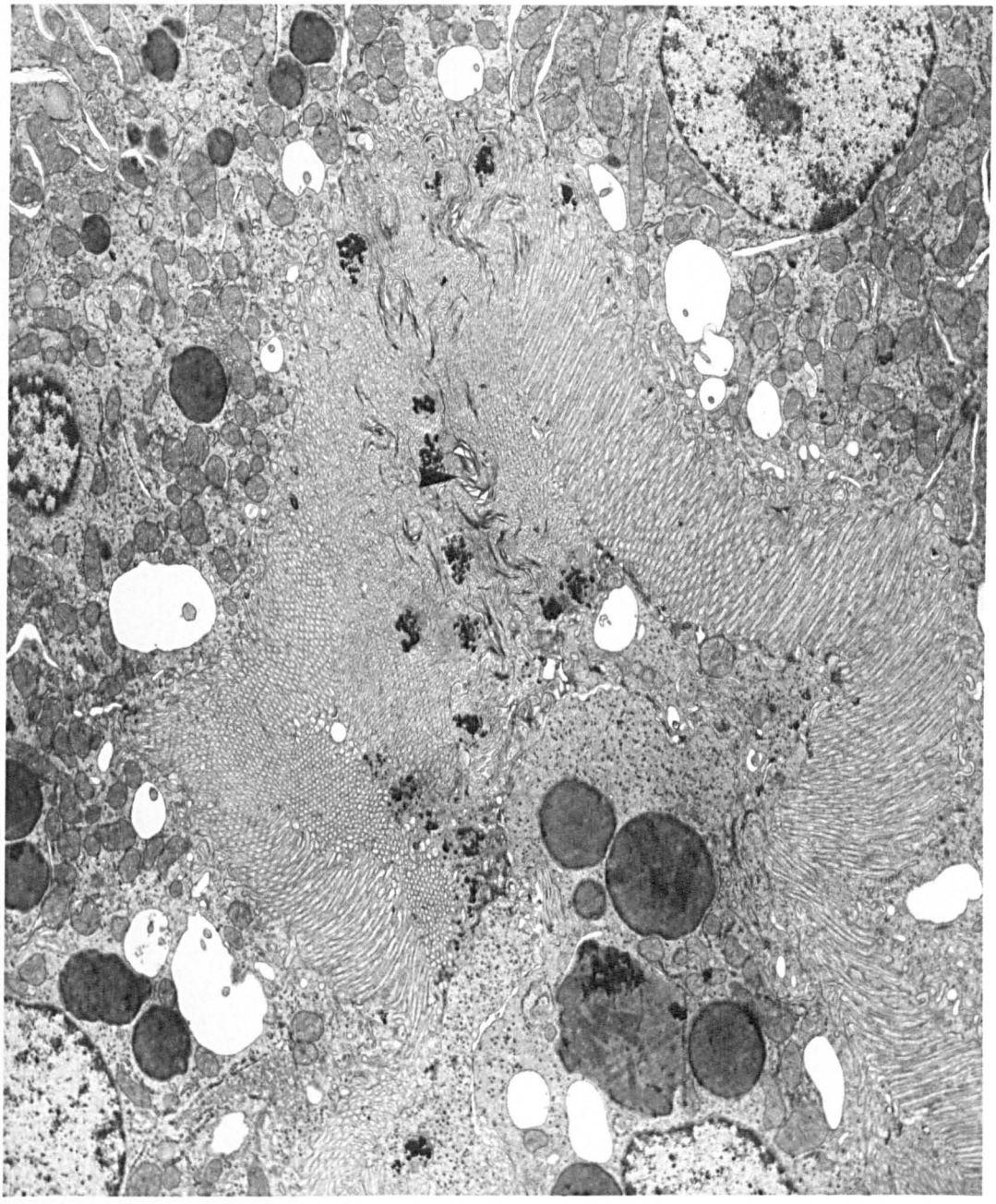


Figure 3.14. Week 6. Copper-loaded rat kidney. Extrusion of apical cytoplasm into the tubule lumen. Also note myelin figures in the microvilli (►). x6,000.

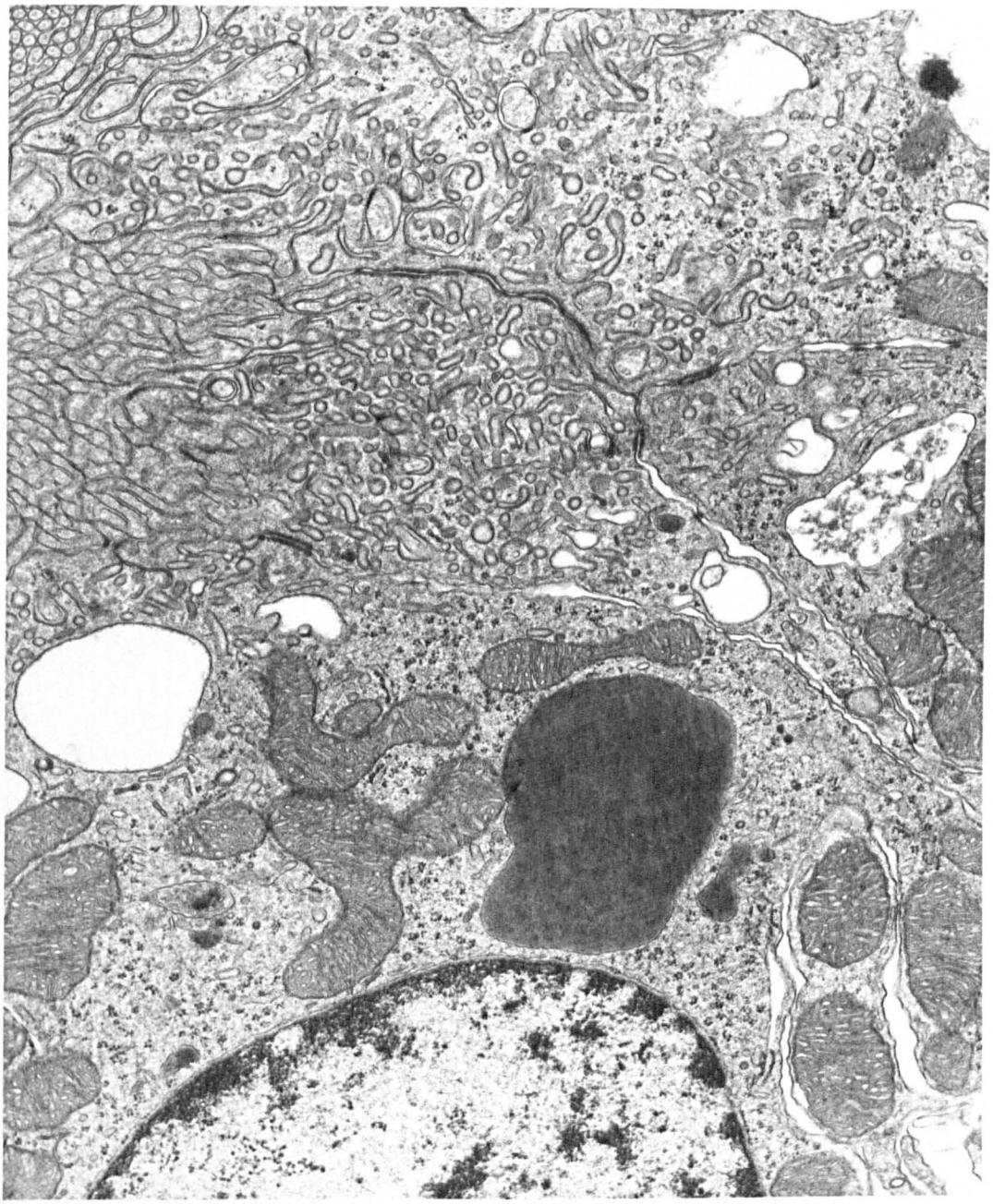


Figure 3.15. Week 6. Copper-loaded rat kidney.
Fusion of microvilli and presence of large pinocytotic vesicles.
x20,000.

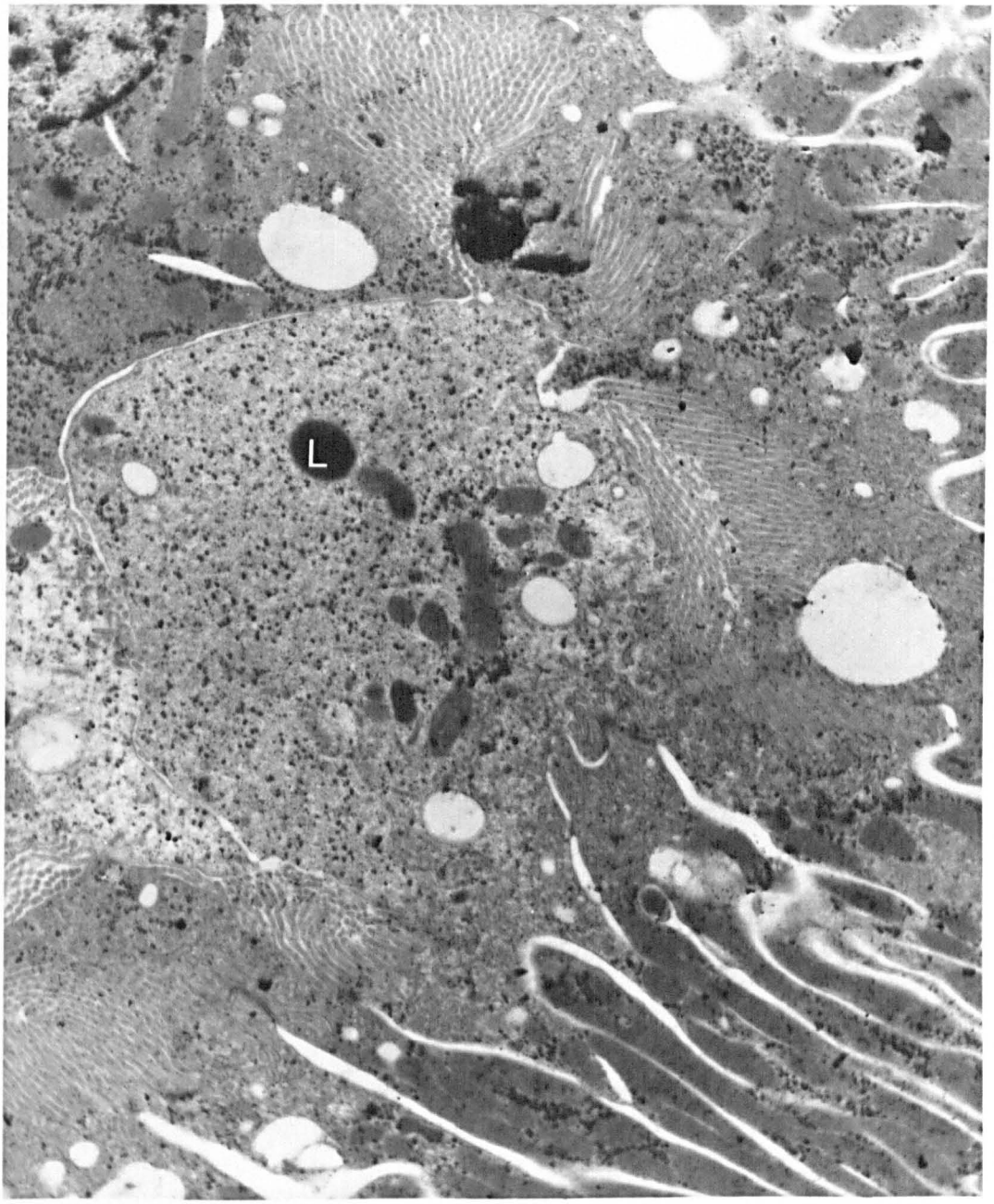


Figure 3.16. Week 8. Copper-loaded rat kidney. Cellular debris in the tubule lumen containing lysosomes and altered cellular organelles. x12,000.

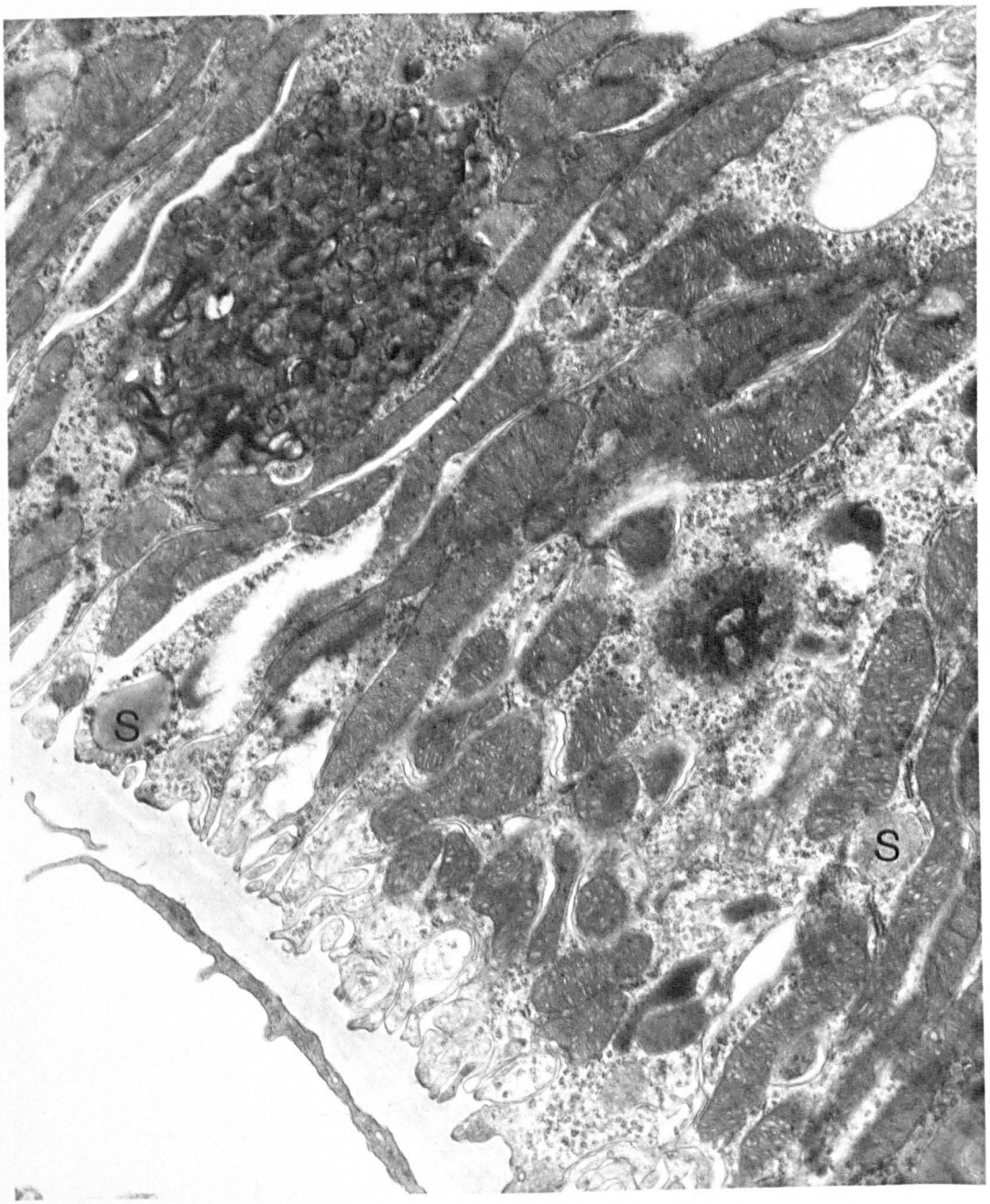


Figure 3.17. Week 8. Copper-loaded rat kidney. Accumulation of myelin figures in the PCT cytoplasm. Spherules are numerous (S). x16,000.

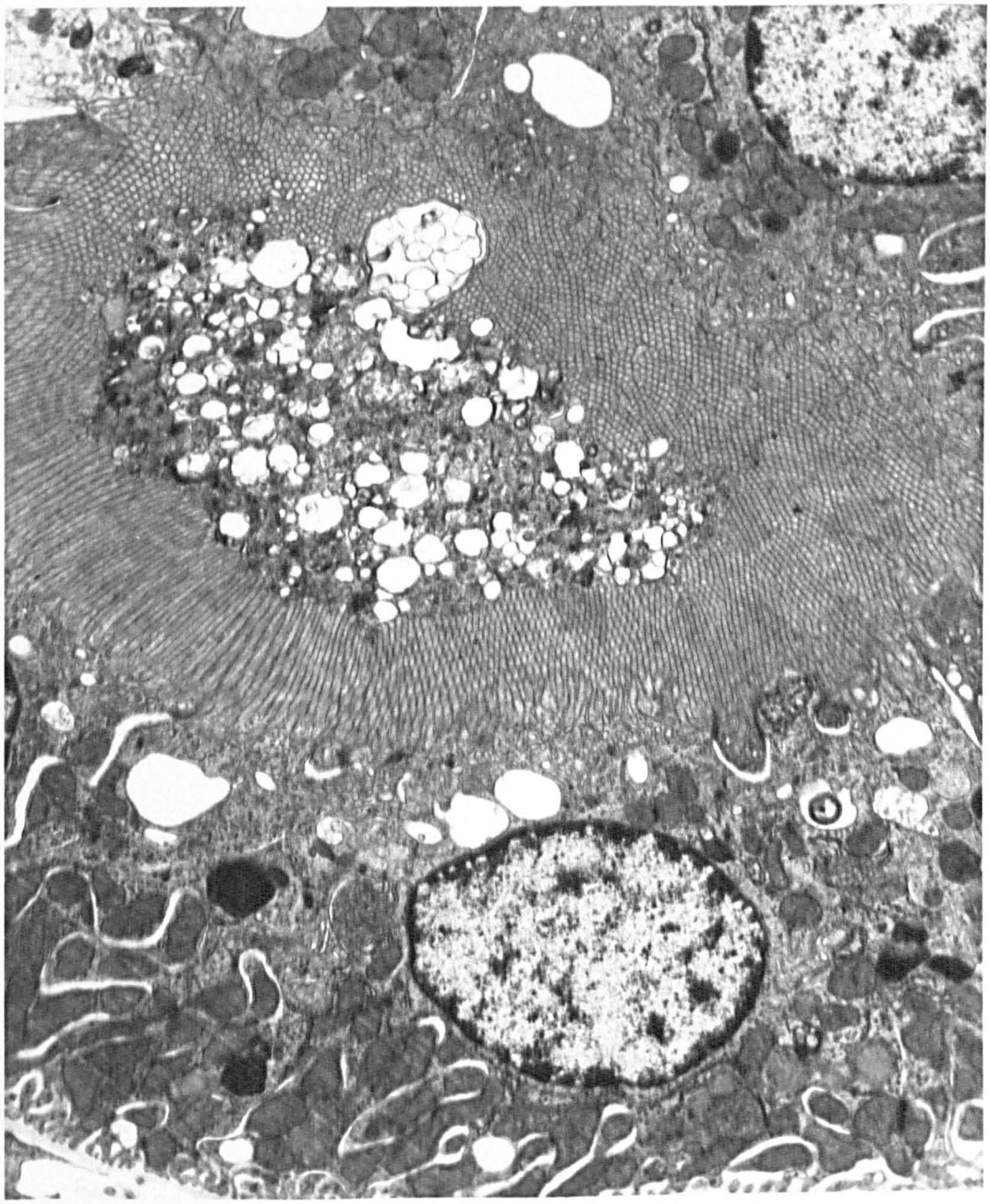


Figure 3.18. Week 16. Copper-loaded rat kidney. Unremarkable brush border microvilli and presence of abundant cellular debris within the tubule lumen. x10,000.

4.- DISCUSSION.

In this study copper accumulated rapidly within the proximal convoluted tubules for the first 4 weeks, accompanied by increasing signs of cellular disturbance, which culminated in nuclear lysis. Subsequently copper concentrations fell associated with tubule cell recovery. The cumulative phase was characterised additionally by increased numbers of lysosomes and other membrane-bound structures which later declined in parallel with the copper content.

Mitochondrial swelling is a nonspecific pathological response, possibly due to anoxia, ion flux or direct action of toxins (Goyer and Krall, 1969).

Swelling of mitochondria has been reported in pulmonary alveolar macrophages as a consequence of acute lead toxicity (DeVries et al, 1983), in PCT after Cadmium-metallothionein administration in rats (Nishizumi, 1972; Cherian et al., 1976) and as a result of hexacloro-1:3-butadiene in mice (Ishmael et al., 1984). Swelling of mitochondria and dilatation of tubular SER have been reported in ischaemic cell injury (Glaumman and Trump, 1975), chronic cadmium-overload in the rat (Goyer and Krall, 1969; Kajikawa et al., 1981).

Cells with condensed or swollen mitochondria can recover (Trump et al., 1974; Glaumman et al, 1975), but when they contain flocculent electron-dense masses the pathological change is irreversible (Trump, 1965). It has been shown that injury to

mitochondria may lead to an alteration in the permeability of mitochondrial membranes resulting in mitochondrial swelling and leakage of calcium (Trump et al., 1978)

Proliferation of the SER has been reported as a common finding after the administration of various nephrotoxic subtances (Wachstein and Bessen, 1964; Suzuki and Mostofi, 1966; Fowler , 1972b). Several investigators have associated proliferation of SER with induction of microsomal detoxification enzyme systems (Jones and Fawcett, 1966; Hutterer et al, 1968; Fowler, 1972b). In consideration of these data and the findings in the liver (Chapter 2) it seems likely that the proliferation of SER found in the present study may represent a similar attempt at the cellular level to detoxicate copper.

Increase in the number and size of endocytotic vacuoles (pinocytotic vesicles), as seen in the present study after 2 weeks of copper-overload, have also been reported in chronic copper poisoning in sheep where it was interpreted as a direct response of the tubules to excess copper (Gooneratne et al., 1986). Pinocytotic vacuoles have also been reported in chronic cadmium poisoning (Kajikawa et al., 1981) and administration of cadmium-metallothionein in the rat (Kajikawa et al., 1981; Cherian et al., 1976). It is generally accepted that cadmium which combines with metallothionein in the liver is transported to the kidney, and reabsorbed in the tubules after glomerular filtration (Kagi and Vallee, 1961; Squiff et al., 1976). Therefore, it has been suggested that pinocytotic vesicles are the mechanism for

cadmium transport into the tubule cells (Cherian et al., 1976) and also for the transport of many other substances (Jacques, 1975).

The spherules which appear plentiful after 3 weeks of copper overload are morphologically distinct from the pinocytotic vesicles. The spherules probably represent the hyaline droplets observed under light microscopy (Chapter1), which have been demonstrated to be composed mainly of the urinary protein $_{2u}$ globulin (Alden, 1986). Alpha $_{2u}$globulin is synthesised in the liver of male rats and rapidly secreted into the circulation without accumulation in the liver. Since alpha $_{2u}$ globulin is a low molecular weight protein (18,600 Daltons) it is rapidly filtered. In the kidney 60% is reabsorbed and poorly hydrolysed, thus accumulating in the PCT epithelium as morphologically visible hyaline droplets (Alden, 1986).

Disruption of ribosomes from the endoplasmic reticulum, has also been reported in renal tubules in cadmium toxicity studies (Kajinava et al., 1987); in pulmonary alveolar macrophages as a consequence of acute lead toxicity (DeVries et al, 1983) and as a consequence of numerous noxious influences (Chapter 2). Degranulation of RER is considered a useful morphological indicator of depressed protein synthesis (Smuckler et al., 1962; Hoffmann et al., 1975).

The changes seen in the microvilli after 4 weeks of copper-overload are similar to those reported in the rat after induced ischaemia (Venkatachalam et al., 1978; Glauman and Trump, 1975) and following hypotension (Kreisberg et al., 1976; Dobyan

et al., 1977). Focal brush border loss also occurs after induced ischaemia (Venkatachalam et al., 1978), exposure to mercuric chloride (Gritzka and Trump , 1968; Ganote et al., 1974) and chromate (Evan and Dail; 1974).

The heterogeneity of PCT lysosomes has been widely discussed (Ericsson et al., 1965; Ghadially, 1975; Gooneratne et al., 1980; Jones et al., 1984). In general two types of secondary lysosomes are found in the normal rat kidney: cytosomes and cytosegresomes (Ericsson et al., 1965); both are acid phosphatase positive and are limited by a membrane. Cytosomes are invariably limited by a single membrane and contain variable amounts of membranes, filaments and dense material of different shapes. The cytosegresomes are single, or occasionally double, membrane-delimited bodies containing clearly recognizable cytoplasmic organelles such as mitochondria and endoplasmic reticulum.

In the present study there was an increase in numbers, size and morphological diversity of lysosomes. Similar findings have been reported in chronic copper poisoning in sheep (Jones et al., 1984; Gooneratne et al., 1986) and in chronic cadmium poisoning in rats (Kajikawa et al., 1981). Increased numbers of heterolysosomes has been reported in the PCT after cadmium administration in rabbits (Fowler and Nordberg, 1978). These lysosomes appeared morphologically as two types, both acid phosphatase positive, one containing dense flocculent membranous material and the other of proteinaceous material and vacuoles. The increase in the number of lysosomes has been attributed to the increased entry of copper

into the cells of the kidney tubules and possibly represents a mechanism of removing excess copper from the cytosol and so preventing copper damage (Gooneratne et al., 1986). Lysosomes have been reported to protect the cell from the cytotoxic effects of copper (Hellman et al., 1985) and the findings from this study support this hypothesis.

The presence of membrane-bound crystalline structures after 5 weeks of copper-overload is interesting. Crystalline structures have been previously seen within lysosomes in acute tubular necrosis caused by glycerin administration (Suzuki and Mostofi, 1966). Similar crystals have also been identified in kidneys of normal male rats in which they exhibited two characteristic features of lysosomes; a distinct limiting membrane and acid phosphatase activity (Maunsbach, 1966). The origin of the crytals is not clear, they could be synthesized within the cell or may represent material absorbed by the cell (Maunsbach, 1966). It is possible that the crystalline structures in the PCT cells of copper-loaded rats are modified lysosomes.

Degeneration and necrosis of tubular cells have been reported in ischaemic cell injury (Glaumann and Trump, 1975), cadmium toxicity (Cherian et al, 1976) and after induced renal tubular necrosis by a chlorinated solvent, hexacloro-1:3-butadiene, in the mouse (Ishmael et al., 1984). Karyolysis has also been reported in pulmonary alveolar macrophages as the result of acute lead toxicity (DeVries et al., 1983).

Whereas the lysosomes were not disturbed in the present study; nuclear changes, detected as early as week 2, progressed to karyolysis after 4 week of copper-overload. The early onset and sequence of nuclear changes identified in the PCT cells during copper-loading are similar to those occurring in hepatocytes (Chapter 2) and are strongly suggestive of nuclear disruption being the primary cytotoxic event rather than lysosomal damage as has hitherto supposed (Gooneratne et al., 1980). This has received support from a study of time-related changes occuring in the kidney after glycerin injection which have shown that advanced nuclear injury preceeded (60 min) lysosomal changes (90 min) (Suzuki and Mostofi, 1966). Furthermore, Sunderman et al. (1987), following parenteral administration of nickel chloride to rats, have also presented evidence that lysosomal damage is the consequence rather that the cause of cellular injury.

Changes have been reported in the glomeruli during the haemolytic phase of chronic copper poisoning in sheep (Gooneratne et al, 1986). In contrast to these observations in sheep, no changes were seen in the glomeruli of the rat kidney in the present study. Haemolysis, which was not present in the rat, has been ascribed as the probable cause of glomerular damage in the sheep (Gooneratne et al., 1986).

Recovery is associated with some loss of copper and reversal of the cellular changes. A notable feature of the tubule cell adaptation is the extrusion of apical cell contents into the lumen. This has also been reported in the mouse after

administration of hexacloro-1:3-butadiene (Ishmael et al, 1984). This is similar to the secretory activity exhibited by various exocrine glands and may represent the means whereby excess copper and possibly damaged organelles are removed from the proximal convoluted tubule cells.

CHAPTER 4.

CELLULAR MECHANISMS OF TOXICITY AND TOLERANCE IN THE COPPER LOADED RAT. IV. SUBCELLULAR LOCALISATION OF COPPER IN THE LIVER AND KIDNEY.

1. INTRODUCTION

The mechanisms by which copper exerts its toxic effect on the cell are still unclear; Lindquist (1968), supported by Gooneratne et al. (1980), suggested that the rupture of copper-loaded lysosomes is responsible for the cytotoxic effect of copper. An alternative theory is that cellular degeneration occurs as a consequence of copper-induced nuclear disorganisation (Haywood et al., 1985a). This receives substantial support from the findings of irreversible nuclear damage in the livers and kidneys of copper-loaded rats with minimal disturbance in other organelles, which are described in Chapters 2 and 3. Possible mechanisms of nuclear disruption are the ability of copper to destabilise DNA (Bryan and Frieden, 1967) and to inhibit RNA polymerase activity (Novello and Stirpe, 1969). It has further been suggested that an influx of copper into the nucleus may disturb zinc homeostasis and displace zinc from this site (Haywood et al., 1985a) and so interfere with nuclear transcriptional activity which is dependant upon zinc (Falchuck and Valle, 1984). The demonstration of chemical elements in biological material by x-ray electron probe microanalysis is laborious and requires highly sophisticated equipment. However, this technique permits the examination of extremely small tissue samples (Birks, 1963; Tousimis, 1963) and even of single organelles (Goldfischer and Moskal, 1966; Fowler, 1983). Therefore, it is particularly useful

in determining differences in elemental concentrations between certain subcellular compartments (Fowler, 1983; Janssens et al., 1984a).

An electron beam, 1 μ in diameter, is focused on a specimen; atoms in the path of the beam emit characteristic x-rays which are analysed by an x-ray spectrometer and recorded (Goldfischer and Moskal, 1966).

X-ray electron probe analysis has detected copper in Descemet's membrane (Tousimis and Adler, 1963) and liver (Gueft et al., 1964) of patients with Wilson's disease; iron in liver (Gueft et al., 1964; Yasuzumi, 1962), intestine (Lever and Duncumb, 1961) and synovial membrane (Mellors and Carroll, 1961) and calcium and phosphorus in normal dental tissue and bone (Boyde et al., 1961; Brooks et al., 1962; Mellors, 1964). Also aluminium has been identified in experimental neurofibrillary degeneration (Terry and Pena, 1965) and cadmium in the renal proximal convoluted tubule cells after its experimental administration (Fowler and Nordberg, 1978; Squibb et al., 1979) using these methods.

Electron probe microanalysis of liver of patients with Wilson's disease has further demonstrated that copper was sequestered in lysosomes (Goldfischer and Moskal, 1966; Tanikawa et al., 1973). Similar localisations of copper within lysosomes have been shown in the livers and kidneys of copper-loaded rats (Verity et al. 1967), and of copper-loaded sheep (Jones et al., 1984). X-ray microanalysis has also been used to detect copper in hepatocellular lysosomes of patients with Primary biliary

cirrhosis both qualitatively (Nakumuna et al., 1979; Epstein et al., 1981a; Humbert et al., 1982) and quantitatively (Janssens et al., 1984a).

In order to further clarify the pathogenesis of copper toxicosis in the copper-loaded rat it was decided to study the intracellular localisation of copper and zinc and associated elements in livers and kidneys of copper-loaded rats by ultrastructural x-ray microanalysis and to relate this to the cytopathological changes that take place in these organs.

2.- MATERIALS AND METHODS

The animals, their feeding and management are described in detail in Chapter 2. Copper-loaded and control rats were killed at the predetermined intervals and their livers and kidneys removed.

METHODS

a.-Copper and zinc analysis:

Triplicate samples of approximately 0.3 g of liver (right median lobe) and kidney cortex were removed with plastic knives. Copper and zinc concentrations were determined by atomic absorption spectrophotometry as described in Chapter 1.

Copper and zinc concentrations are expressed as the mean and the standard error of the mean (M±SEM), in ug/g of tissue on a dry weight basis.

b.-Transmission electron microscopy:

Small pieces of liver and kidney were processed for transmission electron microscopy as described in Chapters 2 and 3.

c.- Electron-probe microanalysis.

Small pieces of liver and kidney were fixed in 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), rinsed in buffer, dehydrated through a graded series of

ethanol, cleared in acetone and infiltrated with TAAB resin. Semi-thin sections (0.5 um) were cut on an ultramicrotome, stained with alkaline toluidine blue to select an area for analysis (periportal area in the case of the liver) (Fuentealba et al., 1988). The blocks were retrimmed before cutting 0.3µm sections which were collected on aluminium grids and analysed for their elemental content by energy dispersive x-ray microanalysis in a JEOL JEM 100 CX transmission electron microscope fitted with a XEVEX detector integrated to a Microplus + multichannel analyser system (Dapple Systems, Sunnyvale, California, USA).

Analyses were performed at an accelerating voltage of 80KV, with a beam current of 30mA, a spot diameter of 0.1nm and an analysis time of 50 seconds, with a specimen take-off angle of 45°.

Repeated analyses were made of all the major organelles and cytosolic background within several cells of a particular section.

3.-RESULTS

A.-Copper and Zinc analysis:

The liver copper and zinc concentrations are shown in Table 4.1; the kidney copper and zinc concentrations are shown in Table 4.2.

Liver copper concentrations rose from 679±40 µg/g at week 1 to 2840±46 µg/g at 4 weeks, falling subsequently to 2271±92 µg/g at 16 weeks (control liver copper concentration was 22±1 µg/g). Similarly, renal copper concentration rose from 56±2 µg/g at 1 week to 1,242 ±88 µg/g at 4 weeks, decreasing later to 500±30 µg/g at 16 weeks (control kidney copper concentration was 34 ±1 µg/g).

Liver and kidney zinc concentrations remained stable at 142±3 and 112±2 µg/g respectively in the copper-loaded group.

B.-Transmission electron microscopy and x-ray microanalysis:

The sequence of ultrastructural changes in the livers and kidneys in which progressive degeneration occurs up until 4 weeks of copper-overload and is then succeeded by regeneration and recovery has already been described in Chapters 2 and 3. In the present study attention was concentrated on altered intracellular organelles in relation to their elemental contents.

a.-The Liver.

Hepatic lysosomes:

Electron-dense, membrane-bound bodies, identified as lysosomes were prominent within the pericanalicular region of hepatocytes after 1 week of copper loading (Fig.4. 1a,b). Their x-ray emission spectra demonstrated markedly elevated Cu peaks and in addition raised iron (Fe) and zinc (Zn) (Fig.4.2). On account of their characteristic morphology and emission profile these were designated as lysosomes- Type I.

At week 3 the lysosomes were larger and irregularly shaped although more heterogeneous with regard to electron density than those observed previously (Fig.4.3). Their emission spectra now included sulphur (S) and phosphorus (P) in addition to Cu and these organelles were named lysosomes- Type II (Fig.4.4).

By week 4 lysosomes were very numerous and displayed marked diversity with respect to their morphology and emission profiles. In addition to Types I and II, a third type of lysosome occurred which had more granular, sparse, electron dense contents (Figs.4.5a and b), and contained much reduced elemental residues on x-ray analyisis (Fig.4.6).

Lysosomal numbers and variety were maintained up to 6 weeks after which lysosomes declined in numbers and at 16 weeks the remaining lysosomes had emission spectra characteristic of Types II and III only. The lysosomal membrane appeared intact at all times and degenerative changes were not observed within these organelles.

Lysosomes were also heterogeneous in Kupffer cells (Fig.4.7) and three different types similar to those identified within hepatocytes were observed (Fig.4.8).

Lysosomes from control rat livers had no raised elemental residues.

Hepatocyte nuclei:

Chromatin condensation (Fig.4.9a) occurred as early as week 2 and by week 3 the nuclei were misshapen and had a crenated nuclear membrane (Fig.4.9b). Irreversible nuclear damage characterised by karyolysis occurred at week 4 (Fig.4.10).

Copper was detected within the nucleus (nucleolus) by x-ray microanalysis coincident with the earliest degenerative changes (Fig.4.11) but was later accompanied by increased concentrations of Zn, Fe, S, P and Ca as nuclear abnormalities became more marked (Fig.4.12). However, the lysed nuclei had no elevated copper.

Nuclei from the recovered cell population at week 16 appeared unremarkable with only very low x-ray emission activity of residual elements (Fig.4.13).

Nuclei from parenchymal cells of control rat livers showed no elevation of elemental residues (Fig.4.14).

Neither copper nor other elements were identified at any sampling time in the Kupffer cell nuclei (Fig.4.15).

Other subcellular constituents:

Copper was not detected in any other organelle or within the cytosol at any sampling point (Figs.4.16).

Copper was detected in apoptotic bodies (Fig.4.17) and sinusoidal cellular debris (Fig. 4.18) but not in the Mallory body-like structures (Fig.4.19a and b).

b.-The Kidney proximal convoluted tubules.

Lysosomes.

Electron-dense membrane bound bodies identified as lysosomes were prominent within the cytoplasm of the proximal convoluted epithelium from week 2 (Fig.4.20). Their emission spectra demonstrated elevated Cu peaks and included also Fe and Zn and were identified as Lysosome-Type I (Fig 4.21).

After 3 weeks of copper-overload lysosomes were more numerous and diverse in both morphology and electron density (Fig.4.22). Degenerative changes were not observed within these organelles. The emission spectra of these lysosomes now included Cu, S, P and were called Lysosome -Type II (Fig.4.23).

Lysosomes Type I and II were consistently observed between weeks 3 and 16 of copper-overload.

Membrane-bound spherules, some with granular contents, (Fig.4.24) and crystalline structures (Fig. 4.25) were observed between weeks 4 and 16 of copper-overload. They differed in their elemental contents, the spherules initially had very little copper (Fig.2.26a) which increased later, coincident with the inclusion

of discrete granules (Fig.2.26b).

The crystalline structures were morphologically different from lysosomes but had some similarities with regard to emission profile with Type II lysosomes (Fig.4.27).

Nuclei.

As early as week 2 there was chromatin condensation and crenation of nuclear membranes of the PCT cell nuclei (Fig.4.28), this progressed to karyolysis (Fig.4.29) at week 3. These degenerative changes persisted up to week 6 . X-ray emission spectra of cells with crenated nuclei identified elevated Cu with the addition of P, S and Ca in the nucleolus (Figs.4.30). Elemental residues could not be detected in lysed nuclei.

At 16 weeks the epithelial cells appeared normal (Fig.4.31) and no x-ray emission activity of residual elements was detected (Fig.4.32).

Other subcellular constituents:

Copper was also detected in the luminal debris (Fig.4.33).

Copper was not detected in any other organelle or within the cytosol at any sampling point in the copper-loaded rats.

Neither copper nor other elemental residues were identified within any of the subcellular compartments of the control kidneys.

TABLE 4.1. Liver copper and zinc concentrations (µg/g) dry weight, M ± SEM) in copper-loaded rats. (n=4)

Time (weeks)	Copper concentrations	Zinc concentrations
1	679 ± 40	121 ± 7
2	1346 ± 72	123 ± 3
3	2697 ± 57	144 ± 2
4	2840 ± 46	156 ± 6
5	2358 ±106	132 ± 5
6	2389 ± 91	142 ± 4
8	2429 ±156	120 ± 3
12	2540 ±147	143 _+1
16	2271 ± 93	114 ± 1

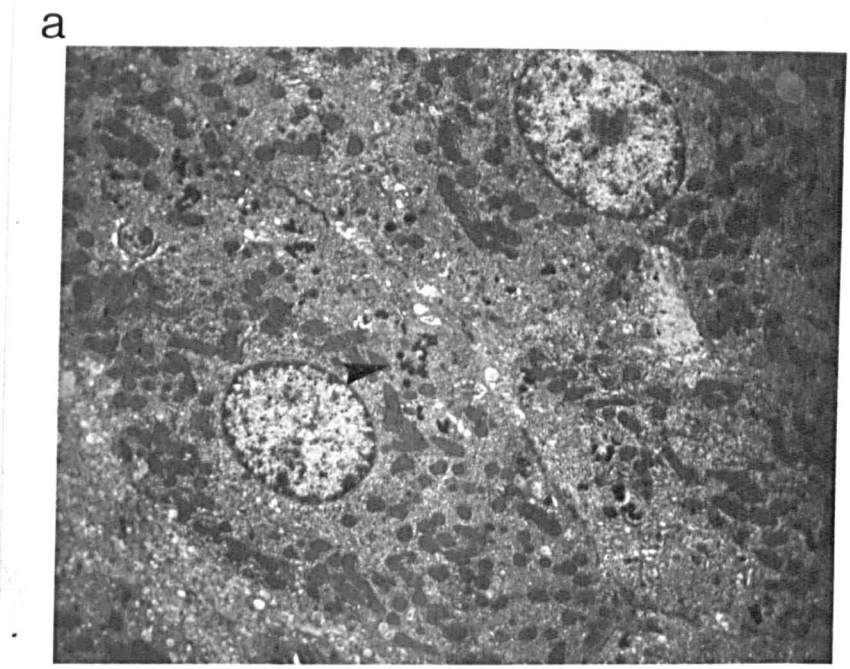
Control copper concentration (n= 20) = 22 ± 1

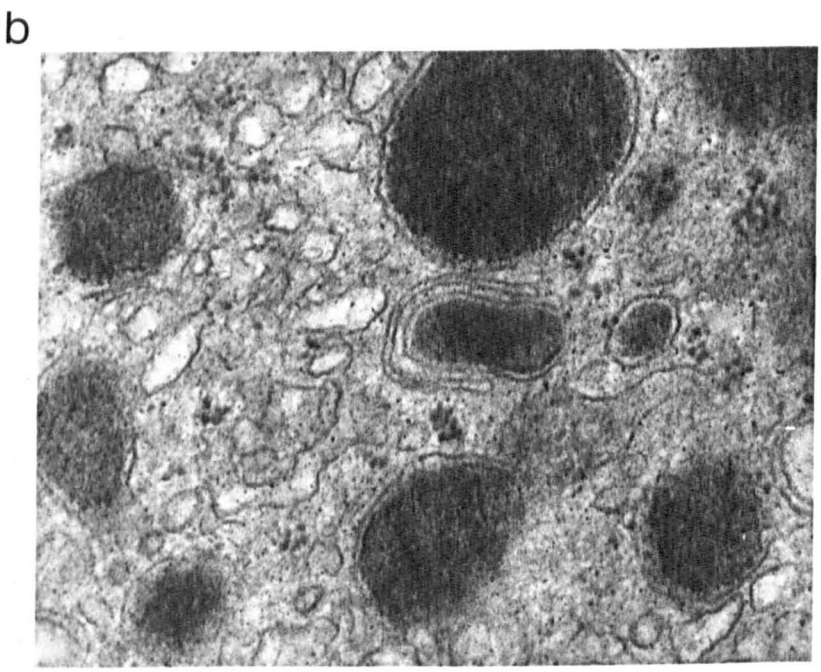
Control zinc concentration (n=20)= 120 ± 2

Table 4.2. Renal copper and zinc concentrations (µg/g) dry weight tissue (M±SEM) of copper-loaded rats. (n=4).

Time (weeks)	Copper concentrations	Zinc concentrations
1	56 ± 2	139 ±2
2	306 ±21	95 ±1
3	458 ±29	113 ±1
4	1,142 ±88	122 ±1
5	1,001 ±64	114 ±3
6	768 ±72	124 ±3
8	526 ±48	121 ±1
12	702 ±50	129 ±2
16	500 ± 30	136 ±3

Control copper concentration (n=20)	34 ± 1
Control zinc concentration (n=20)	134 ± 2





Figures 4.1a and b. Week 1. Copper-loaded rat liver. Electron-dense pericanalicular lysosomes (►). a) x4,200, b) x60,000.

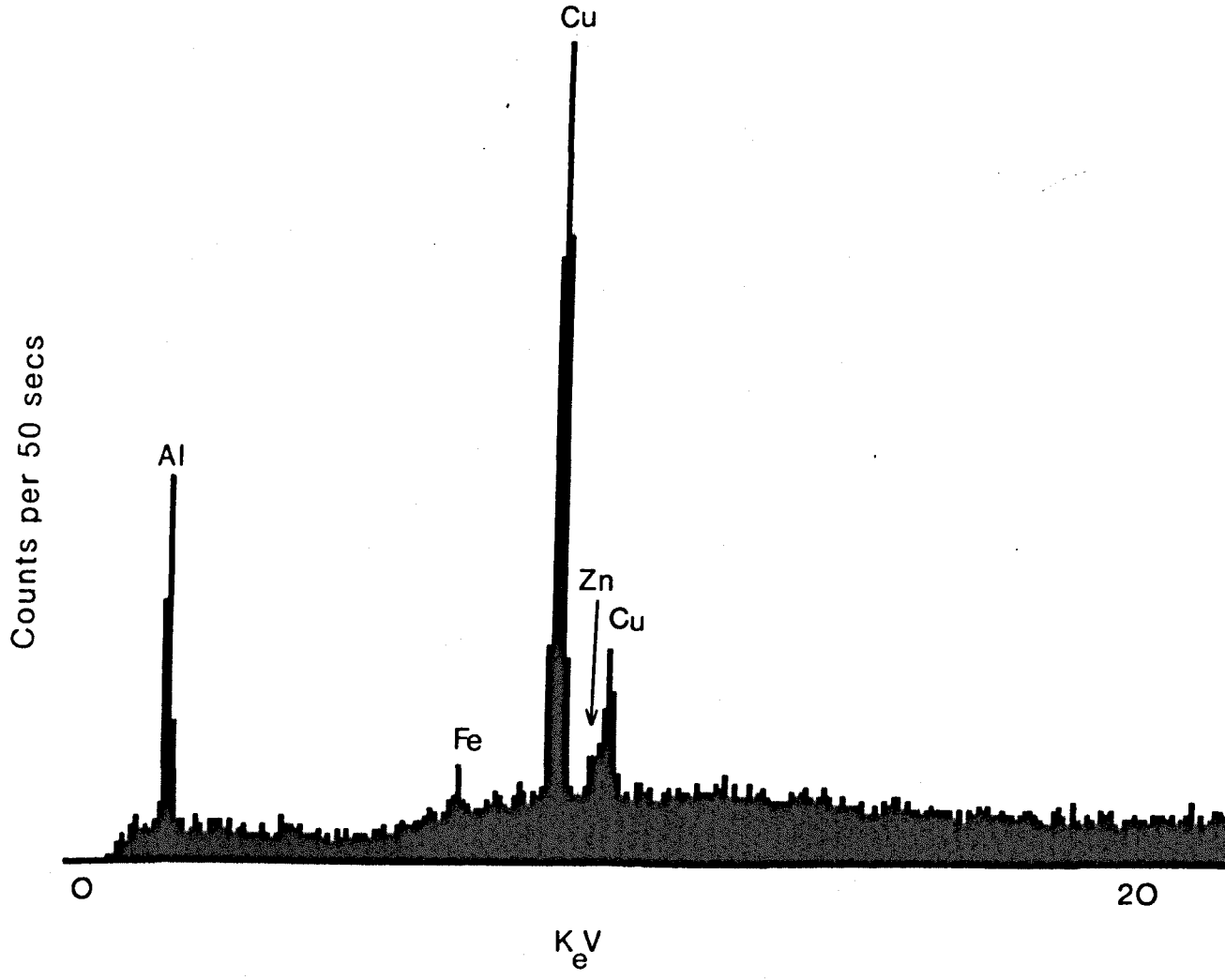


Figure 4.2. Week 1. Copper-loaded rat liver. X-ray emission spectra of lysosomes type I. Presence of copper in addition to Zn and Fe. The source of Al is the grid.

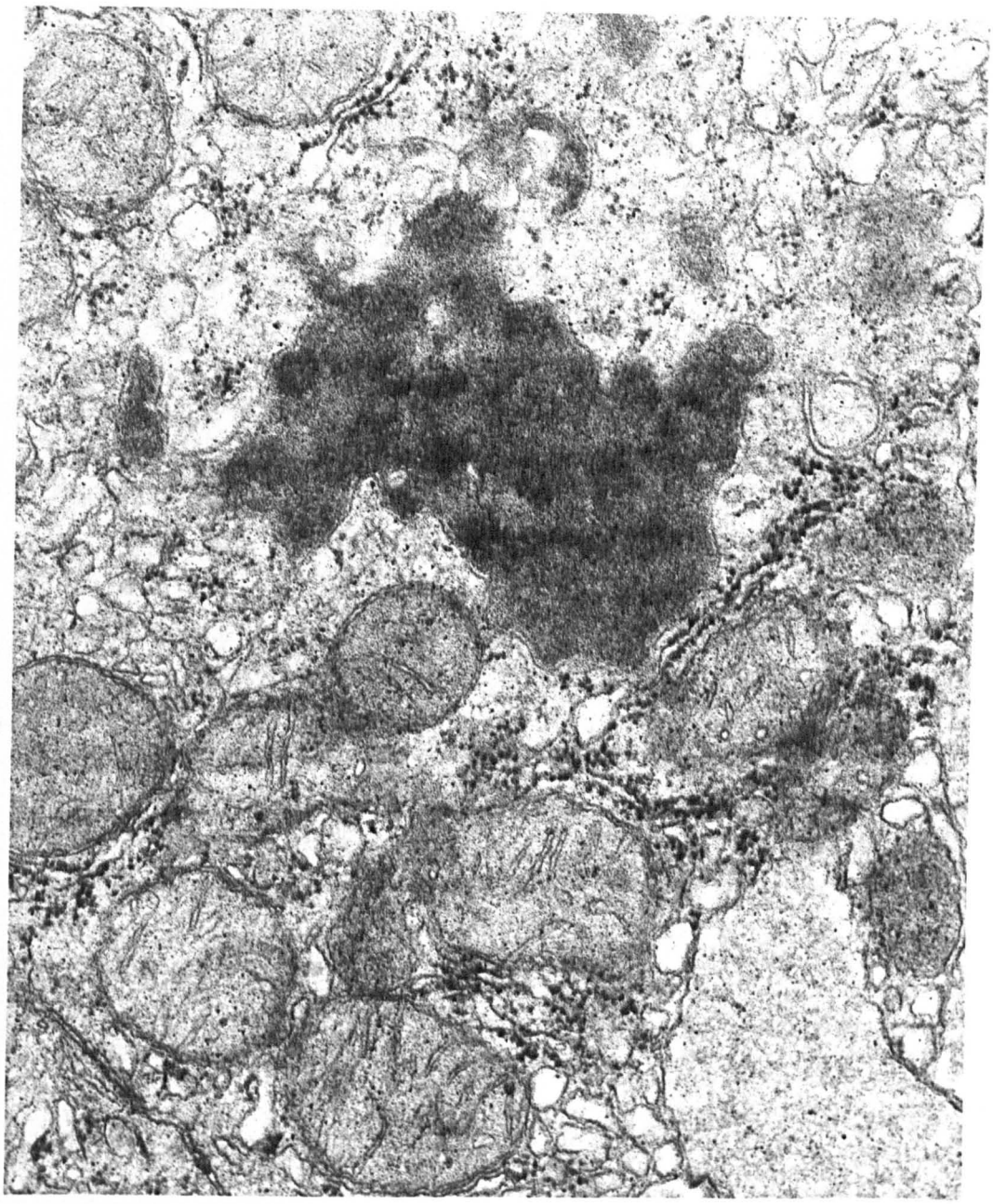


Figure 4.3. Week 3. Copper-loaded rat liver. Irregularly shaped heterogeneous lysosome. x50,000.

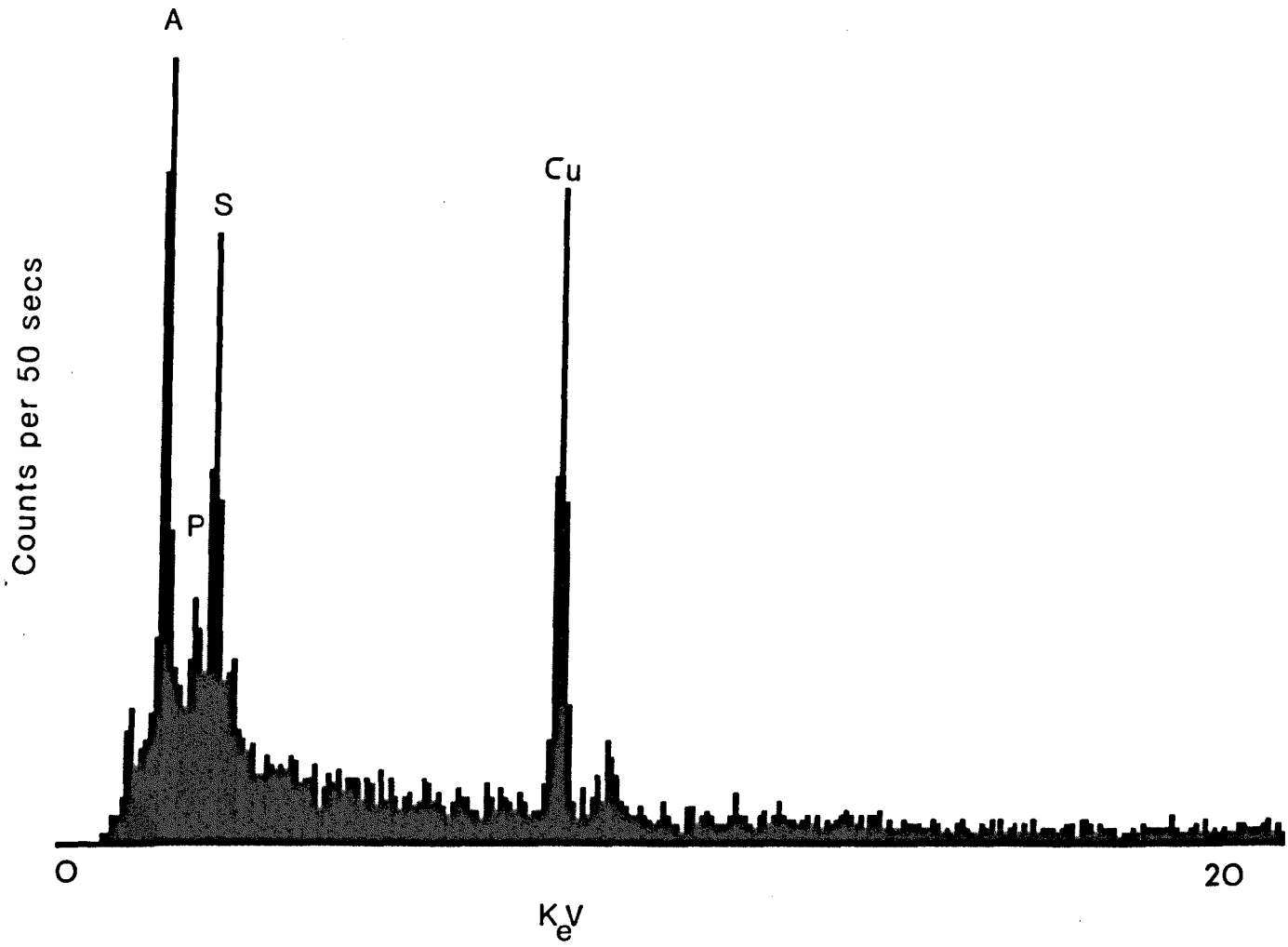
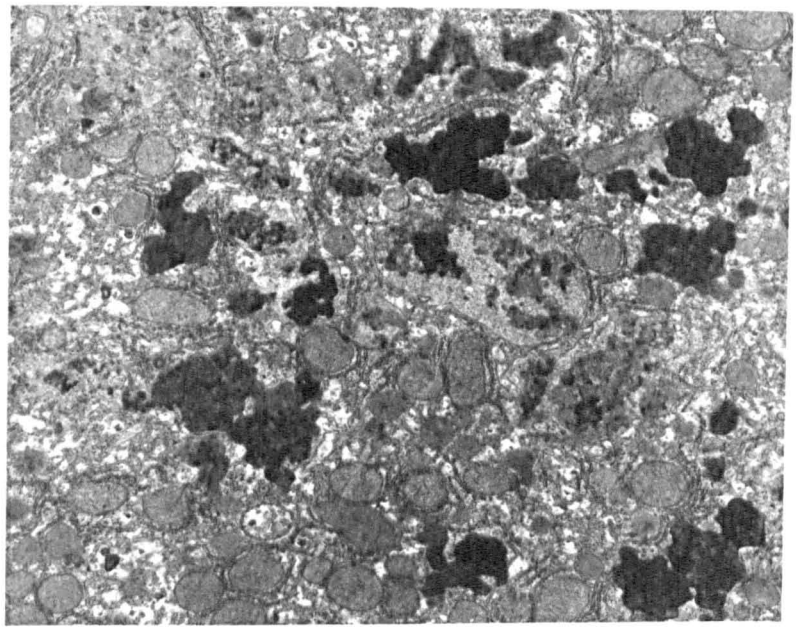
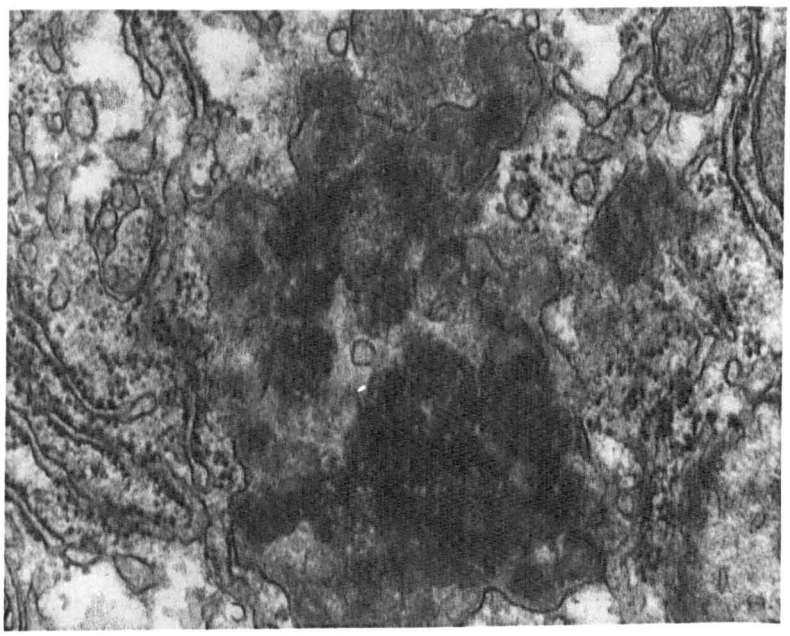


Figure 4.4. Week 3. Copper-loaded rat liver. X-ray emission spectra of lysosome type II within hepatocyte includes P and S in addition to Cu.



a



b

Figure 4.5a and b. Week 4. Copper-loaded rat liver. Irregular shaped lysosomes with granular, sparse, electron-dense contents. a) x9,600, b)x58,000.

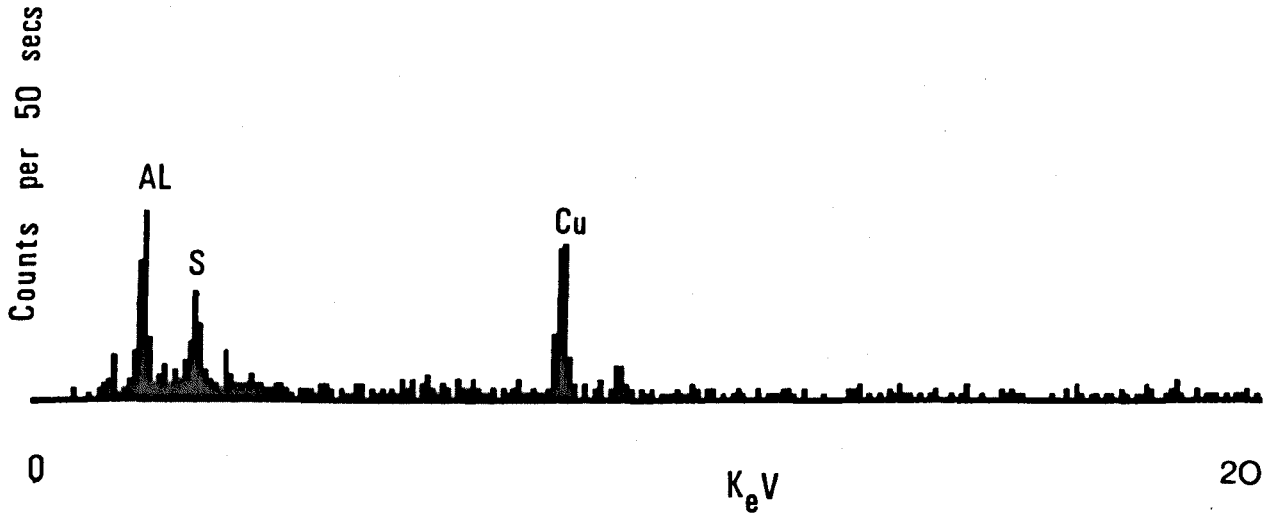


Figure 4.6. Week 4. Copper-loaded rat liver. X-ray emission spectra of lysosome type III. Low levels of copper and other residual elements.

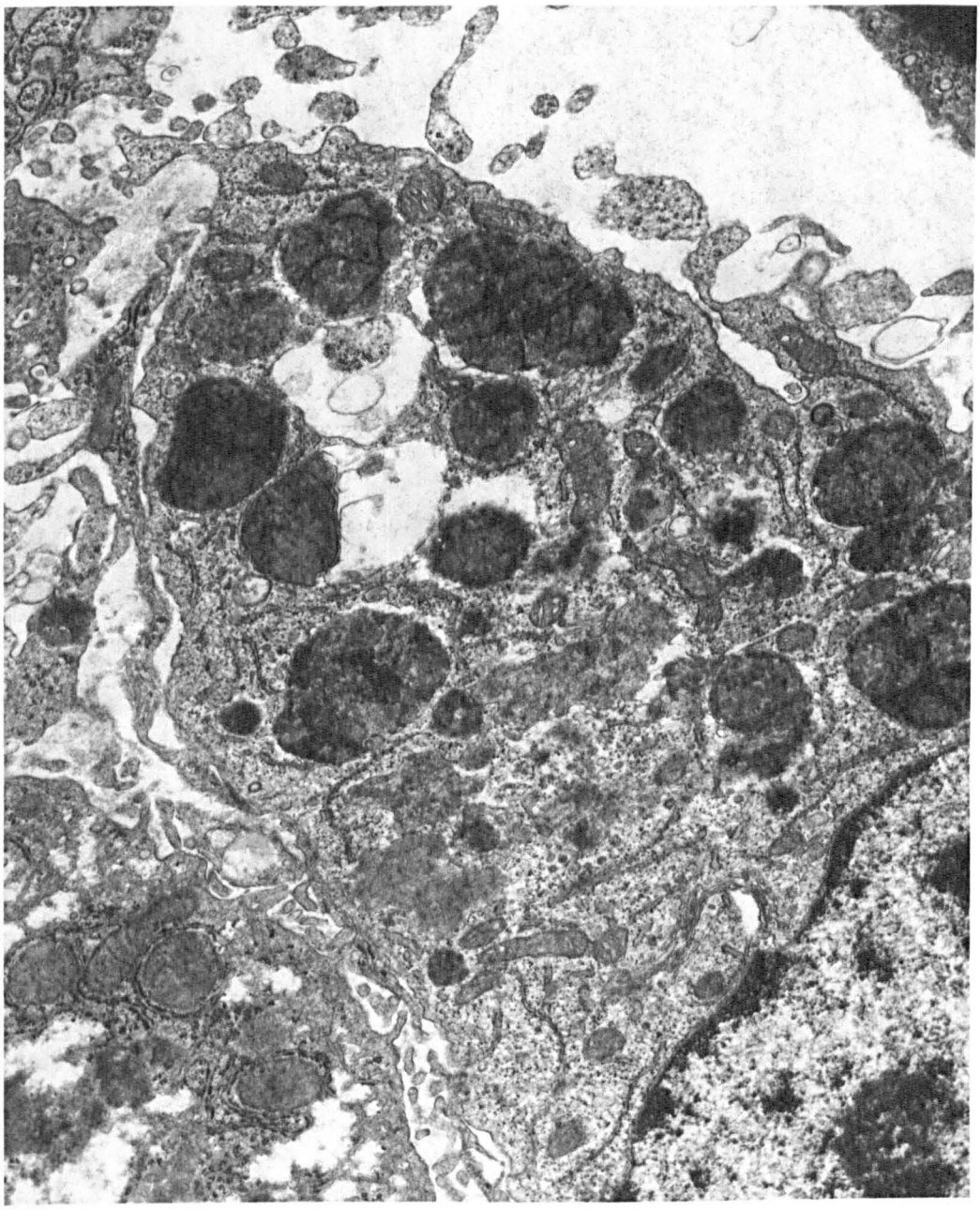


Figure 4.7. Week 5. Copper-loaded rat liver. Presence of a heterogeneous population of lysosomes within a Kupffer cell. x16,000.

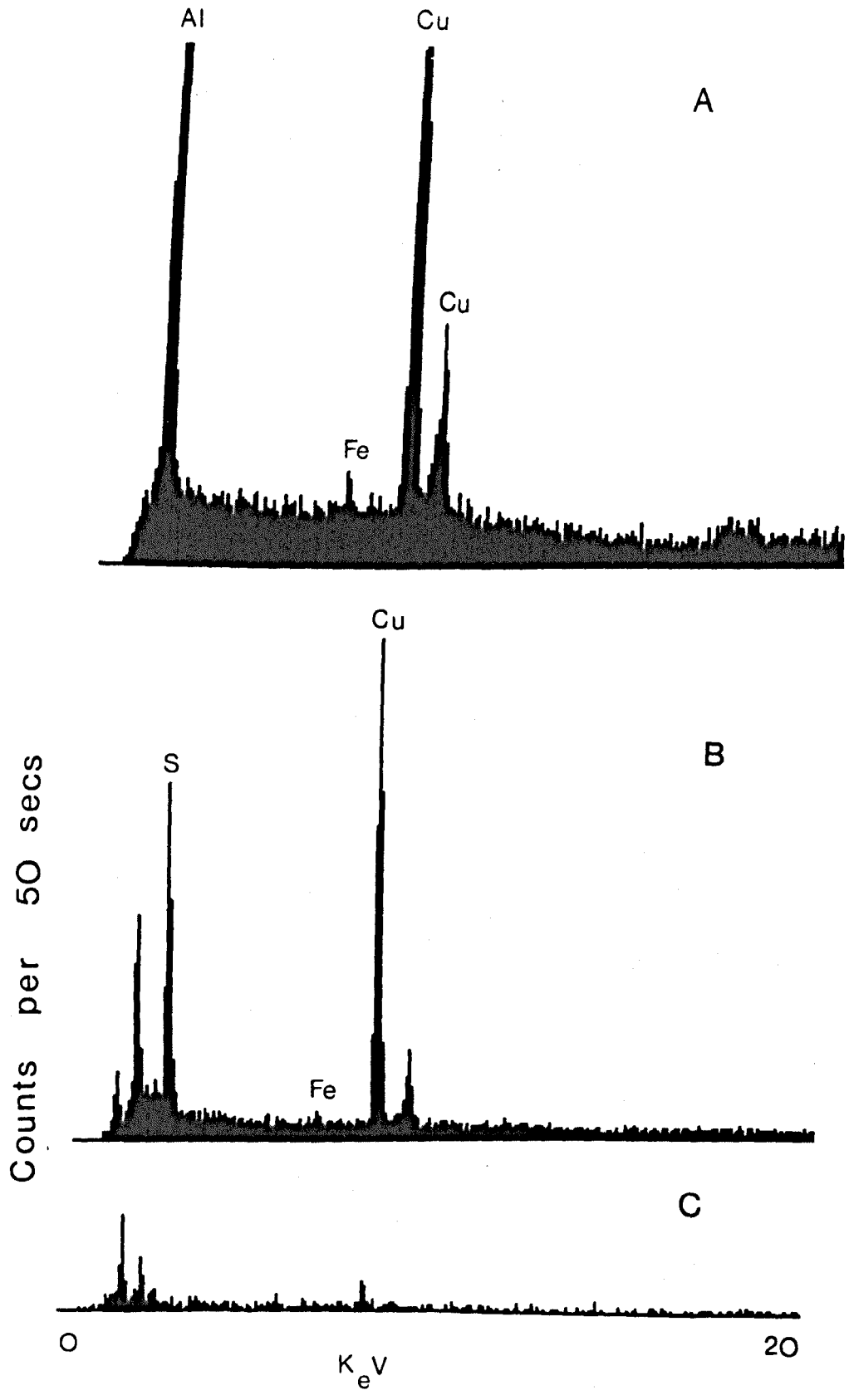


Figure 4.8. Week 1 (A), 3 (B) and 8 (C). Copper-loaded rat liver. X- ray emission spectra of lysosome Types I, II and III in Kupffer cells.

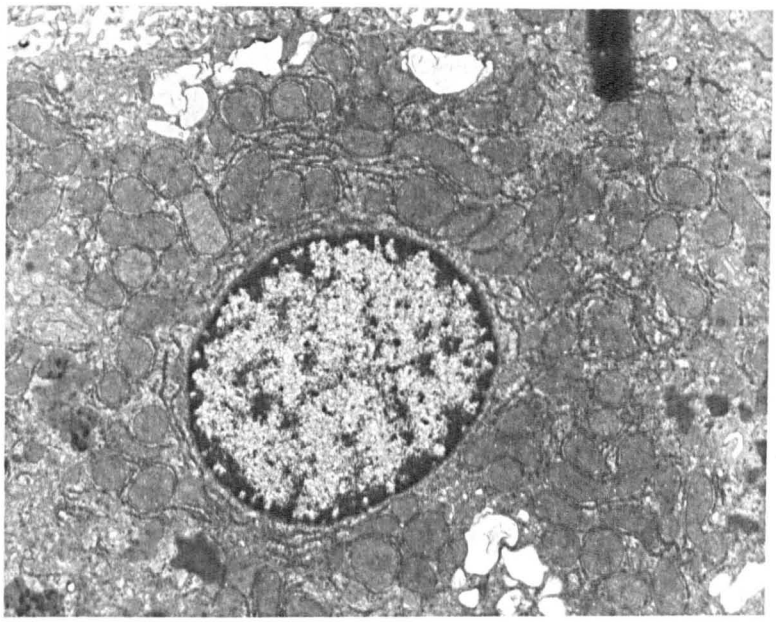


Figure 4.9a. Week 2. Copper-loaded rat liver, Condensation of chromatin in a hepatic cell nucleus. x9,600.

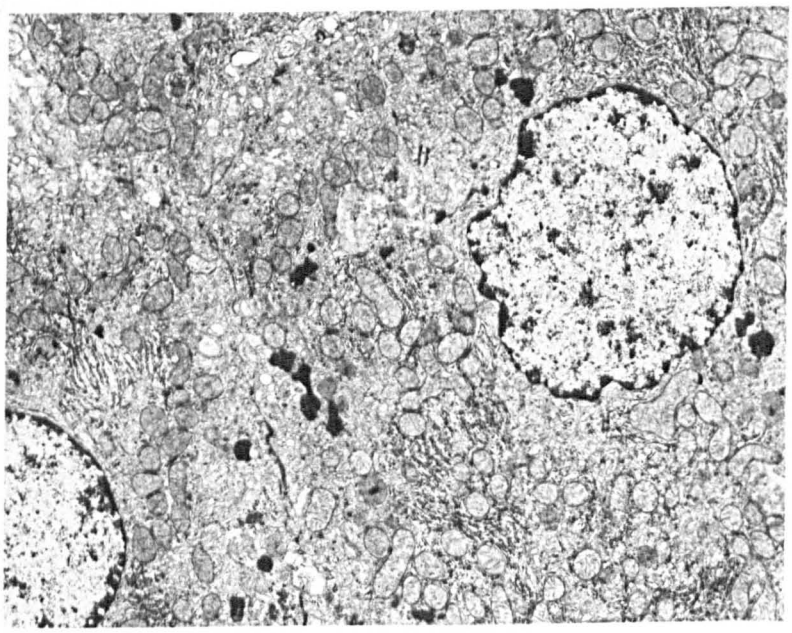


Figure 4.9b. Week 3. Copper-loaded rat liver. Crenated appearance of hepatic cell nucleus. x3,600.

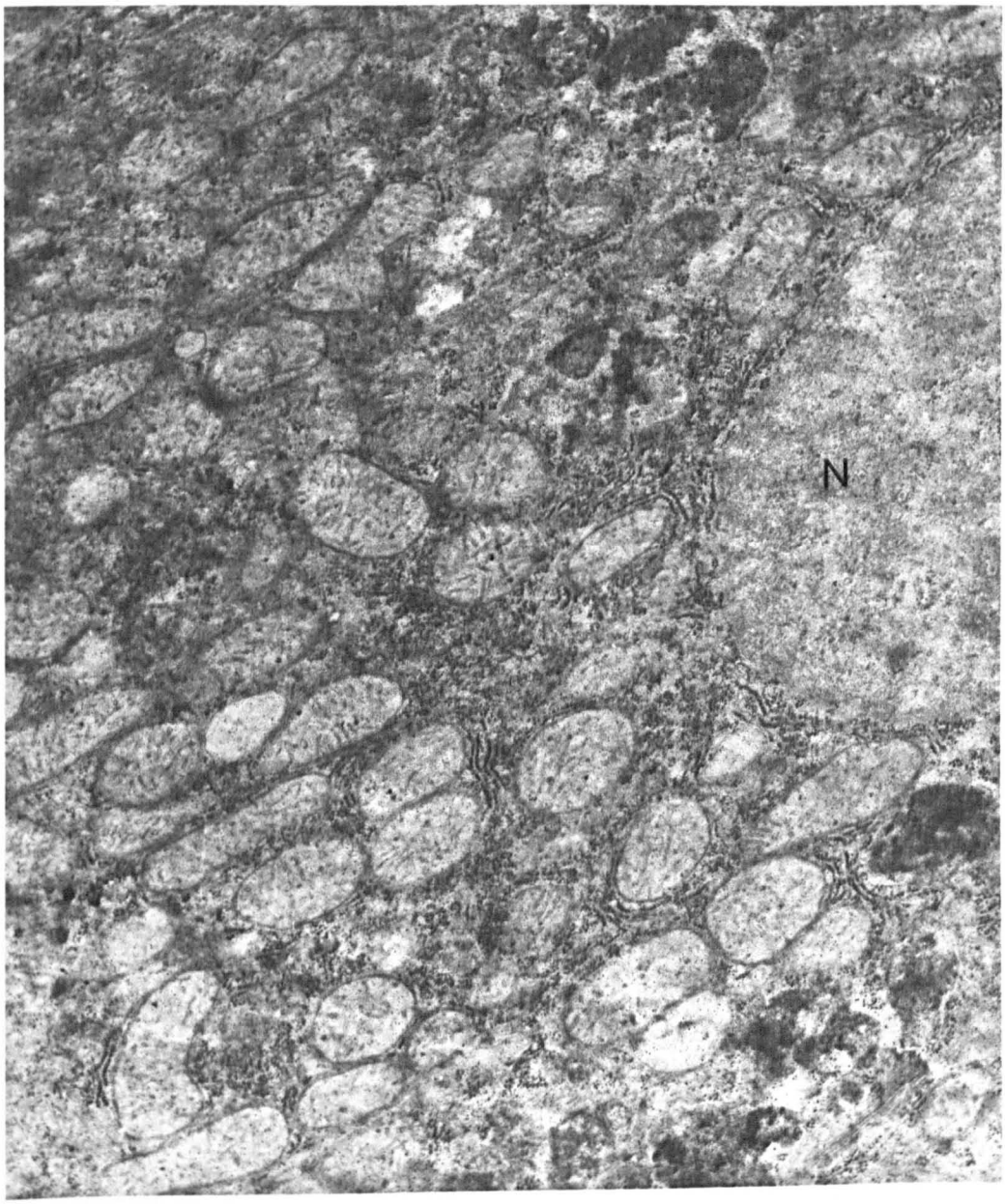


Figure 4.10. Week 4. Copper-loaded rat liver. Karyolysis of hepatic cell nucleus x24,000.

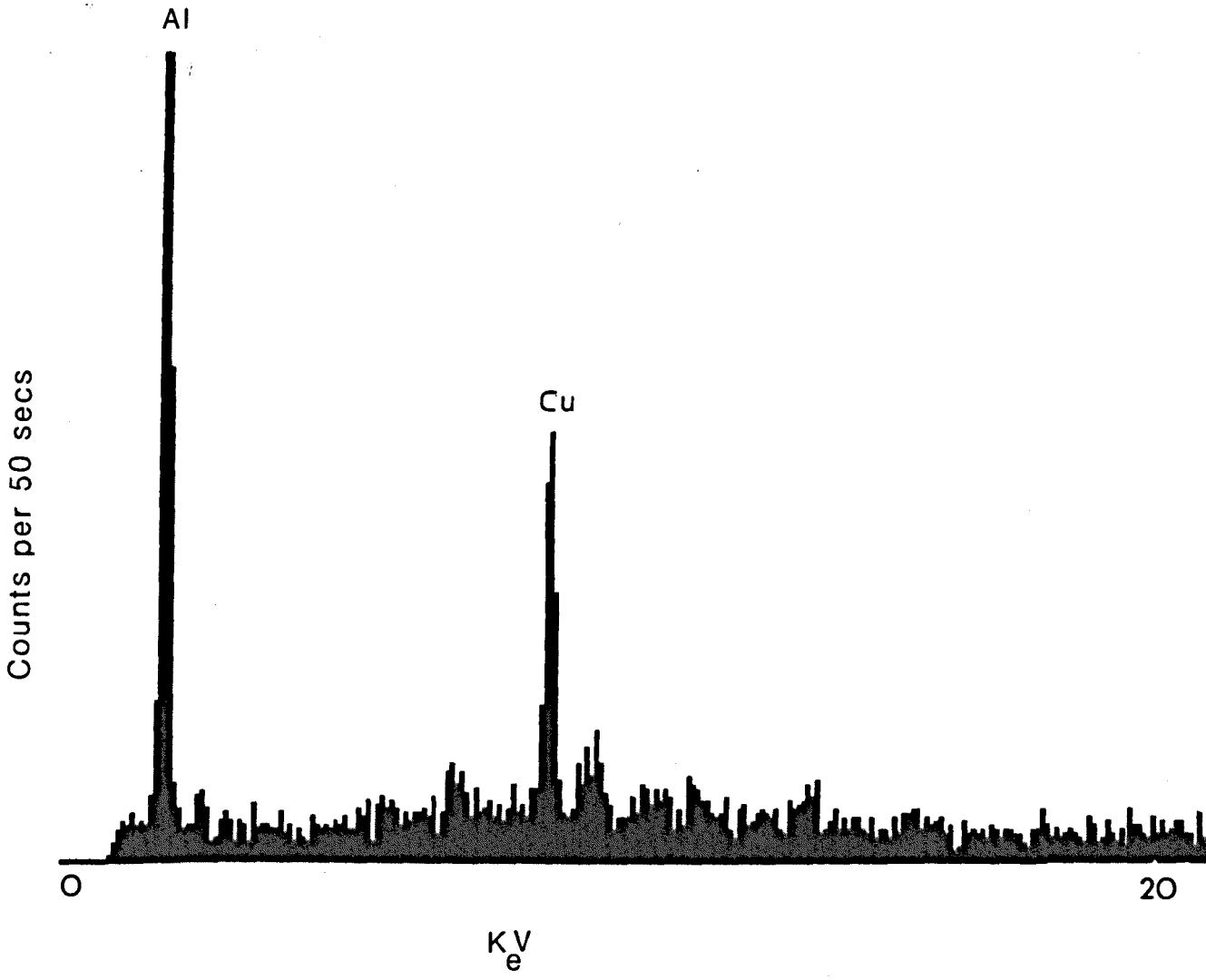


Figure 4.11 . Week 2. Copper-loaded rat liver. X-ray emission spectra of a hepatic cell nucleus with condensation of chromatin.

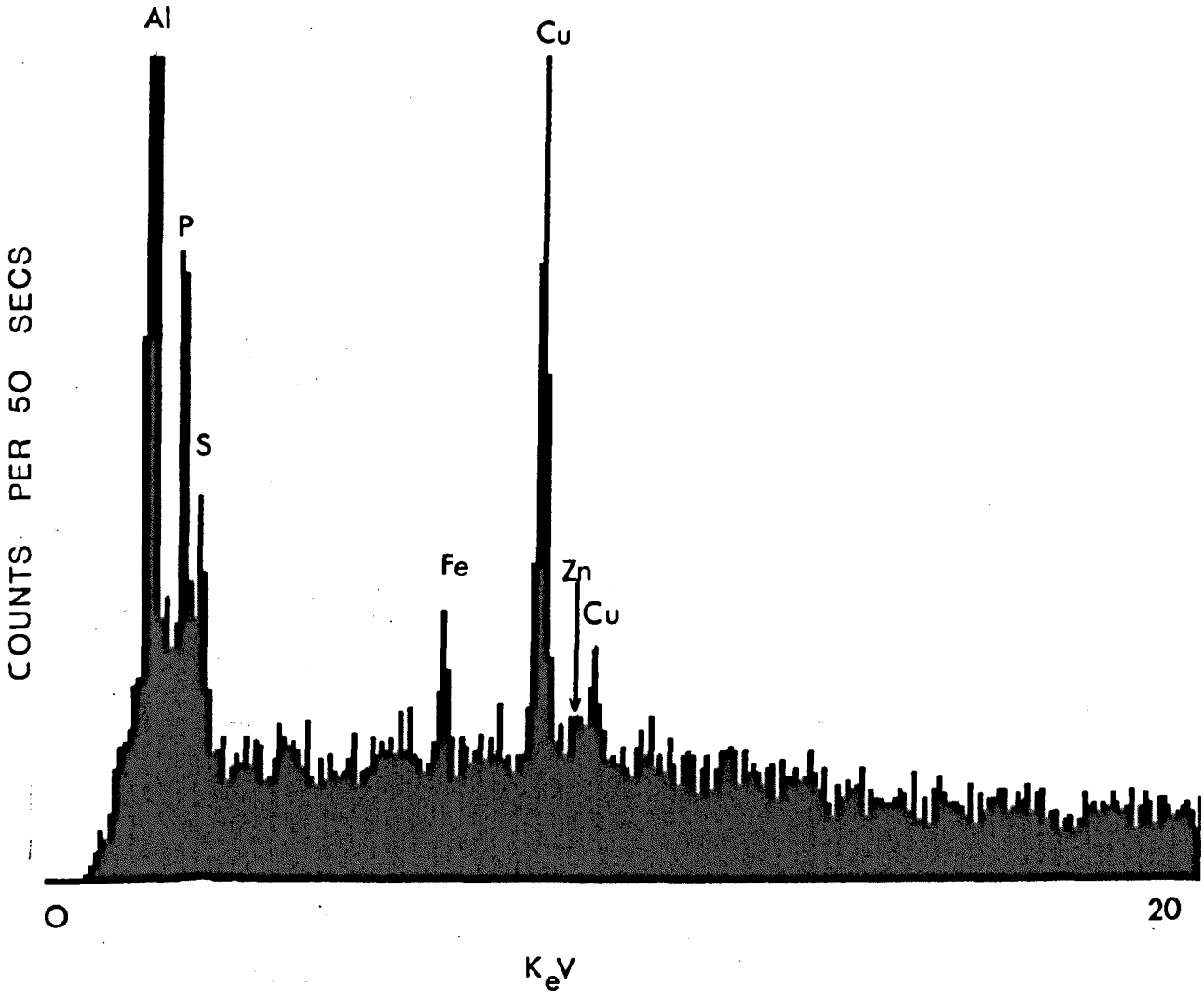


Figure 4.12. Week 4. Copper-loaded rat liver. X-ray emission spectra of nucleolus in hepatic cell degenerated nucleus. In addition to high levels of copper there is an increase in P, S, Fe and a small peak of Zn.

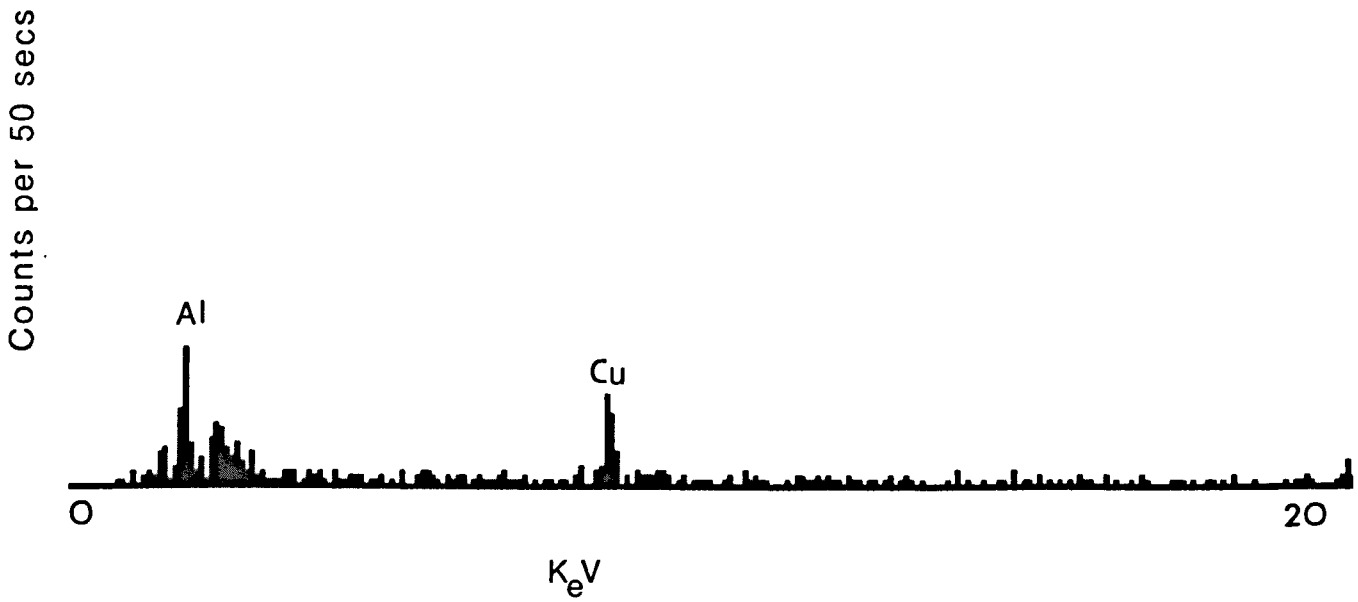


Figure 4.13. Week 16. Copper-loaded rat liver. X-ray emission spectra of an hepatic cell nucleolus. Low levels of copper and other residual elements

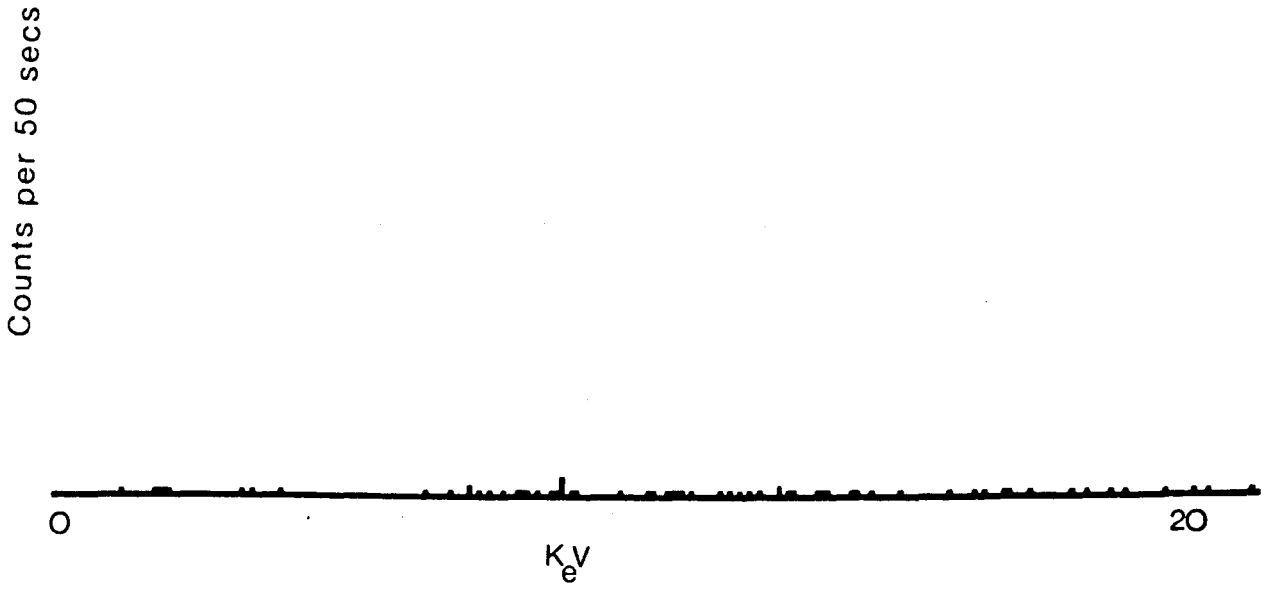


Figure 4.14. X-ray emission spectra of a control rat liver nucleus.

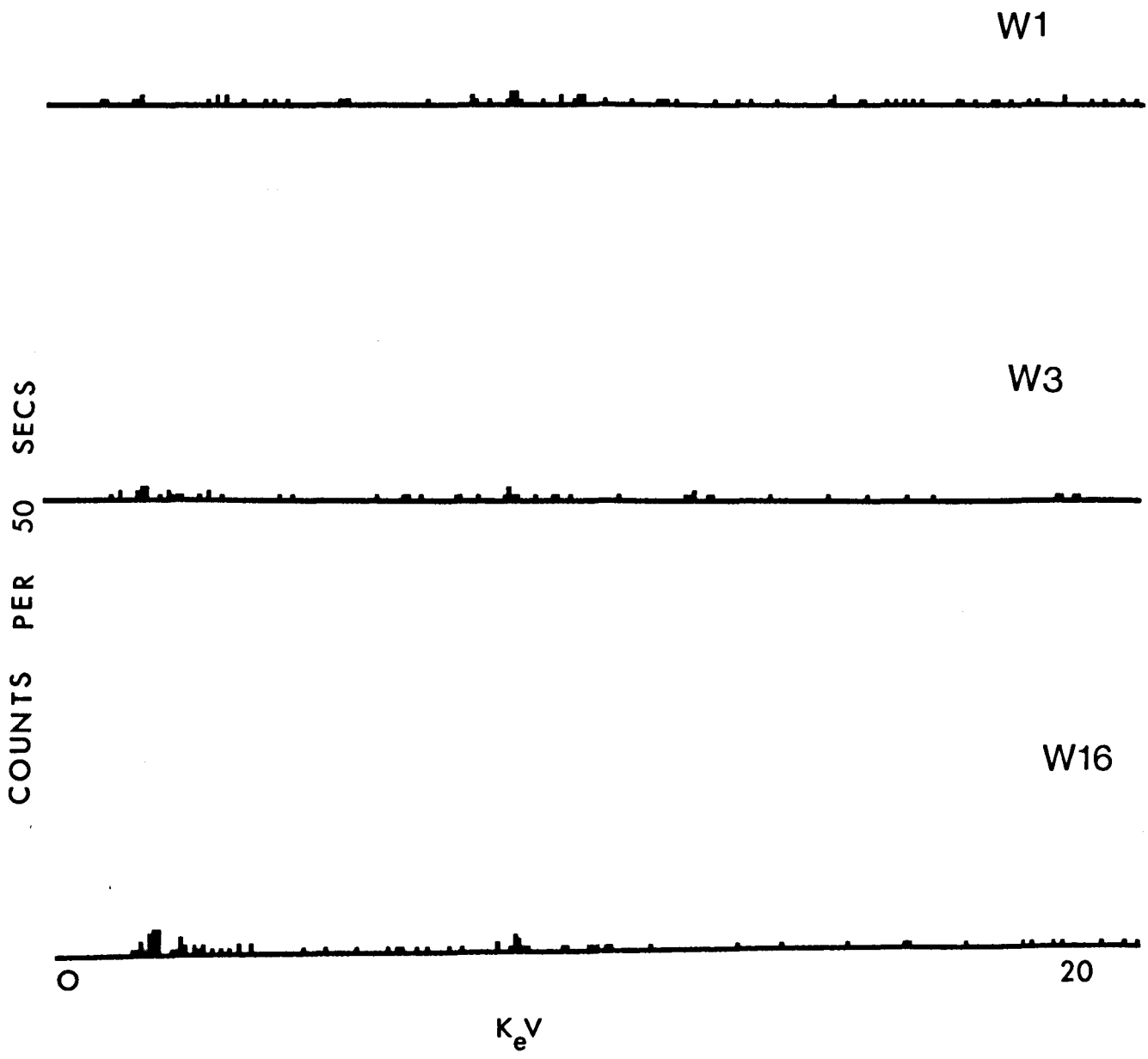


Figure 4.15. Weeks 1,3 and 16. Copper-loaded rat liver. X-ray emission spectra of a Kupffer cell nucleus after 1, 3 and 16 weeks of copper overload. In all of them there are insignificant levels of copper and other residual elements.

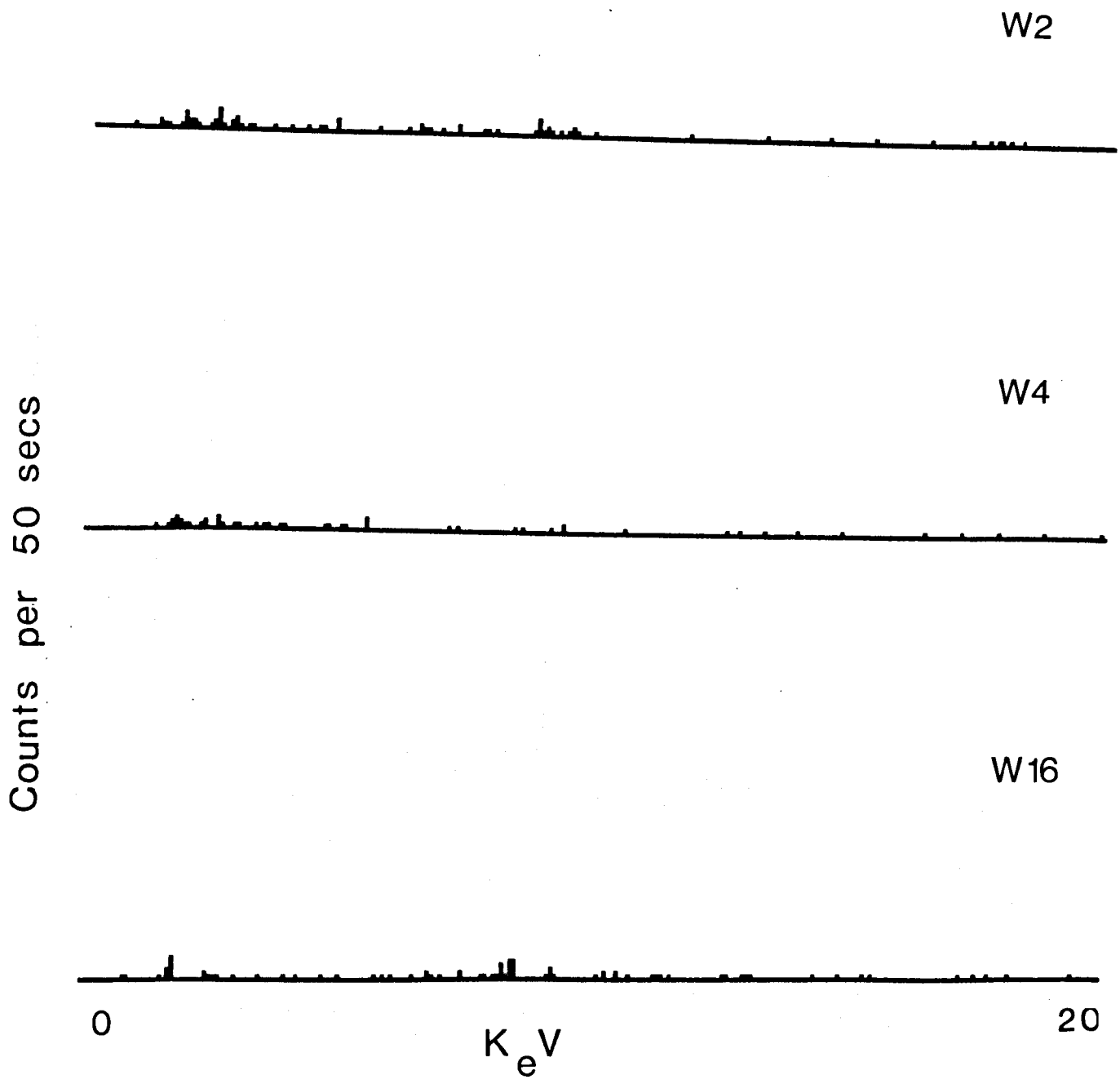


Figure 4.16. Weeks 2, 4, 16. Copper-loaded rat liver. X-ray emission spectra of hepatic cell cytoplasm.

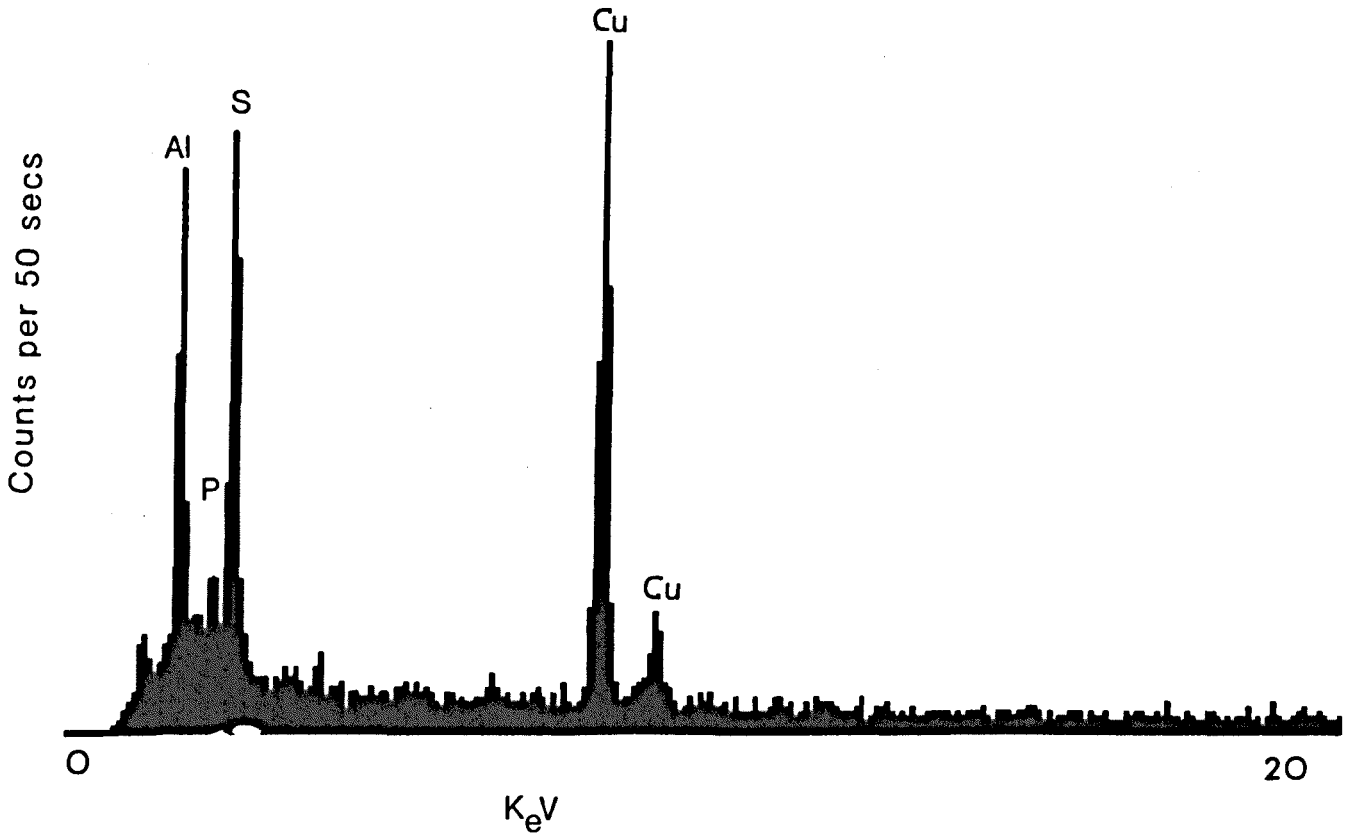


Figure 4.17. Week 5. Copper-loaded rat liver. X-ray emission spectra of apoptotic body. High levels of copper, sulphur and presence of phosphorus.

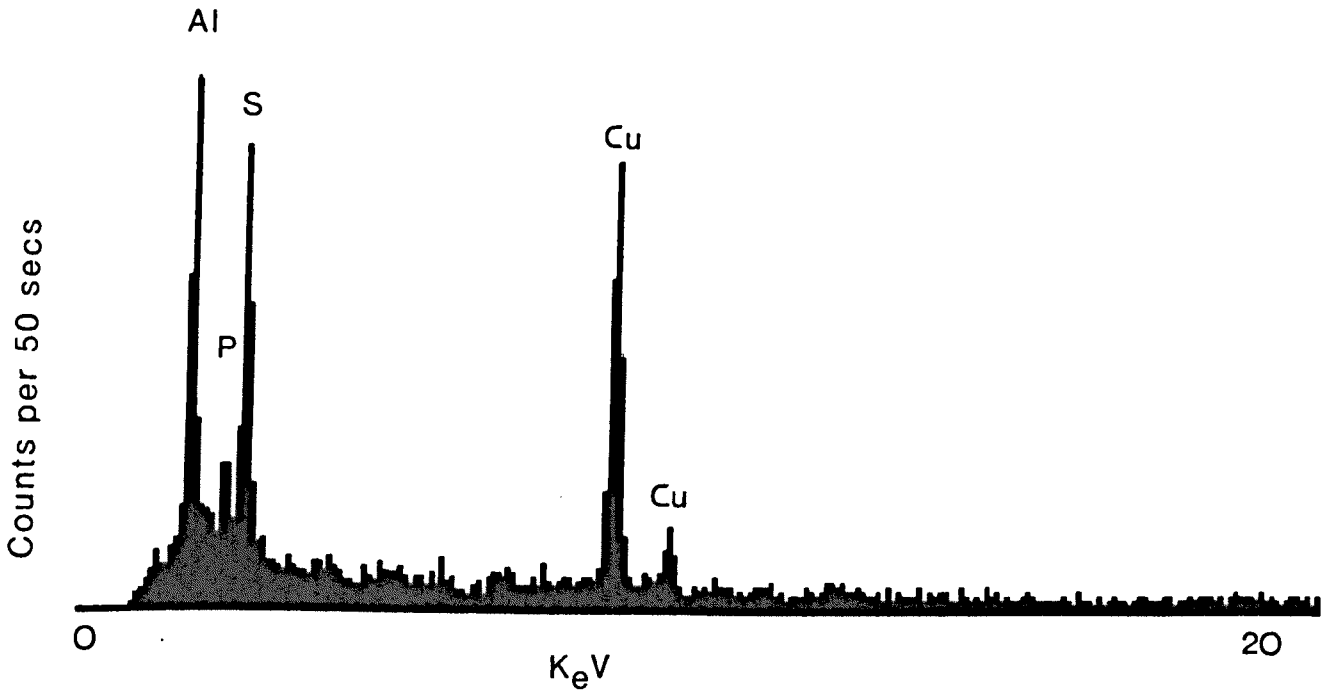


Figure 4.18. Week 16. Copper-loaded rat liver. X-ray emission spectra of cellular debris within sinusoids.

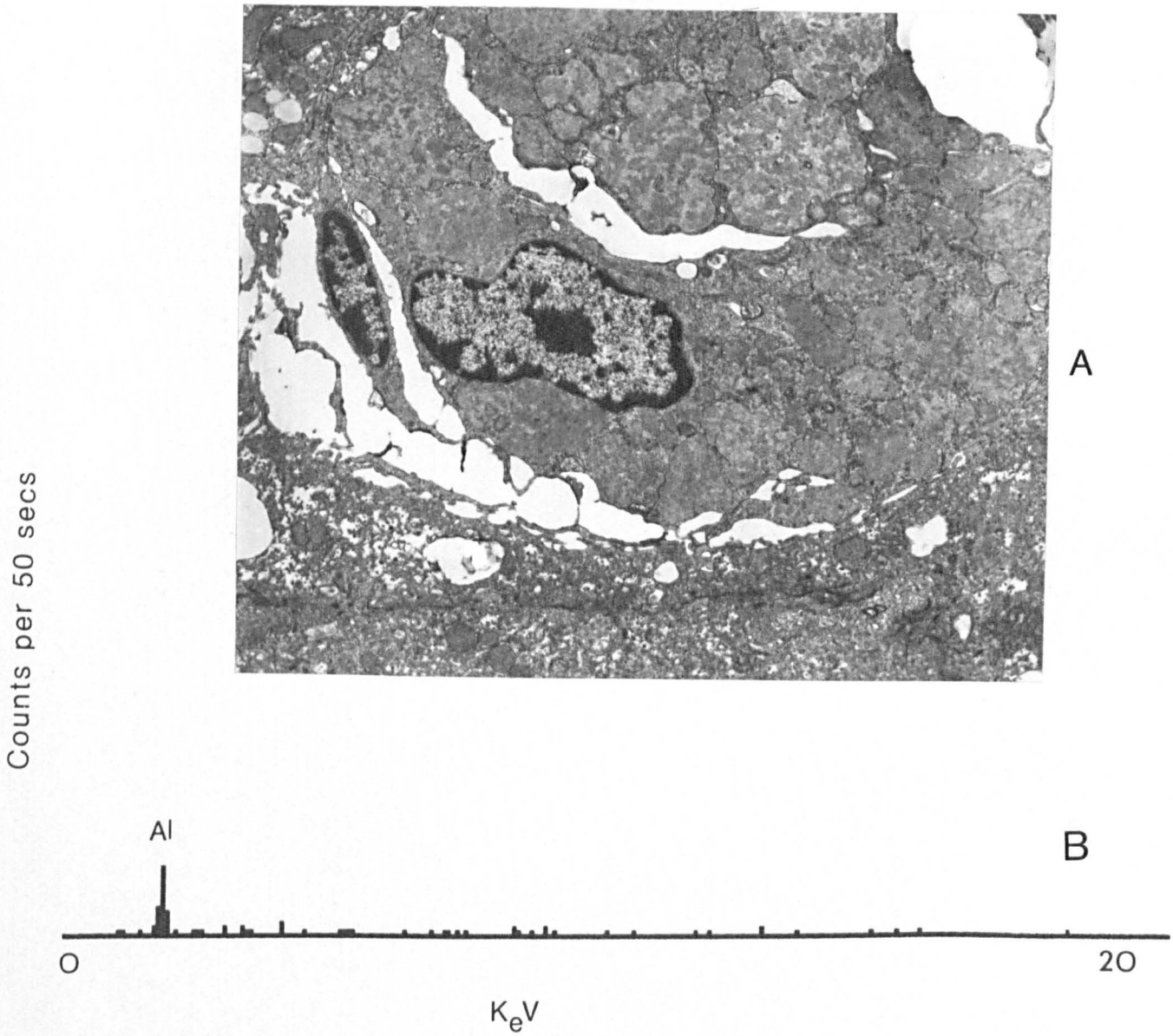


Figure 4.19. Week 12. Copper-loaded rat liver. A) Mallory body-like structure. B) X-ray emission spectra of Mallory body-like structures. No metal was detected.

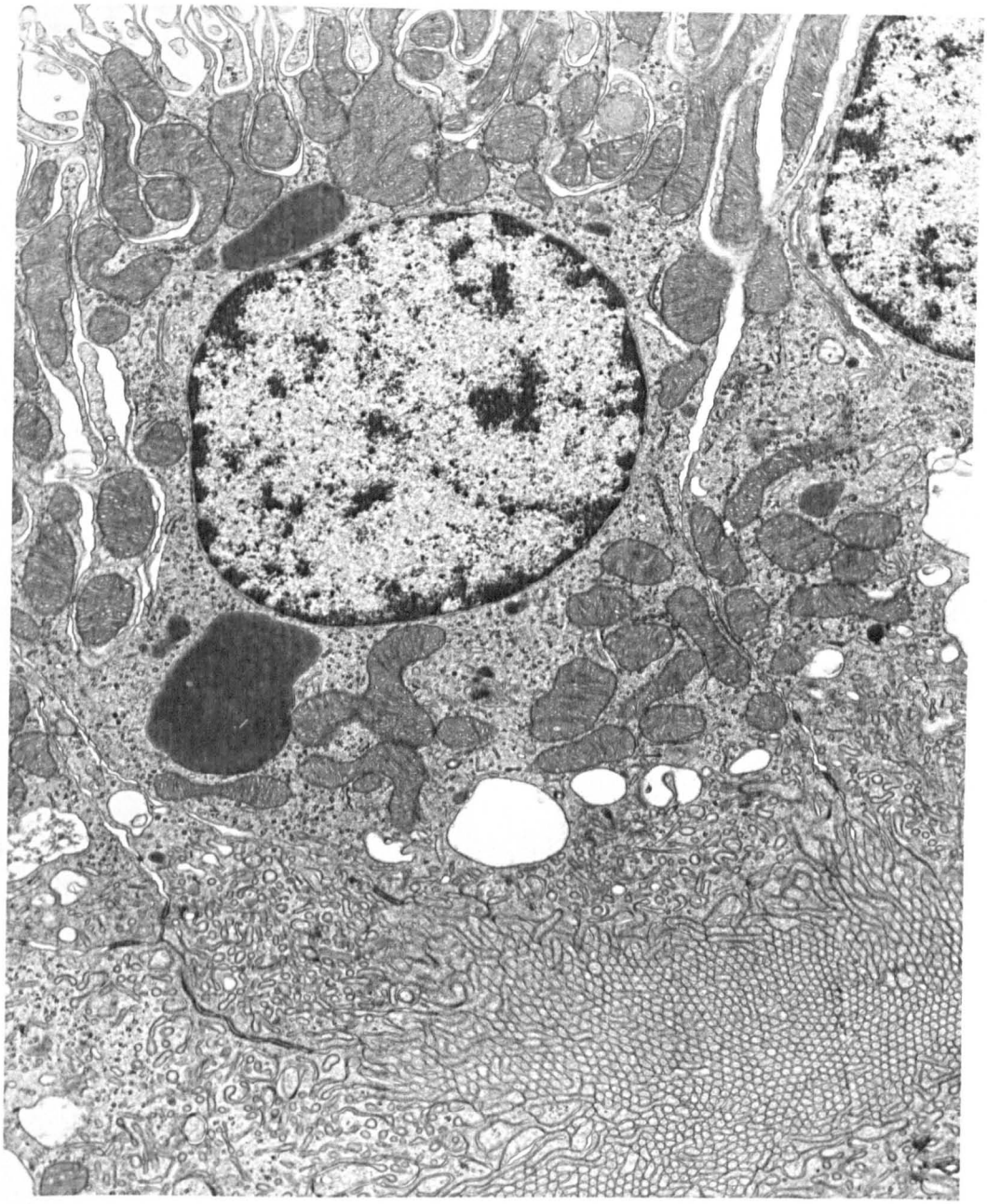


Figure 4.20. Week 1. Copper-loaded rat kidney. Proximal convoluted tubular cell. Presence of electron dense lysosomes. x12,000.

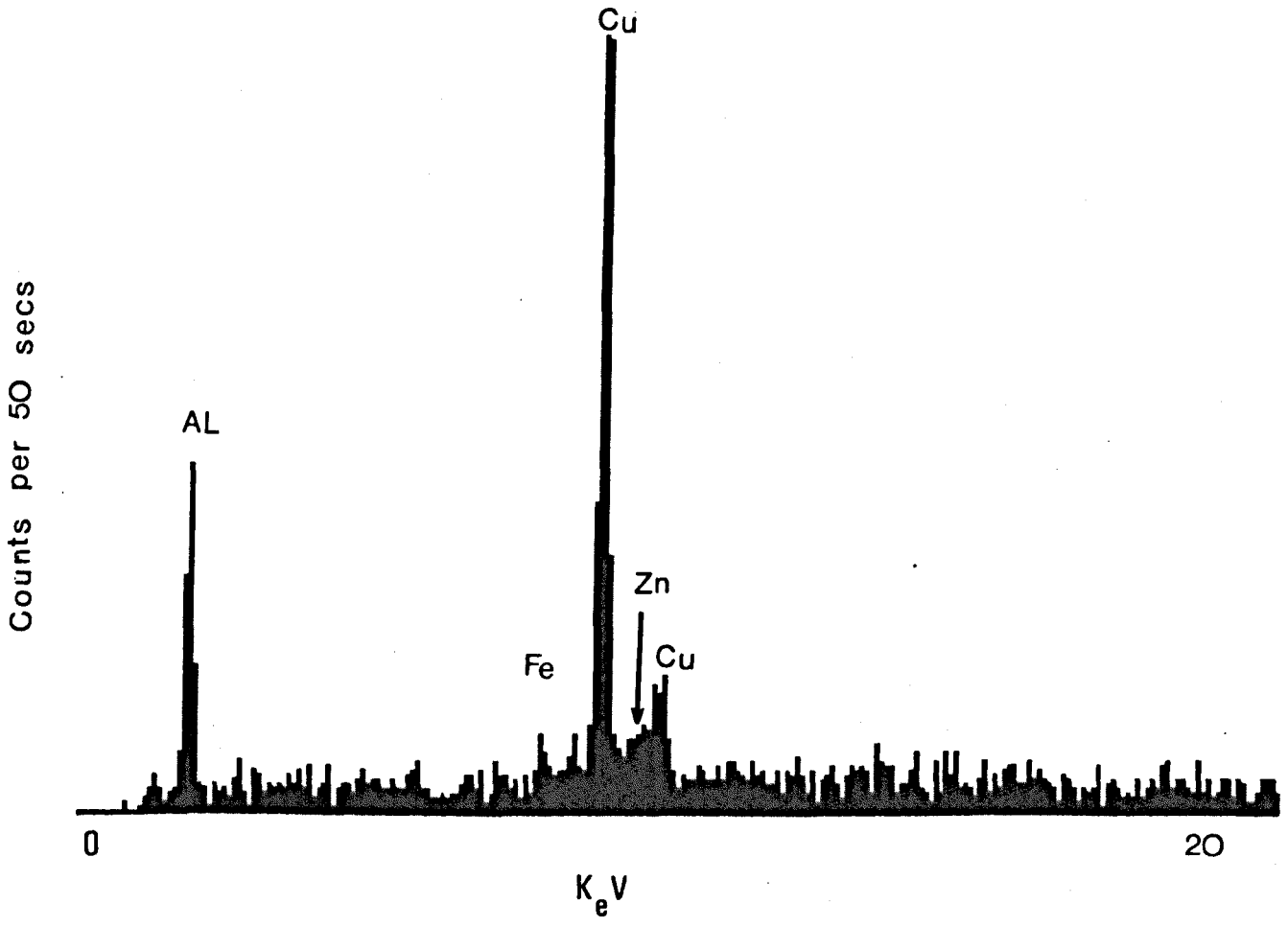


Figure 4.21. Week 2. Copper-loaded rat kidney. X-ray emission spectra of Lysosome type I in a PCT epithelial cell.

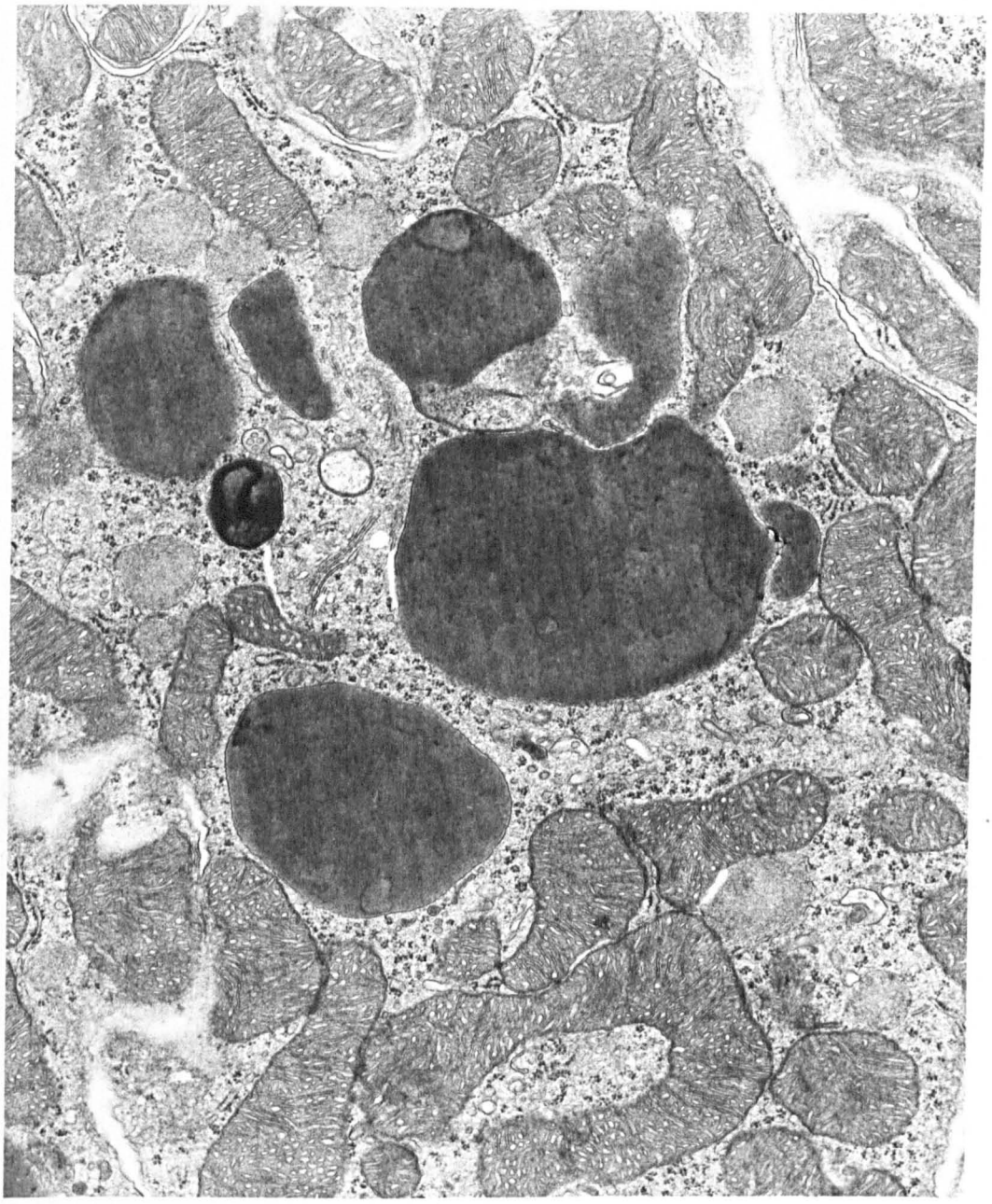


Figure 4.22. Week 4. Copper-loaded rat kidney. Lysosomes in a PCT epithelial cell. Lysosomes are bigger and more numerous than those observed during the previous weeks. x20,000.

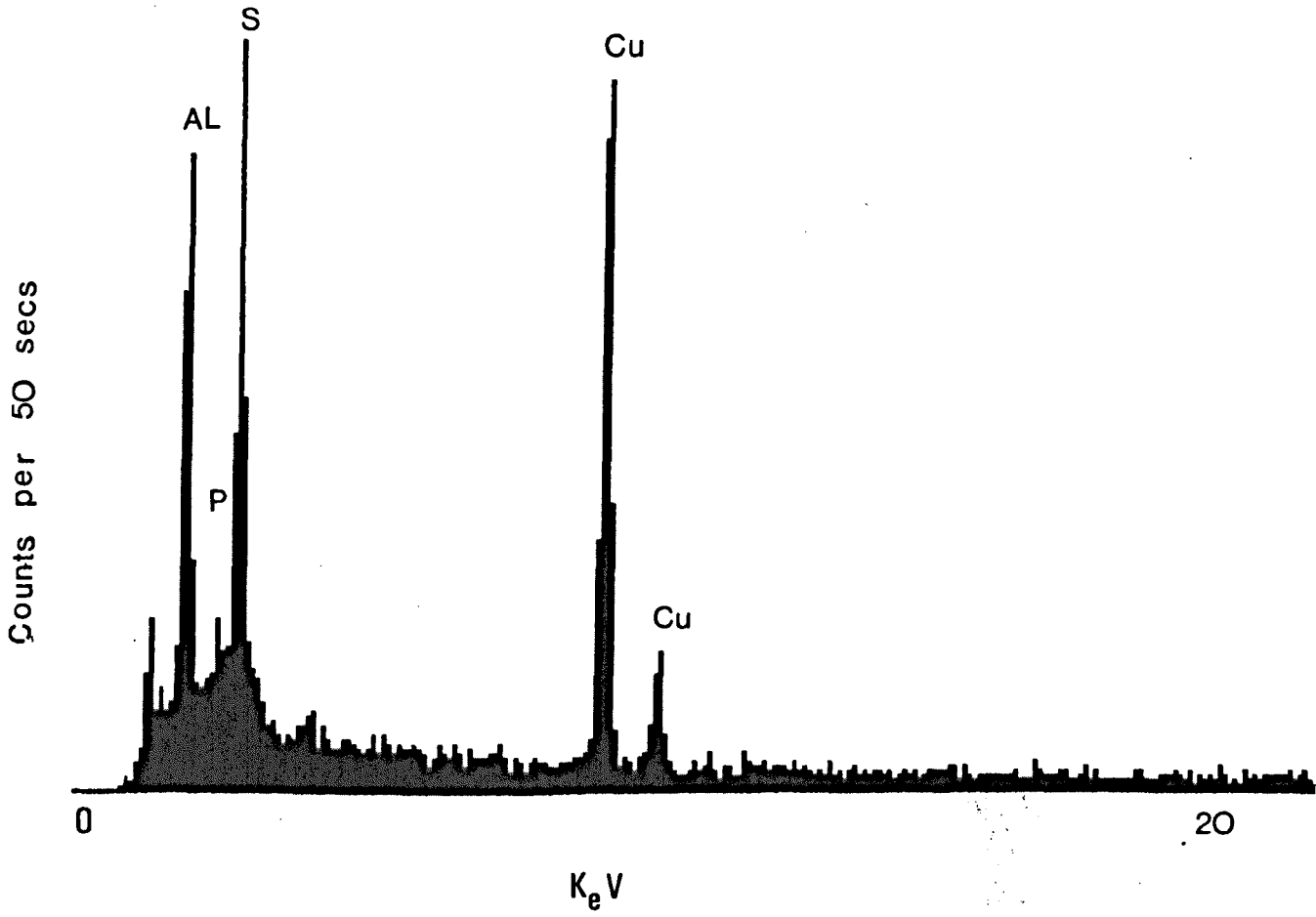


Figure 4.23. Week 5. Copper-loaded rat kidney. X-ray emission spectra of Lysosome type II in a PCT epithelial cell. High levels of Cu, S, and P.

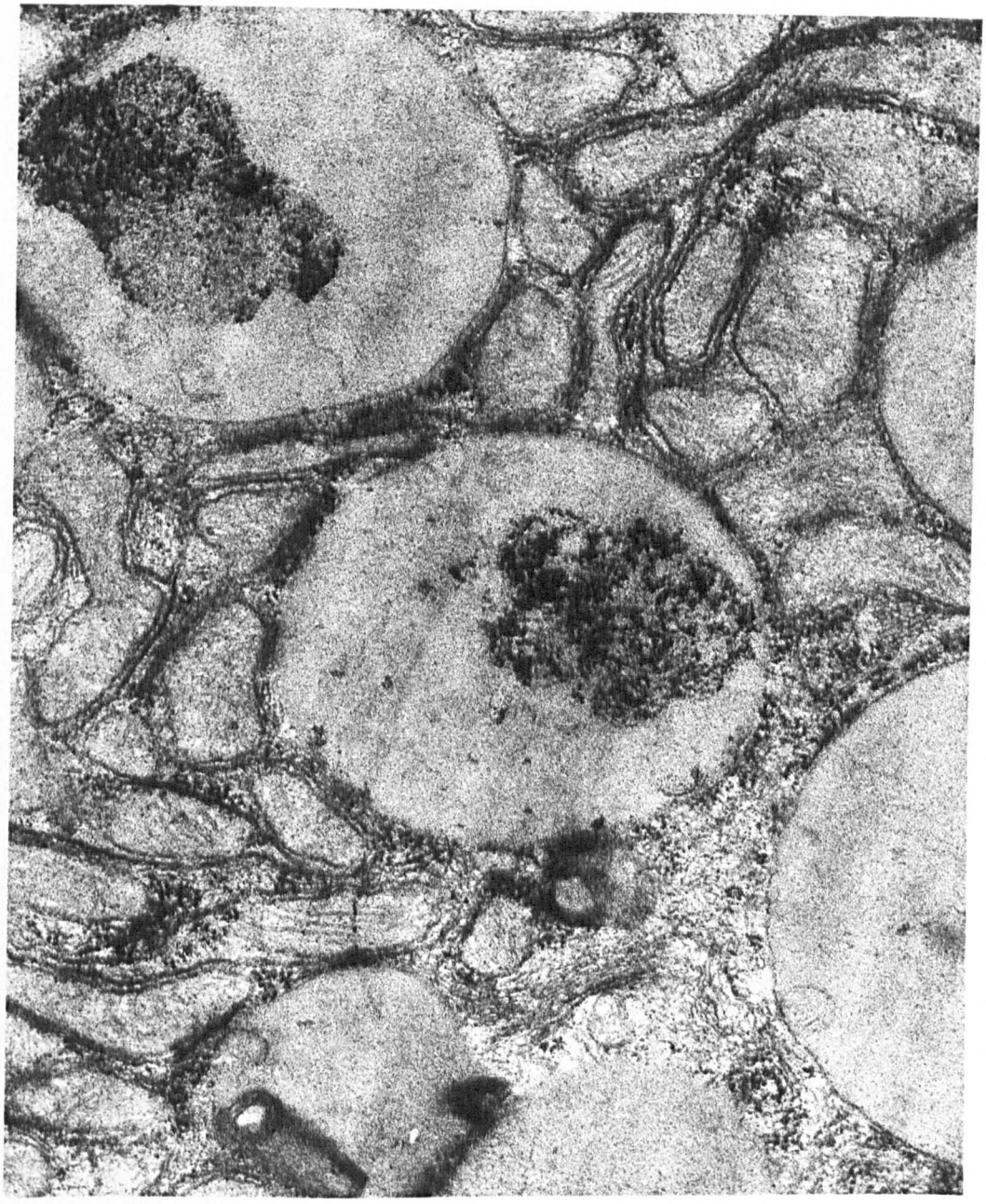


Figure 4.24. Week 4. Copper-loaded rat kidney. Electron-lucent spherules with granular content in the PCT epithelium. x40,000.

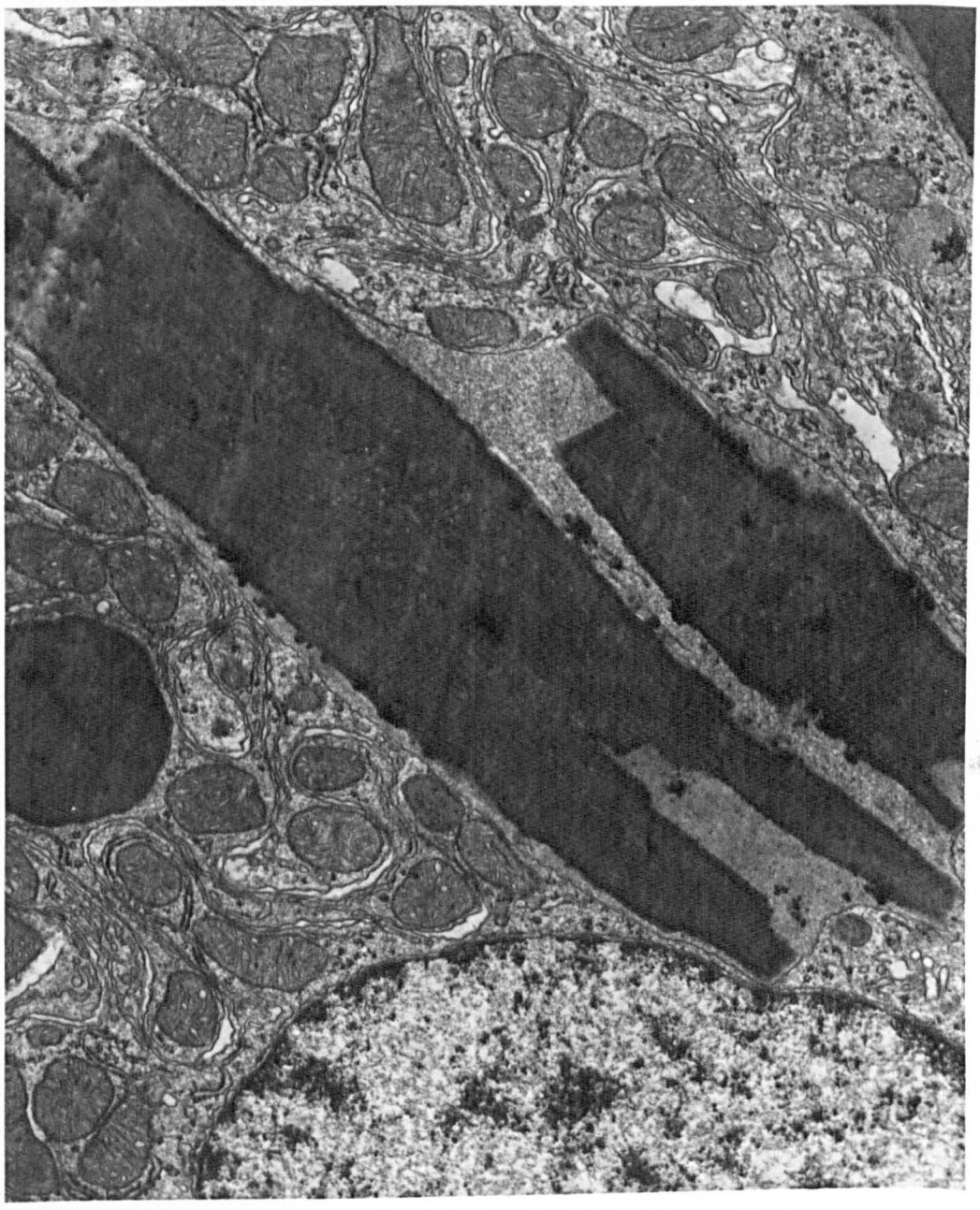


Figure 4.25. Week 5. Copper-loaded rat kidney. Membrane-bounded crystalline structures in the PCT epithelial cells. x20,000.

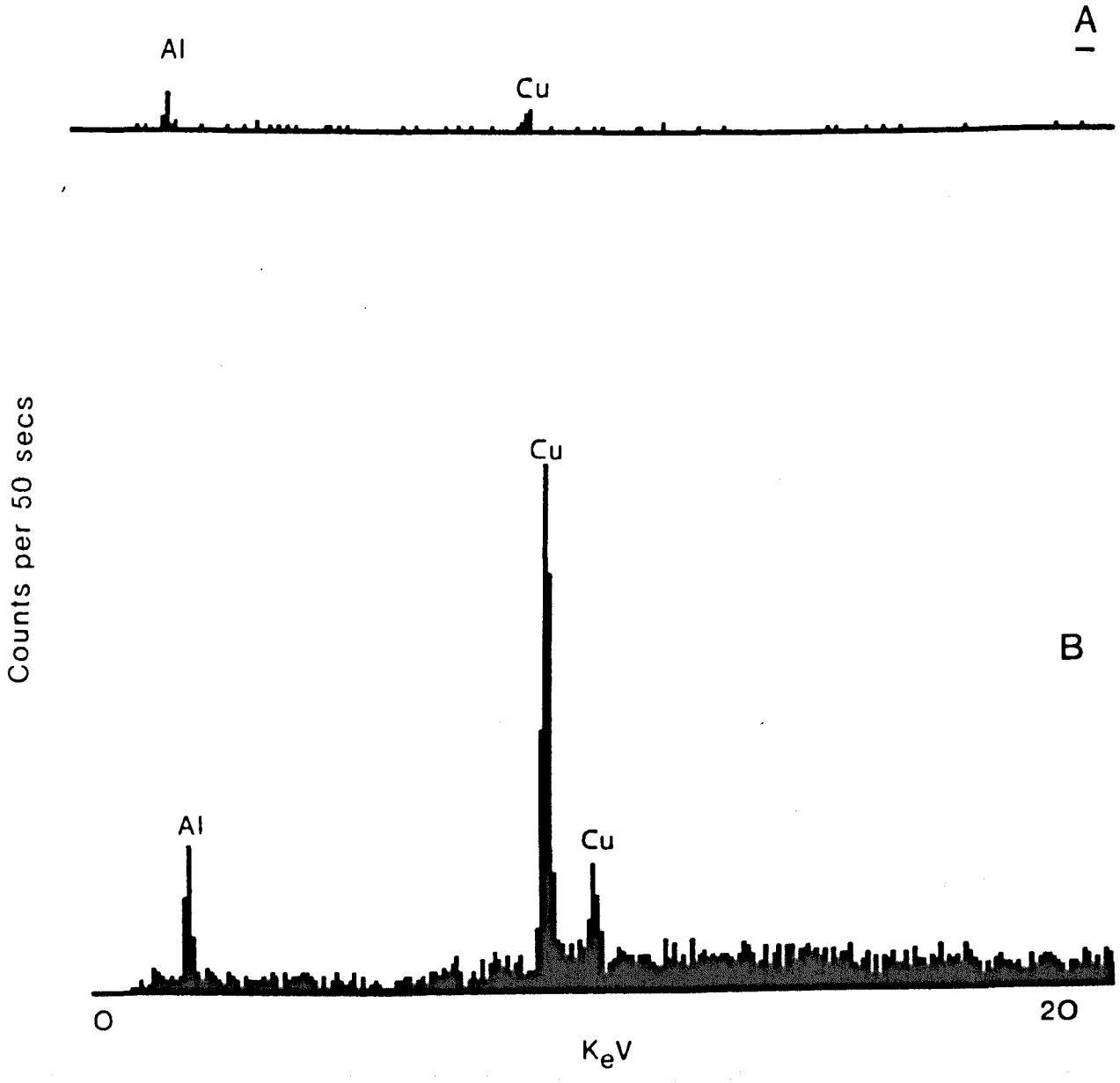


Figure 4.26. Week 4. Copper-loaded rat kidney. X-ray emission spectra of electron-lucent spherules (A). Low levels of copper are detected in spherules containing granular material (B).

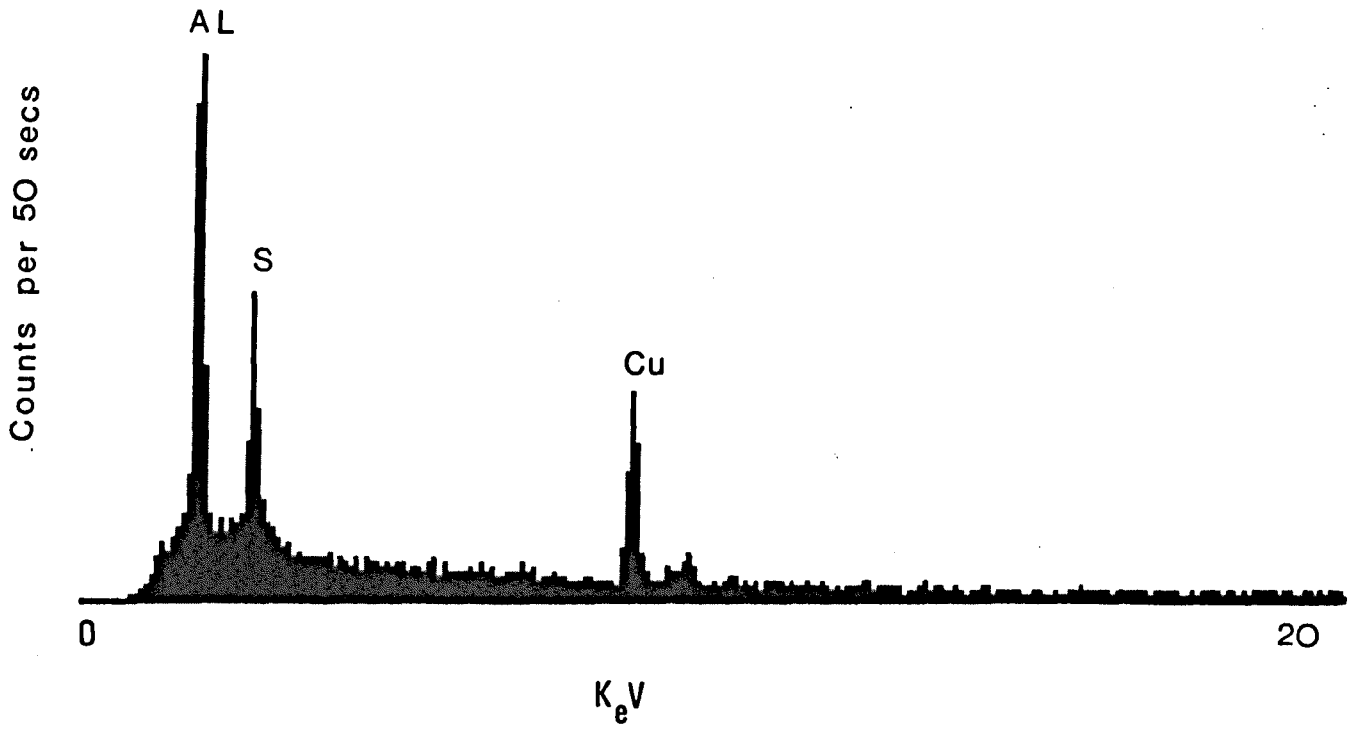


Figure 4.27. Week 5. Copper-loaded rat kidney. X-ray emission spectra of crystalline structures. High levels of copper and sulphur, but in contrast to lysosome type II, there is no phosphorus.

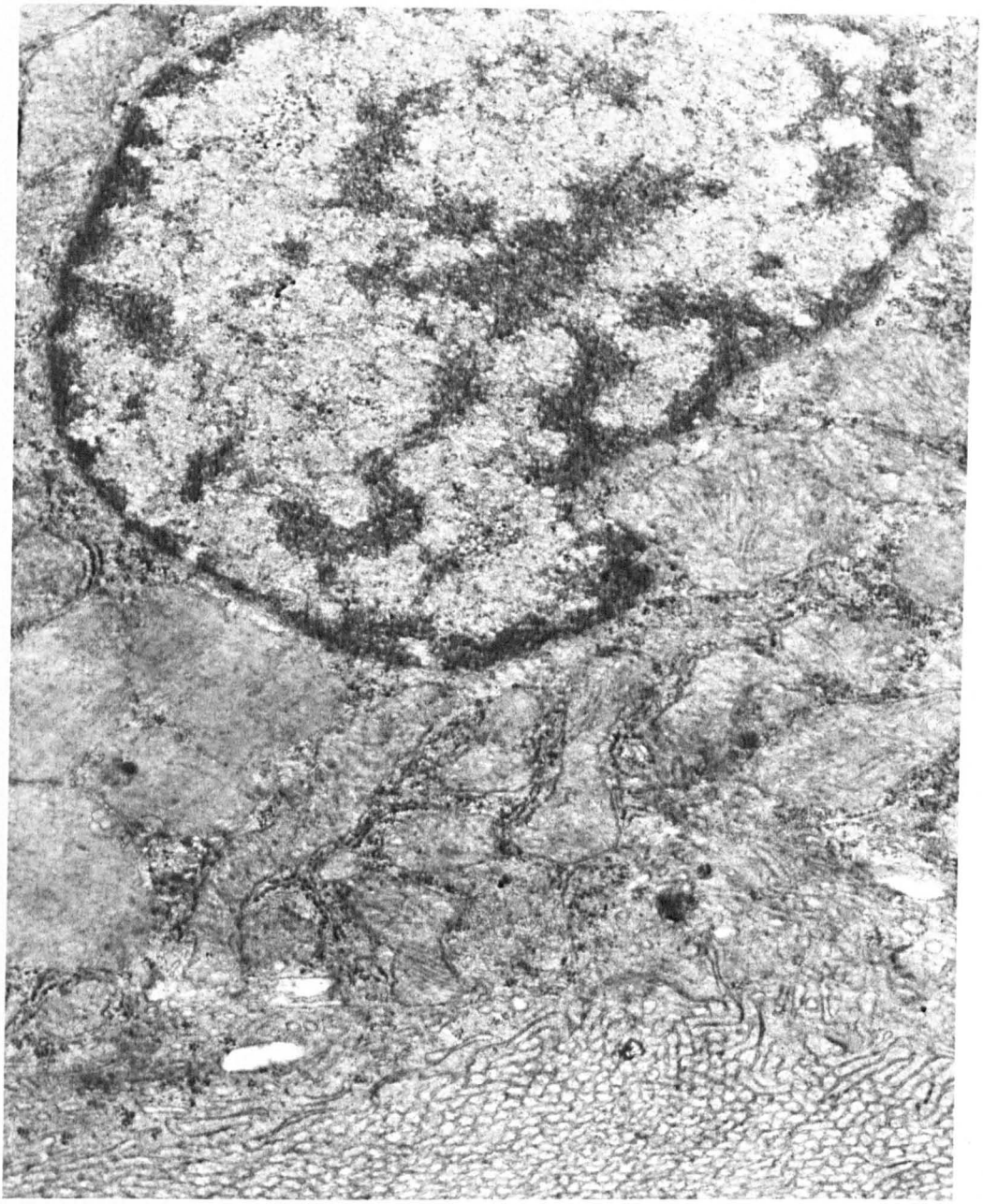


Figure 4.28. Week 3. Copper-loaded rat kidney. PCT epithelial cell nucleus with condensation of chromatin and crenated appearance. x24,000.

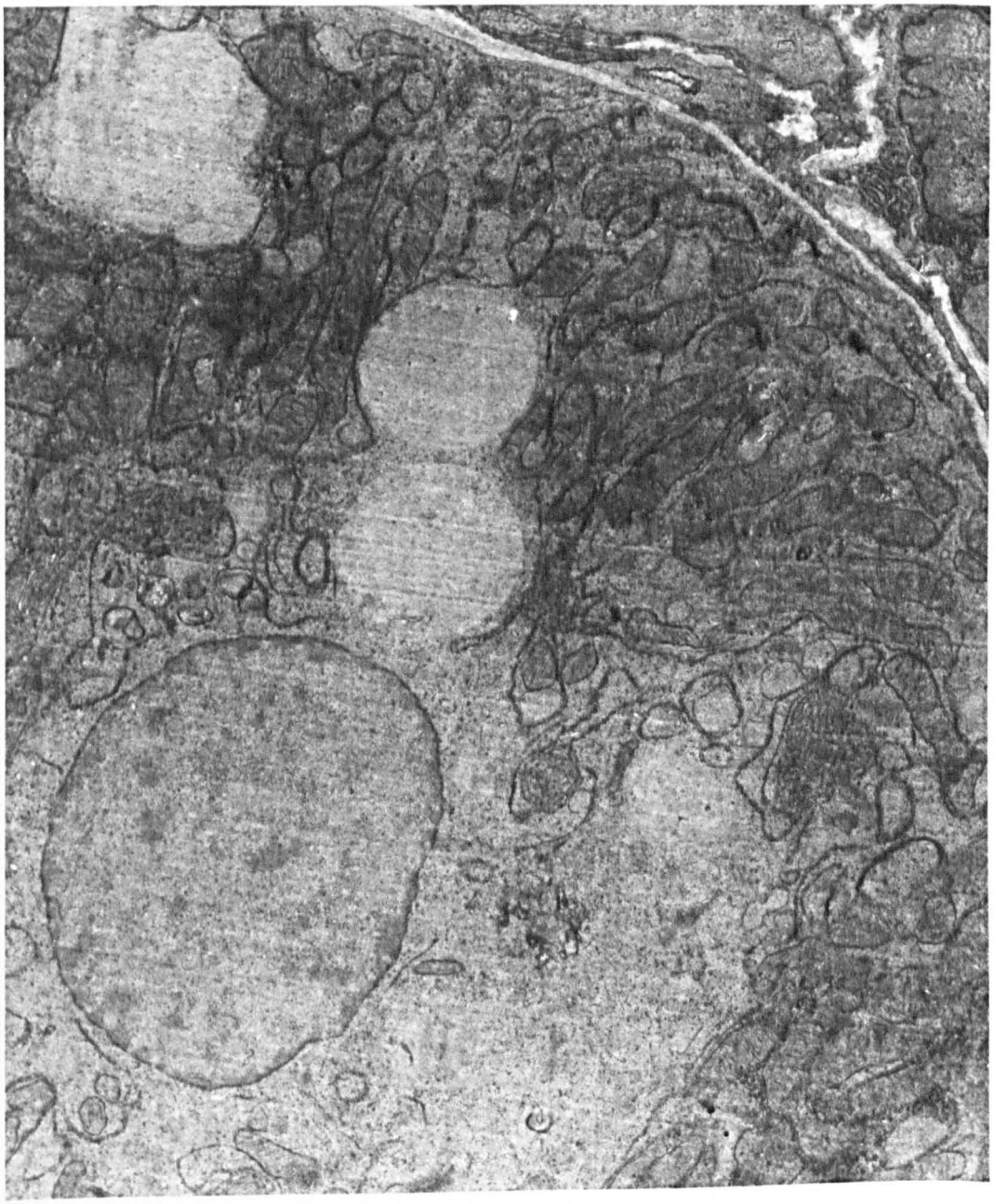


Figure 4.20. Week 4. Copper-loaded rat kidney. Karyolysis of a PCT epithelial cell nucleus. x12,000.

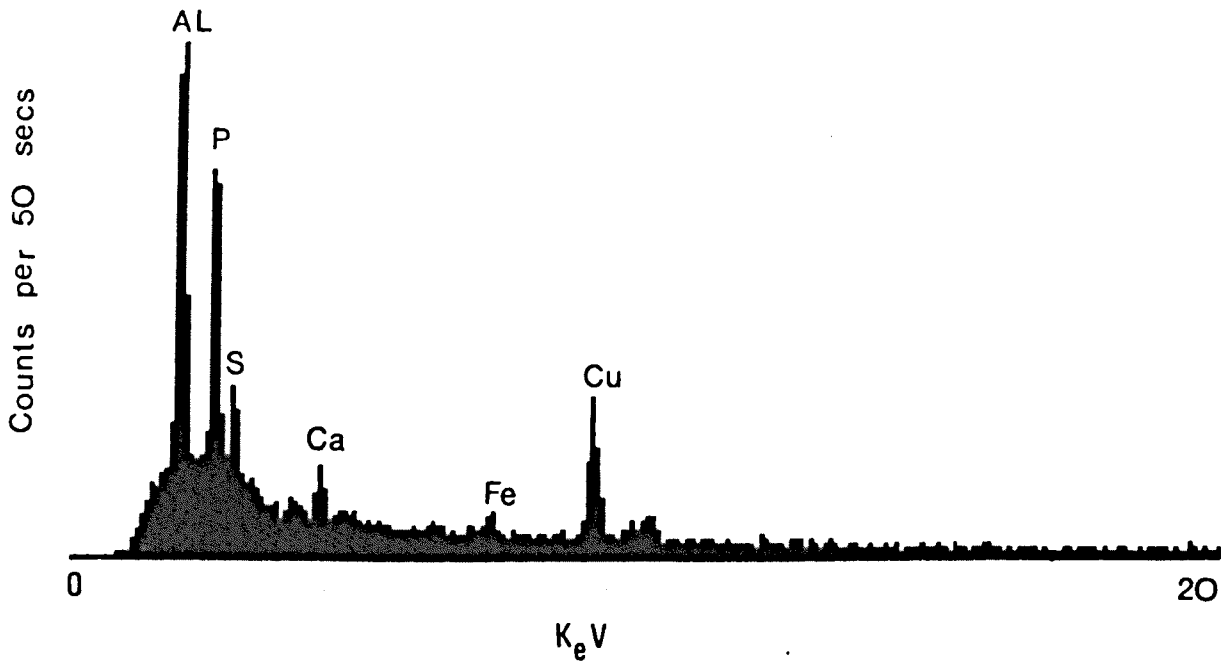


Figure 4.30. Week 3. Copper-loaded rat kidney. X-ray emission spectra of a nucleolus from a crenated nucleus. High levels of phosphorus, sulphur and calcium, in addition to copper.

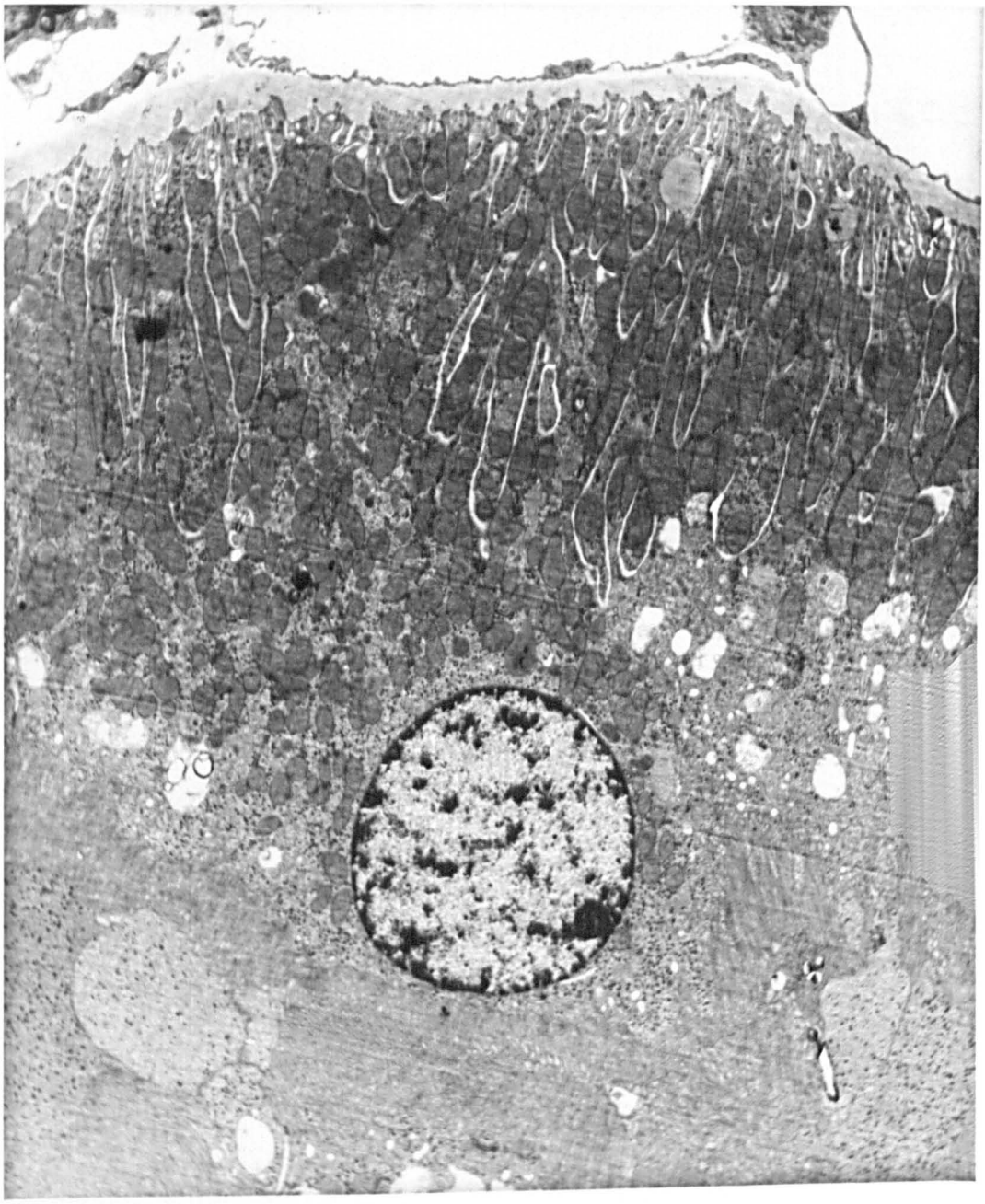


Figure 4.31. Week 16. Copper loaded rat kidney. Recovered PCT cell. x7,000.

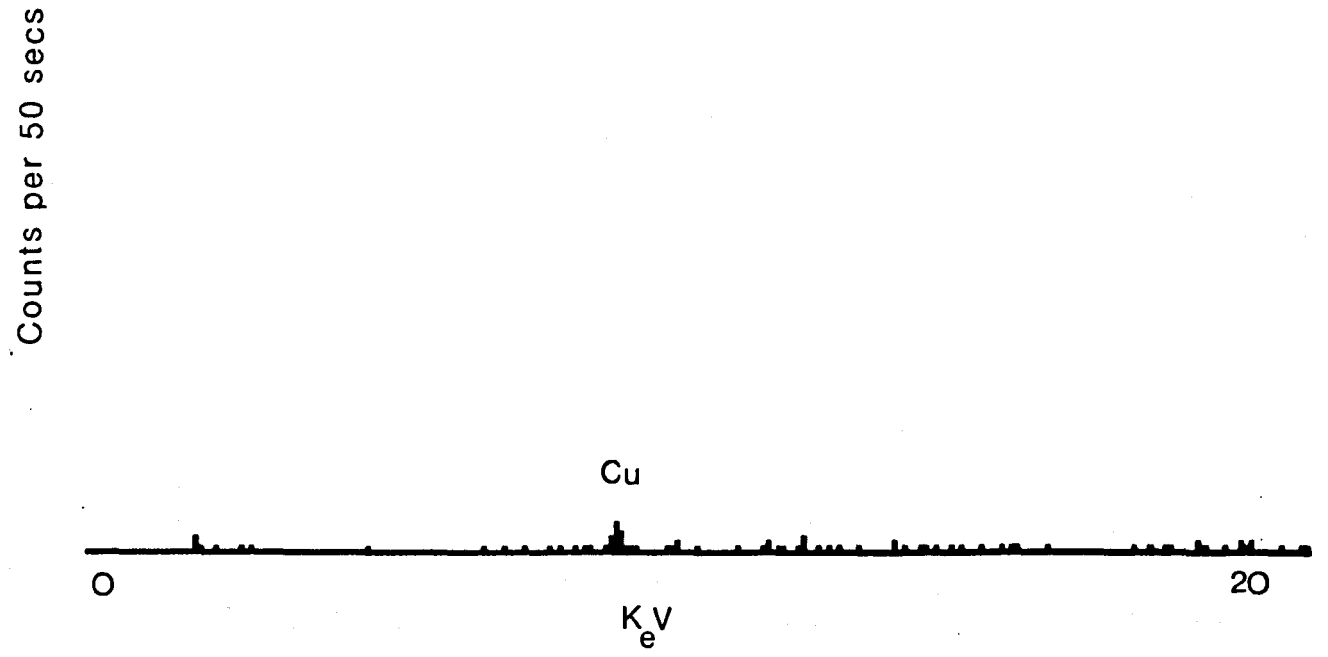


Figure 4.32. Week 16. Copper-loaded rat kidney. X-ray emission spectra of PCT cell nucleus.

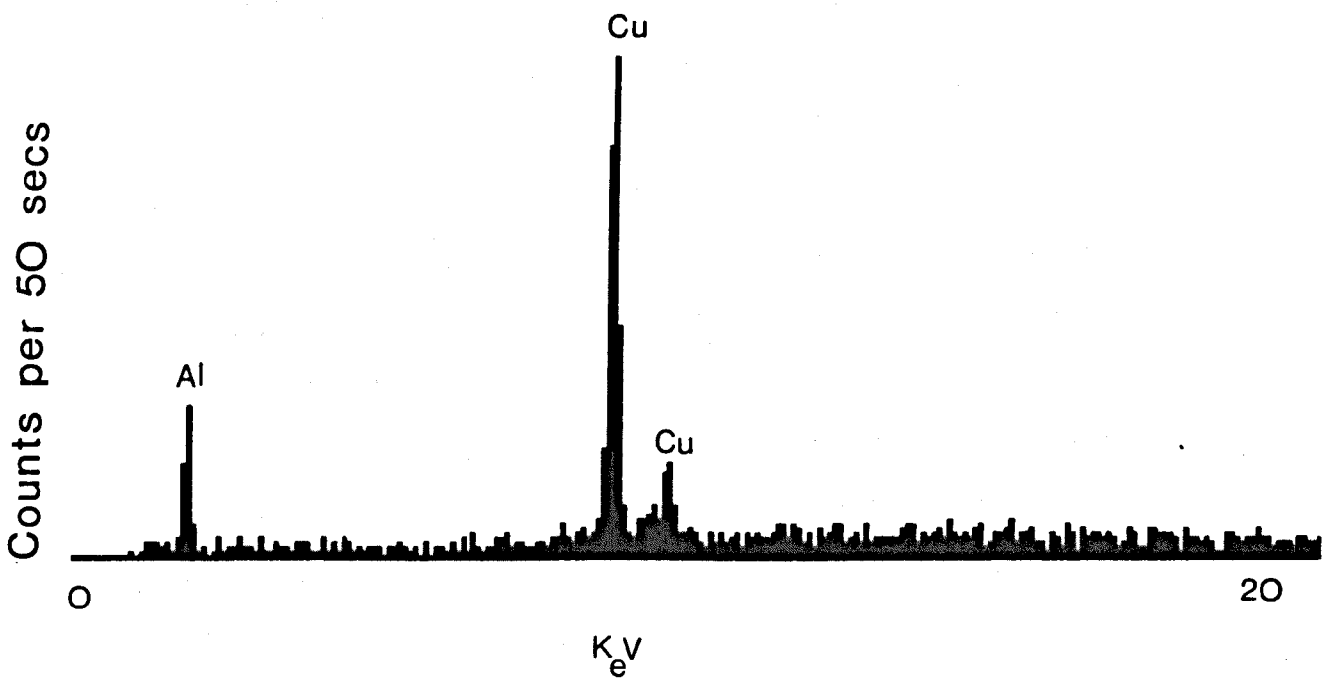


Figure 4.33. Week 16. Copper-loaded rat kidney. X-ray emission spectra of lumen debris. Presence of copper only.

4. DISCUSSION.

This investigation has shown that excess copper is stored, with other elements, in three different forms of rat liver lysosomes and two forms only of proximal convoluted tubule cell lysosomes, none of which showed evidence of membrane damage. Copper was also demonstrated, with other elemental residues, within the hepatocyte and PCT cell nuclei, in which it was associated with profound injury to these organelles.

The localisation of copper in hepatic cytoplasmic granules (lysosomes) has been demonstrated histochemically in liver biopsies from patients with Wilson's disease (Goldfischer, 1965) and Primary biliary cirrhosis (Fleming et al., 1974) and in animals following chronic administration of copper in the diet (Howell, 1959, McNary, 1963). These observations were confirmed by electron microscopic studies (Schaffner et al., 1962, Goldfischer, 1963, Barka et al., 1964) and by the detection of copper in lysosomes by electron probe microanalysis (Goldfischer and Moskal, 1966; Jones et al., 1984).

The significance of lysosomal copper accumulation has been much debated; Gooneratne et al. (1980) suggested that copper-induced lipid peroxidation of lysosomal membranes resulted in damage with release of hydrolytic enzymes. However, Kumaratilake (1985) in a similar study could not demonstrate rupture of lysosomal membranes and claimed that the presence of non-membrane bound electron-dense areas may be due to the plane of section.

Furthermore, he reported degenerative and necrotic changes in cells with intact and membrane-bound lysosomes. Additional arguments against the hypothesis of lysosomal damage can be deduced from the fact that large amounts of copper accumulate in the lysosomes of toads (Goldfischer et al., 1970, swans (Molnar, 1983) and human neonates (Sternlieb, 1980) with no evidence of hepatocellular injury. Moreover, it has been demonstrated that the defective incorporation of copper into lysosomes in beige mice is responsible for the enhanced hepatotoxicity shown by this strain and hence that lysosomal sequestration of copper is protective (Helman et al., 1985). Moreover, Sunderman et al. (1987), following parenteral administration of nickel chloride to rats, have also presented evidence that lipid peroxidation in alveolar macrophages is a consequence of lysosomal damage rather than a cause of cellular injury.

The designation lysosome is used to describe a group of membrane-bound bodies containing a number of hydrolytic enzymes. It is difficult to present a useful morphological description of these organelles because of their structural heterogeneity (Fawcett, 1966). Due to the variety of functionally different structures the lysosomal system shows great complexity (Schellens et al., 1977). Many functional and morphological forms of lysosomes have been identified (de Duve and Walliaux, 1966). The heterogeneity is caused by the divergent functional activity of lysosomes in different cell types as well as in the same type (Daems et al., 1969). A primary lysosome is a structure

containing lysosomal hydrolases which has not yet encountered material to be degraded. The content is usually of low or medium electron density so it cannot unequivocally be distinguished from other vesicular structures (Ghadially, 1975).

The clasification of lysosomes by de Duve and Walliaux (1966) divides secondary lysosomes into two main classes; phagolysosomes (heterolysosomes) where exogenous material is digested and cytolysosomes (autolysosome) where endogenous material is digested. As a result of phagocytosis, pinocytosis or endocytosis, ingested material comes to lie within a single membrane-bound structure called a phagosome. Fusion of a primary lysosome with a phagosome leads to the formation of phagolysosome.

Cytolysosomes usually present as single-membrane-bound bodies containing a portion of cytoplasm bearing organelles such as mitochodria and endoplasmic reticulum and also inclusions such as glycogen and lipid. The sequestered material may be well preserved and easily identifiable, or in various states of breakdown or degradation, until a point is reached when one cannot confidently assert whether a given lysosome started as a cytolysosome or a phagolysosome. In practice it is difficult to classify all secondary lysosomes as heterolysosomes or autolysosomes. Finally, a residual body containing undigested electron-dense lipidic residues (lipofuscin) is produced (Ghadially, 1975).

The lysosomes identified in the liver in this study all fall into the category of secondary lysosomes. They display

diverse morphology which correlates in most instances with distinct x-ray emission profiles with respect to copper and other elements (Lysosomes types I, II and III).

Type I lysosomes appear early in copper-loading and seem to include simple copper complexes associated with some iron and zinc, the latter probably as result of displacement of cations due to influx of copper.

Type II lysosomes appear later and represent a more enduring form of lysosomal copper sequestration. Their complement of sulphur and phosphorus suggests that copper occurs in the form of metallothionein. Metallothionein is known to have an induction threshold of 600 ppm wet tissue in rat (Bremner, 1986) and is a stable copper-complex.

Type III lysosomes are a late occurring form and persist throughout the phase of recovery; they contain low amounts of copper and could either represent a newly generated population of lysosomes or, more likely, lysosomes which have released copper as part of the unloading process. It is interesting that all three lysosomal types occurred in both hepatocytes and Kupffer cells.

The variability of the lysosomal population in the proximal convoluted tubules of the kidney has been widely discussed by many authors (Ericsson et al., 1965; Ghadially, 1975). Secondary lysosomes of varying shape and density have also been described in the copper-loaded sheep (Gooneratne et al., 1980; Jones et al., 1985). Distinct differences between the chemical composition of irregularly shaped electron- dense

lysosomes have also been noted (Jones et al., 1984), but mainly in relation to the non-copper residual elements. X-ray emission spectra of copper-laden lysosomes in Wilson's disease (Goldfischer and Moskal, 1966) and chronic active hepatitis (De Santi et al., 1986), are similar to Type I lysosomes. The presence of copper and sulphur in lysosomes, similar to Type II lysosome have been reported in Primary biliary cirrhosis (Humbert et al., 1982; Janssen et al., 1984a), chronic copper poisoning in sheep (Jones et al., 1984) and in abnormal hepatic copper storage in the teleost fish Morone americana (Bunton et al., 1987).

There is considerable evidence that some lysosomes contain metallothionein (MT) (Johnson et al., 1981; Mehra and Bremner, 1983). MT characteristically has a high cysteine content (Kagi and Nordberg, 1979) and sulphur is present in cysteine and cysteine-rich proteins (Moore et al., 1984). The greatly elevated sulphur concentrations identified in parallel with copper in Type II lysosomes in both liver and kidney in this study supports the possibility of its being copper-metallothionein (Cu-MT). The concept of lysosomal unloading of copper (as Cu-MT) possibly as a form of exocytosis, is very intriguing. MT is known to be present in blood and urine (Bremner et al., 1986) of copper-loaded rats but it has not been possible to present direct evidence from this study that lysosomal contents are discharged into the bile canaliculi or sinusoids.

Copper has also been identified within spherules, structures corresponding to the hyaline droplets of light

microscopy; crystalline structures and luminal debris. The presence of copper within spherules is variable depending on the inclusion or otherwise of granular material; this corresponds in some measure to the variable copper staining of these structures and it is possibly bound quite lightly to the alpha $_{2u}$ globulin urinary protein of which they are most likely composed (Alden, 1986). It is interesting that the luminal debris shows a similar simple copper emission spectra; perhaps this represents the same complex identified in earlier works as excreted from the PCT of male rat (Haywood et al., 1985b). It is not known whether or not this occurs in the female rat.

By way of contrast, copper occurs associated with sulphur in the crystalline structures, similar to a type II lysosome profile. It is probable that these structures do represent an end stage (residual body) of Type II lysosomes in which the contained material, putative MT, has crystallised. If this is so then there arises the possibility of different pathways existing in the liver and kidney with respect to the lysosomal life cycles.

Accumulation of copper into the nuclear fraction has been reported during copper-loading (Lal and Sourkes, 1971; Haywood et al., 1985) and Hardy and Bryan, (1975) demonstrated the in-vitro and in-vivo avidity of nuclear heterochromatin for copper. The confirmatory findings from this study of the nuclear accumulation of copper in both livers and kidneys of copper loaded rats are increased evidence of the biological importance of this event.

The bound forms in which copper occurs within the nucleus are of considerable interest. Metallothionein bound to copper in the hepatic nuclei has been demonstrated in mature rats (Clarkson et al., 1984). In Primary biliary cirrhosis it has been demonstrated that the accumulation of copper induces synthesis of cytoplasmic and nuclear MT (Janssen et al., 1984b). The presence of sulphur in addition to copper in this study supports this particular conjugation of copper in the nuclei. Moreover, immunocytochemical studies on similar copper-loaded rats have identified copper in the nuclei (Elmes et al., 1987). MT has been demonstrated in the hepatic nuclei of fetal and neonatal rats in which it has been ascribed to an increased synthesis of this protein in these cells (Banerjee et al., 1982; Panemangalore et al., 1983; Nartey et al., 1988). In pathological situations nuclear MT has been demonstrated in hepatocytes and PCT of cadmium-loaded rats (Banerjee et al., 1982). Furthermore, MT has been demonstrated in the nuclei of hepatic parenchymal cells of patients with Wilson's disease and Primary biliary cirrhosis (Elmes and Jasani, 1987; Nartey et al., 1987). There is a close parallel with the results from this study in which putative MT occurs also in the nuclei. The significance of nuclear MT however is puzzling and further clarification of its role within the nucleus is needed.

The presence of zinc in the nucleus requires comment and shows that it is not displaced by copper from this organelle; therefore zinc deficiency can be discounted in the pathogenesis of

copper toxicosis. It is known that nucleic acids contain covalently bound phosphorus (Moore et al., 1984), and increased phosphorus has been detected in necrotic macrophages as a result of lead toxicity (De Vries et al., 1983). However, the role of excess phosphorus in the nucleus is not understood.

It can be argued that the influx of copper and other elements into the nucleus is a consequence of damage rather than its precursor; certainly the presence of calcium is regarded as a pathological indicator of irreversible membrane breakdown (Trump et al., 1980) which could account for the passive accumulation of sulphur and phosphorus. However, the clear demonstration that copper accumulation within less disturbed nuclei precedes the influx of other elements into the more degenerate forms, is firm evidence of the directly injurious effects of the metal on nuclear structure and function; although the mechanism by which it exerts this effect is not yet known.

Although x-ray microanalysis has proved to be a most useful technique for the detection of the intracellular accumulation of copper, it lacks sensitivity to low levels of copper and other metals (Galle and Berry, 1986). The detection limit for most x-ray microanalytical units is about 100 metal/g of tissue (Beaman and Isasi, 1972), therefore it is relatively easy to get a false negative result (Fowler, 1983). It is possible that some loss of copper occurs from the different organelles during the processing of tissue for electron microscopy; hence absence of copper from the cytoplasm when other studies have shown

high copper (Lal and Sourkes, 1971). There also may be a loss from other nuclear constituents and only residual copper left bound to the nucleolus. It seems therefore necessary to investigate the real threshold and localisation of copper by means of cryotechniques in addition to electron probe x-ray microanalysis. However this technique may also have its disadvantages for although the use of ultracryomicrotomy on freeze dried sections may help minimise the effects of extraction, it is not entirely clear that such procedures prevent intracellular translocation effects for all elements because different cellular compartments may not freeze at exactly equal rates even using rapid freezing techniques (Fowler, 1983).

In conclusion this study has shown unequivocally that, in the copper-loaded rat liver and kidney, lysosomes are essentially protective and do not on any significant recognisable scale contribute to cell death. However, copper, accumulating within the nucleus is apparently directly injurious to this organelle which is ultimately responsible for the death of the whole cell.

CONCLUSIONS

CONCLUSIONS.

The overall aim of this investigation has been to explore and clarify the pathogenesis of copper-induced damage and subsequent adaptation in the livers and kidneys of copper-loaded rats and to relate these findings towards the understanding of copper toxicosis in man and animals. Particular objectives have been to examine the respective contributions of possible nuclear or lysosomal impairment to cell death.

The early studies confirmed the pattern of copper accumulation with injury in liver and kidneys followed by copper unloading, and recovery as reported in previous investigations (Haywood, 1980; 1985). The depressive effect of excess copper on growth rate has been established as not being associated with a reduced food intake and appears to reflect more the requirements of detoxification and removal of copper than toxic interference with vital processes. Finally overall zinc balance in the livers and kidneys remained unaltered by the influx of copper into these organs.

The findings additionally extended the earlier reports by identifying the histochemical localisation of copper in the liver to the periportal and midzones only, in contrast to naturally occurring copper toxicosis in dogs and sheep where copper has been primarily identified in the central zones. This has suggested that overloading or obstruction of the biliary excretion mechanisms as occurs in Primary biliary cirrhosis and Indian childhood cirrhosis

may not be the sole explanation for the copper-overload that occurs in canine and ovine livers. Secondly, the unreliability of histochemical stains for the quantitative identification of copper in the overloaded rat liver has been demonstrated unequivocally in this study and is similar in this respect to Wilson's disease.

The ultrastructural examinations of both liver and kidney cortex showed that severe and irreversible changes occurred primarily, only, in the nuclei of the hepatocytes and proximal convoluted tubule cells. X-ray microanalysis confirmed the coincidence of progressive copper accumulation with nuclear injury in these sites. Changes within cytoplasmic organelles reflected only mild perturbations consistent with detoxification processes; the lysosomes, although they became very numerous and morphologically diverse, did not disintegrate despite very high copper burdens.

The mechanisms of copper-induced injury are unclear. It has recently been suggested that copper generated free radicals are highly injurious to proteins and furthermore that histones which are responsible for the stabilisation of DNA may be vulnerable to this effect (Halliwell and Gutteridge, 1985). Although this remains to be established it receives support from earlier findings (Bryan and Frieden, 1967) of the ability of copper to destabilise DNA.

However it can be stated with some confidence that since exclusion of Zn from the nucleus apparently does not occur, an

induced Zn deficiency does not contribute to the cytotoxic effect of copper.

The lysosomal population of both liver and kidney (PCT) cells displayed an increase in both numbers and morphological diversity associated with the magnitude of the copper burden. Furthermore X-ray analysis identified three types of lysosomes with respect to their emission profiles. Type I lysosomes exhibited a comparatively simple profile confined to copper (iron and zinc), which appeared early on in both liver and kidney and probably represented a simple copper conjugate. Type II lysosomes included sulphur in addition to copper which strongly suggested the presence of Cu-MT. These lysosomes occurred later on during copper loading and were more enduring, eventually replacing Type I lysosomes. Morphologically they displayed a more heterogeneous appearance and even exhibited crystalline forms in the kidney.

Type III lysosomes occurred only in the liver with qualitatively similar emission spectra to Type II lysosomes although their elemental content was very greatly reduced. Morphologically they were less electron dense. They were considered most likely to be unloading lysosomes, that is lysosomes discharging their copper (MT) content. It was interesting that copper (MT) within the liver and kidney lysosomes apparently suffered different fates. Evidence pointed to a certain flexibility of copper movement in and out of the hepatic lysosomes whereas within kidney lysosomes copper appeared to be sequestered in a more permanent form. However, it can not be stressed too

strongly that the lysosomes appeared remarkably resistant to damage and even in advanced cellular breakdown lysosomal integrity was most striking.

Apoptosis or cell deletion is a distinct biological entity distinguishable from other forms of cell death such as "high amplitude swelling" characterised by membrane dysfunction and failure to exclude Ca^{++} with cellular swelling. Apoptosis is associated initially with nuclear changes characterised by chromatin condensation and also includes changes on the cell surface which permits the recognition by macrophages or parenchymal cells and consequent phagocytosis (Wyllie, 1987).

This description is consistent with the phenomenon of apoptosis and apoptotic body formation observed in the liver in this study. Moreover, copper-induced injury can now be added to the growing list of cell deletions or deaths occurring by this process: atrophy, killing by cytotoxic T cells, tumor regression and some tumor chemotherapeutic agents in contrast to high amplitude swelling of cells subjected to hypoxia, oxidative stress, extremes of pH and temperature, high toxin concentrations, lytic virus infections or membrane damaging agents such as complement (Wyllie, 1987).

It may be that a similar form of cell death did occur in the kidney and that the anucleate remains were discharged into the lumina of proximal convoluted tubules rather than phagocytosis taking place. It was observed that as well as phagocytosis of apoptotic bodies occurring in the liver many were discharged

directly into the sinusoids. This could be an alternative method of disposal.

Membrane bound spherules occurring in PCT cells of the kidney have been identified with the hyaline droplets of light microscopy. These droplets are peculiar to the male rat and have been found to be composed of alpha $_{2u}$ globulin synthesised by the liver, filtered and reabsorbed by the kidney as discussed earlier. Their precise biological function is not understood, however their accumulation has been associated with certain xenobiotics and accompanied by a nephropathy (Neuhaus, 1980; 1987). That such droplet accumulation occurs with copper excess has not hitherto been reported nor with any other metal. The evidence of alpha 2 urinary protein-copper binding is an additional feature which may facilitate its excretion by reverse pinocytosis as this and earlier studies (Haywood et al, 1985b) have suggested.

Adaptation to, and recovery from, the cytotoxic effects of copper has been confirmed by the reestablishment of structural normality of liver and kidney with falling copper concentrations. Functional adaptation of the recovered cell population is displayed by the Cu:S (Cu-MT) association in lysosomes and its subsequent "unloading" from the liver; in the kidney copper balance seems to be maintained by extrusion of cellular contents into the PCT lumen. Understanding concerning the mechanisms of adaptation and tolerance is however far from complete and requires further study.

However, it is possible to construct a probable sequence of events occurring in the liver and kidney due to the influx of copper. The metal is initially stored within lysosomes as a simple conjugate but later is bound predominantly to a S-rich moiety - metallothionein. Lysosomal copper is innocuous and the organelle itself is highly resistant to damage. Excess copper transfers into the nucleus in which it is responsible for irreversible nuclear injury and ultimately cell death by apoptosis. Subsequently regeneration (tolerance) takes place and functional adaptation occurs in which excess copper is discharged from both liver and kidney, albeit by different mechanisms and nuclear copper uptake averted.

Whether or not the sequence of events described in this study is a model of copper-induced damage in the naturally occurring copper-associated diseases in man and animals is not known. There is no reason to suppose that the underlying molecular basis of injury should differ in other species, although it would seem that the recovery which occurs so successfully in the rat may have a genetic basis not present in copper sensitive species.

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APPENDIX 1.

AVERAGE CALCULATED COMPOSITION OF DIET

PROXIMATE ANALYSIS		%
Ash		5.3
Crude Oil		2.4
Crude Protein		17.9
Crude Fibre		3.6
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
Starch		45
Digestible energy, MJ/Kg		13.3

TRACE ELEMENTS		mg/kg
Manganese	(Mn)	63.0
Copper	(Cu)	1,500.0
Cobalt	(Co)	0.40
Iron	(Fe)	106.0
Iodine (added)	(I)	0.28
Zinc	(Zn)	61.0
Selenium	(Se)	0.17
Chromium (added)	(Cr)	0.49

AMINO ACIDS	g/kg
Threonine	6.6
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	1.0
Histidine	4.4
Arginine	11.1
Tryptophan	2.1

VITAMINS	per kg
Vitamin A (Retinol) iu	15000
Vitamin D3 (Cholecalciferol) iu	2400
Vitamin E (α-Tocopherol) iu	88
Vitamin K3 added mg	150
Vitamin B1 (Thiamin) mg	12
Vitamin B2 (Riboflavin) mg	13
Vitamin B6 (Pyridoxine) mg	17
Niacin (Nicotinic Acid) mg	38
Pantothenic Acid mg	20
Folic Acid mg	3.1
Vitamin B12 (Cyanocobalamin) mg	0.079
Choline mg	1200
Biotin (available) mg	0.30

APPENDIX 2.

Haematoxylin and eosin staining.

The sections were:

1. Stained in Mayers haemalum (Mayer 1903, described by Lendrum and Mc Farlane 1940) for 5 minutes.
2. Washed in running water 2 to 3 minutes.
3. Differentiated in 0.5- 1.0% HCl in 70% alcohol for 5 to 10 seconds.
4. Washed again in running water for 5 minutes.
5. Stained in 0.25% aqueous eosin with acetic acid for 10 minutes.
6. Washed.

APPENDIX 3.

Growth rate of 6 week old male rats (M± SEM) on high copper diet compared with control for 16 weeks. (N=Number of rats)

	Experiment N°1		Control	
Week	N	Body weight (g)	N	Body weight (g)
0	40	173.20 ± 0.40	20	216.6 ± 0.79
1	40	199.07 ± 0.40	20	267.9 ± 0.75
2	36	232.38 ± 0.54	16	314.06 ± 1.12
3	32	256.37 ± 0.76	16	344.87 ± 1.22
4	28	269.07 ± 1.02	16	374.25 ± 1.47
5	24	279.62 ± 1.48	12	398.25 ± 2.18
6	20	283.15 ± 1.92	12	418.08 ± 2.37
7	16	285.8 ± 2.42	12	438.25 ± 2.54
8	12	300.58 ± 3.24	12	451.66 ± 2.74
9	8	315.75 ± 4.91	8	465.00 ± 4.19
10	8	331.37 ± 5.19	8	484.25 ± 4.43
11	8	341.37 ± 5.02	8	502.75 ± 5.74
12	8	350.75 ± 5.23	8	506.50 ± 4.45
13	4	349.75 ±12.82	4	522.50 ± 8.76
14	4	358.00 ±13.41	4	531.25 ± 7.69
15	4	366.5 ±14.13	4	541.5 ± 7.65
16	4	377.00 ±14.36	4	555.5 ± 8.09

APPENDIX 4

Weekly food consumption of groups of 4 rats (M±SEM) on high copper diet compared with control for 16 weeks. (N=number of groups)

	Experiment N°1		Control	
Week	N	Food intake (g)	N	Food intake (g)
1	10	611.0 ± 2.4649	5	632.6 ± 15.64
2	9	858.33 ± 6.3895	4	719.25 ± 17.43
3	8	862.12 ± 5.8112	4	659.25 ± 8.59
4	7	788.42 ± 8.1764	4	754.25 ± 9.13
5	6	736.0 ± 9.4446	3	706.0 ± 9.8
6	5	707.0 ± 10.2479	3	692.66 ± 13.65
7	4	655.75 ± 8.4210	3	717.66 ± 10.57
8	3	723.0 ± 3.684	2	691.33 ± 19.98
9	2	775.0 ± 4.5 0	2	679.00 ± 3.50
10	2	742.0 ± 30.0 0	2	703.00 ± 10.00
11	2	788.5 ± 0.755	2	677.5 ± 23.75
12	2	750.5 ± 33.255	2	690.5 ± 14.25
13	1	756	1	626
14	1	783	1	667
15	1	761	1	702
16	1	780	1	785

APPENDIX 5.

Copper concentration (µg/g) dry weight tissue (M±SEM) of livers of rats on high copper diet. (N=4)

Week	Experiment N°1	Control
1	411.0 ± 40.12	19.0 ± 0.77
2	943.0 ± 34.78	
3	1478.25 ± 59.46	
4	1967.75 ± 63.54	19.0 ± 0.39
5	2899.50 ± 60.07	
6	2888.0 ± 57.32	
8	2957.54 ± 134.14	28.75 ± 2.3
12	2645.75 ± 107.83	22.25 ± 0.71
16	1929.25 ± 90.90	22.00 ± 0.61

APPENDIX 6.

Copper concentration (µg/g) dry weight tissue (M±SEM) of kidneys of rats on high copper diet. (N=4)

Week	Experiment N°1	Control
1	89.75 ± 6.67	21.75 ± 0.81
2	190.00 ± 18.22	
3	206.00 ± 20.44	
4	475.5 ± 59.22	24.0 ± 0.91
5	820.0 ±135.16	
6	1003.25 ±116.9	
8	919.5 ± 45.50	43.25 ± 3.38
12	923.25 ± 45.8	32.75 ± 0.95
16	829.5 ± 65.7	47.00 ± 0.70

APPENDIX. 7

Zinc concentration (µg/g)dry weight tissue (M±SEM) of livers of rats on high copper diet. (N=4).

Week	Experiment N°1	Control
1	93.0 ± 1.57	134.5 ± 0.37
2	99.75 ± 1.12	
3	124.25 ± 4.13	
4	116.5 ± 4.26	114.75 ± 3.79
5	163.25 ± 4.90	
6	152.75 ± 5.19	
8	159.5 ± 2.69	135.5 ± 2.13
12	132.5 ± 1.50	131.0 ± 0.30
16	146.25 ± 1.99	123.75 ± 3.26

APPENDIX 8

Zinc concentration (µg/g) dry weight tissue (M±SEM) of kidneys of rats on high copper diet. (N=4)

Week	Experiment N°1	Control
1	87.75 ± 1.44	104.5 ± 1.13
2	101.5 ± 1.63	
3	95.75 ± 3.9	
4	107.75 ± 3.19	94.5 ± 1.64
5	105.75 ± 4.10	
6	108.75 ± 1.52	
8	134.5 ± 4.79	135.5 ± 2.13
12	120.75 ± 1.44	113.0 ± 0.53
16	143.75 ± 3.92	115.0 ± 2.11

APPENDIX 9

Growth rate of 6 week old male rats (M± SEM) on high copper diet compared with control for 16 weeks. (N=Number of rats)

	Experiment N°2		Control	
Week	N	Body weight (g)	N	Body weight (g)
0	40	231.30 ± 0.32	20	216.6 ± 0.79
1	40	246.35 ± 0.34	20	267.9 ± 0.75
2	36	281.33 ± 0.37	16	314.06 ± 1.12
3	32	310.15 ± 0.49	16	344.87 ± 1.22
4	28	328.14 ± 0.67	16	374.25 ± 1.47
5	24	337.87 ± 0.96	12	398.25 ± 2.18
6	20	358.95 ± 1.13	12	418.08 ± 2.37
7	16	371.00 ± 1.46	12	438.25 ± 2.54
8	12	375.16 ± 1.95	12	451.66 ± 2.74
9	8	387.25 ± 2.90	8	465.00 ± 4.19
10	8	403.12 ± 2.47	8	484.25 ± 4.43
11	8	401.00 ± 3.26	8	502.75 ± 5.74
12	8	418.00 ± 3.00	8	506.50 ± 4.45
13	4	431.25 ± 4.33	4	522.50 ± 8.76
14	4	434.00 ± 3.72	4	531.25 ± 7.69
15	4	429.75 ± 4.74	4	541.5 ± 7.65
16	4	442.25 ± 3.36	4	555.5 ± 8.09

APPENDIX 10

Weekly food consumption of groups of 4 rats (M±SEM) on high copper diet compared with control for 16 weeks. (N=number of groups)

	Experiment N°2		Control	
Week	N	Food intake (g)	N	Food intake (g)
1	10	580.8 ± 9.49	5	632.6 ± 15.64
2	9	910.55 ± 8.95	4	719.25 ± 17.43
3	8	937.00 ± 9.12	4	659.25 ± 8.59
4	7	845.42 ± 9.64	4	754.25 ± 9.13
5	6	904.00 ±14.46	3	706.0 ± 9.8
6	5	894.4 ± 7.79	3	692.66 ± 13.65
7	4	717.5 ± 8.10	3	717.66 ± 10.57
8	3	731.16 ±11.4	2	691.33 ± 19.98
9	2	704.00 ±12.0	2	679.00 ± 3.50
10	2	807.00 ±30.0	2	703.00 ± 10.00
11	2	879.00 ± 7.5	2	677.5 ± 23.75
12	2	882.00 ±19.5	2	690.5 ± 14.25
13	1	798	1	626
14	1	770	1	667
15	1	638	1	702
16	1	766	1	785

APPENDIX 11

Fixative Solution.

4% Paraformaldehyde/2% glutaraldehyde (x240ml)

1. 8 g Paraformaldehyde powder in 100 ml distilled water were heated to 65 ^{o} C and stired continuously until dissolved when a few drops (4-12) of 1M (1N) Na OH were added to clear.
2. Phosphate buffer. pH 7.4

a) $NaHPO_4H_2O$ (monobasic)

b) Na_2HPO_4 (dibasic)

0.55 g of monobasic phosphate and 2.275g of dibasic phosphate were added to 100 ml distilled water, stirring constantly.

3. Immediately before fixation 40 ml of 25% glutaraldehyde were added to 100 ml of paraformaldehyde solution and 100 ml of phosphate buffer.

APPENDIX 12.

Procedure for Resin-embedding tissue.

1. Dehydration in 70% ethanol 30 min.
2. " 90% " 30 min.
3. " absolute ethanol 30min. x2
4. Acetone 30 mins x2
5. 50% Acetone: 50% resin 15-17 hours.
6. Pure Resin 4-6 hours.
7. The tissues were placed in embedding moulds with resin and polymerised in the oven at 60· C for 17-24 hours.

APPENDIX 13.

Preparation of TAAB Resin (TAAB Laboratories U.K.)

1. 50 ml TAAB embedding resin.
2. 40 ml DDSA (dodecenyl succinic anhydride hardener).
3. 10 ml MNA (methyl nadic anhydride)
4. 2 ml DMP 30 [2,2,6-tri (dimethyl aminomethyl) phenol]

The mixture was agitated for 10-15 min in sealed container and left to stand to allow air bubbles to separate out.

PUBLICATIONS

During the course of this work a number of papers have been presented at scientific meetings, and some others have been published, accepted or submitted for publication. Those which have been published are attached at the end.

1. Fuentealba I (1985). Copper toxicity in sheep. A review. **Arch. Med. Vet.** 17:69-74.
2. Fuentealba I, Haywood S, Trafford J (1987). Evaluation of histochemical methods for the detection of copper overload in rat liver. **Liver** 7:277-282.
3. Fuentealba I, Haywood S, Trafford J (1988). Variations in the intralobular distribution of copper in the livers of copper-loaded rats in relation to the pathogenesis of copper storage diseases. **J. Comp. Path.** (in press).
4. Fuentealba I, Haywood S (1988). Cellular Mechanisms of Toxicity and Tolerance in the Copper-loaded Rat. I. Ultrastructural changes in the liver. **Liver** (in press).
5. Fuentealba I, Haywood S, Foster J (1988). Cellular Mechanisms of Toxicity and Tolerance in the Copper-loaded Rat. II. Pathogenesis of copper toxicity in the liver. **Exp. Mol.Pathol.** (in press).
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7. Fuentealba I, Haywood S, Trafford J (1986). Evaluacion de los metodos histoquimicos en el diagnostico de intoxicacion por cobre en ratas.**Proc. IV Congreso Nacional de Medicina Veterinaria.** Santiago, Chile.

8. Fuentealba I, Haywood S (1987). Evaluation of histochemical methods fpr the demonstration of copper overload in rats compared with Wilson's disease.**Proceedings 154th Meeting of the Pathological Society of Great Britain and Ireland.** Oxford. 7-9 January, 1987.

9. Haywood S, Fuentealba I (1987). Variations in the zonal distribution of accumulated copper in the livers of rats and Bedlington terriers in relation to disease. **Proceedings 154th Meeting of the Pathological Society of Great Britain and Ireland.** Oxford. 7-9 January, 1987.

10. Fuentealba I, Haywood S (1987). Subcellular changes and metal mobilisation in the livers of copper-loaded rats. **Proc. TEMA-6 Conference.** California.

11. Elmes M, Haywood S, Clarkson JP, Fuentealba I, Jasani B (1987). Copper tolerance in rats. A histochemical and immunocytochemical study. **Proc. TEMA-6 Conference.** California.

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14. Fuentealba I, Haywood S, Foster J (1988). The application of x-ray probe microanalysis to the investigation of the pathogenesis of copper toxicosis. **Proc. Annual Conference of the Association of Veterinary Teachers and Research Workers**.Scarborough.

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Revisión bibliográfica

INTOXICACION POR COBRE EN OVINOS

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SUMMARY

COPPER TOXICITY IN SHEEP

Copper (Cu), an essential trace element, may be toxic if accumulated in excessive amounts. Copper toxicity can be acute or chronic, the latter being the most common form which is primarily caused by nutritional and/or genetic factors. The best examples of genetically mediated Cu toxicity are Wilson's disease in humans and copper toxicosis in Bedlington Terriers.

Sheep appear to be especially susceptible to Cu poisoning compared to other species and in these animals it is mainly nutritional disease, although there are also genetic differences in Cu metabolism between breeds of sheep. The factors responsible for chronic Cu poisoning have been divided into three categories: (a) high intake of Cu with normal intake of molibdenum, (b) low intake of molibdenum with normal or high intake of Cu and, (c) consumption of plants containing hepatotoxins such as pyrrolizidine alkaloids.

Chronic copper poisoning in sheep is considered to have three phases. Initially, in the pre-haemolytic phase Cu accumulates in the liver over a period of weeks or months and clinical signs are absent. However, the primary accumulation of Cu in the liver and kidney causes progressive histopathological and histochemical changes in these organs. During this phase elevation of the activity of liver enzymes in the serum has been detected. The second phase, or haemolytic crisis, last from 2 to 6 days and is characterized by the sudden onset of severe haemolysis, there is also haemoglobinaemia, methaemoglobinaemia, haemoglobinuria and jaundice.

Post mortem examination reveals severe liver and kidney damage. Animals usually die during or shortly after the haemolytic crisis, but some may survive and go to the third phase known as post-haemolytic phase. These animals may subsequently develop further haemolytic episodes and die.

In addition to the pathogenesis and clinical signs, this review also describes aspects of clinical pathology, light and electron microscopic changes, prevention and treatment of copper toxicity in sheep.

INTRODUCCION

El cobre es el elemento prostético de varias enzimas esenciales en el funcionamiento biológico de los mamíferos, es un elemento bivalente y puede interactuar con otros que contengan una configuración electrónica igual o similar (Hill y Matrone, 1970). Estas interacciones pueden ocurrir en el sitio de absorción, metabolismo o excreción, lo cual implica que el aporte efectivo del cobre en la ración de un animal depende de la concentración relativa de los otros elementos interactuantes. Por ejemplo, las dosis altas de zinc inhiben la absorción de

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cobre en ratas (Van Campen y Scaife, 1967). Dick y col. (1975) sugieren que en el rumen el molibdeno y el sulfato inorgánico pueden formar tiomolibdato y reaccionar con el cobre formando complejos de tiomolibdato de cobre, el cual no es absorbible. Por esta razón, la concentración de molibdeno y sulfuro, a nivel ruminal, influye sobre la disponibilidad de cobre, condicionando el desarrollo de intoxicación o deficiencia (Suttle, 1975).

La deficiencia de cobre puede originar anormalidades clínicas y su acumulación excesiva en los tejidos produce toxicidad. La intoxicación por cobre puede ser aguda o crónica. El cuadro agudo es poco frecuente y sólo se ha informado en ovinos, después de haberles administrado sulfato de cobre como antihelmíntico (Sholl, 1957) y por la administración subcutánea (Mahmoud y Ford, 1981) e intravenosa de compuestos de cobre (Ishmael y Gopinath, 1972). La intoxicación crónica es la forma más común en el hombre y los animales. En animales es causada en forma primaria por factores nutricionales (Soli, 1980) y genéticos, como en la toxicosis de los perros Bedlington Terriers (Hardy y col., 1975). En comparación con otras especies, los ovinos presentan una mayor predisposición a la intoxicación por cobre (Todd, 1969), existiendo diferencias de susceptibilidad genéticamente determinadas según la raza (Wiener y col., 1978). Es así como ovinos de raza merino sobreviven una serie de crisis hemolíticas (Marston, 1950), mientras que determinadas razas británicas sucumben casi invariablemente a la primera crisis y la recuperación ocurre sólo ocasionalmente (Gracey y Todd, 1960).

En el ovino existen tres factores responsables de intoxicación crónica: consumo alto de cobre y normal de molibdeno; consumo bajo de molibdeno con niveles normales o altos de cobre y consumo de plantas que contienen hepatotoxinas.

La intoxicación crónica, debido a consumo alto de cobre y normal de molibdeno, puede ocurrir al pastorear forrajes contaminados con pesticidas que contienen compuestos de cobre (Tomov, 1964), y posterior al uso de sulfato de cobre como fertilizante o en el control de caracoles (Gracey y Todd, 1960). Bull '1951) describió este problema al estudiar praderas contaminadas con polvo de minas cercanas de cobre y en praderas con alto contenido de cobre. También se han informado casos de intoxicación posterior al acceso libre a mezclas minerales con alto contenido de cobre (Bracewell, 1958) y a la suplementación con mezclas específicamente preparadas para otras especies, tales como porcinos (Pearson, 1956) y bovinos (Kennedy, 1957).

En ovinos, cuando el nivel de molibdeno en la ración es bajo, en el rumen se forma menor cantidad de complejos de tiomolibdato de cobre, aumentando de esta manera el cobre disponible para la absorción, lo cual puede resultar en intoxicación crónica (Dick y col., 1975). Si esto se acompaña con niveles altos de cobre en la dieta, las posibilidades de intoxicación son mayores. Es así como se han informado casos de intoxicación posteriores al consumo de plantas (tales como el trébol subterráneo) que contienen niveles bajos de molibdeno (Toxaemic Jaundice Investigation Committee Report, 1956; Froslie y Norheim, 1983) y por el consumo de concentrados con niveles pobres en dicho mineral (Adamson y Valks, 1969).

El tercer factor responsable de intoxicación crónica por cobre es el consumo de plantas que contienen hepatotoxinas. Los alcaloides pirrolizidínicos dañan las células hepáticas y pueden causar la muerte; pero cuando el daño es menos severo las células sobrevivientes parecen tener una mayor avidez por el cobre (Toxaemic Jaundice Investigation Committee Report, 1956). Este tipo de complicación se ha visto en casos de seneciosis y lupinosis (Gardiner, 1966; Bull y col. 1968).

SIGNOS CLINICOS

En ovinos la intoxicación crónica por cobre es una enfermedad nutricional, cuya principal característica clínica es la hemólisis. Se reconocen tres fases: pre-hemolítica, hemolítica y post-hemolítica (Ishmael y col., 1971).

La fase pre-hemolítica es asintomática y puede durar desde varias semanas hasta más de

un año. En esta etapa el cobre se acumula primariamente en el hígado y riñón, provocando cambios histopatológicos e histoquímicos en estos órganos (Ishmael y col., 1971; Gopinath y Howell, 1975).

La aparición súbita de hemólisis marca el comienzo de la fase clínica, denominada generalmente crisis hemolítica. Esta se caracteriza por apatía, depresión general, inapetencia, disnea, polidipsia, membranas mucosas de coloración café, presencia de fecas de consistencia blanda y orina café rojiza, incluso café-negruzca. En los estados posteriores, las mucosas se observan amarillas (Ishmael y col., 1971; Gopinath y Howell, 1975; Arora y col., 1977). Durante la crisis hemolítica existe hemoglobinemia, metahemoglobinemia, hemoglobinuria e ictericia (Ishmael y col., 1972). Los animales pueden morir a las pocas horas, o entre el segundo y cuarto día posterior a la aparición de la hemólisis (Todd, 1969; Arora y col., 1977). Algunos ovinos sobreviven y pasan a la fase post-hemolítica; posteriormente desarrollan nuevos episodios hemolíticos y mueren (Ishmael y col., 1972; Gopinath y Howell, 1975; Arora y col., 1977). En los ovinos que mueren durante o poco tiempo después de la crisis hemolítica hay daño hepático y renal severo (Ishmael y col., 1972; Gopinath y col., 1974).

Durante la fase post-hemolítica las membranas mucosas se observan pálidas, posteriormente su color y el de la orina vuelven a la normalidad; recuperándose también el apetito. Con el desarrollo de crisis hemolíticas subsecuentes, los ovinos enferman en forma aguda presentando signos clínicos similares a los observados durante la primera fase hemolítica (Gopinath y Howell, 1975).

PATOLOGIA CLINICA

Existen discrepancias respecto al momento en que aumenta el cobre sanguíneo durante el período pre-hemolítico. Esto se debe a que el exceso de cobre es rápidamente removido de la sangre (Neethling y col., 1968), por lo que su detección depende de la frecuencia con que se realice el muestreo (Kumaratilake, 1984). Bremmen y col. (1976) y Gooneratne y Howell (1980) observaron hipercupremia sólo 1-2 días previos a la hemólisis. Durante la crisis hemolítica el aumento de cobre plasmático precede al de leucocitos y eritrocitos (Mc Cosker, 1968). El cobre se moviliza desde el hígado al plasma y luego a los eritrocitos, originando hemólisis (Kumaratilake, 1984). No está claro el mecanismo involucrado en la captación de cobre por parte de los glóbulos rojos y su posterior lisis. Se ha sugerido que se requieren niveles elevados de cobre sanguíneo durante 24-48 horas para causar hemólisis (Todd y Thompson, 1964).

En la fase hemolítica se observa depleción de glutatión reducido, formación de metahemoglobina y presencia de cuerpos de Heinz (Todd y Thompson, 1963; Ishmael y col., 1972). Soli y Nafstad (1976) sugieren que en ovinos intoxicados crónicamente la lisis de los eritrocitos se debería a la acción mecánica de los cuerpos de Heinz adheridos a la superficie interna de la membrana, o a la producción de daño oxidativo directo. Otros autores han realizado estudios *in vitro* que han permitido demostrar que el efecto primario del cobre es la lisis del eritrocito provocada por peroxidación lipídica de la membrana (Hochstein y col., 1980; Pfafferott y col., 1982).

En las distintas etapas de la intoxicación crónica por cobre en ovinos, el hematocrito, hemoglobina total y número de eritrocitos siguen la misma tendencia. A partir de los 7-10 días previos a la hemólisis ocurre un marcado incremento de estos parámetros, causado por el aumento del número de glóbulos rojos y disminución en el volumen sanguíneo, debido a deshidratación (Todd y Thompson, 1963; Ishmael y col., 1972).

Durante la hemólisis se produce rápida reducción en el hematocrito, hemoglobina total y número de glóbulos rojos, indicando que el 50-75% de éstos han sido lisados (Todd y Thompson, 1963; Ishmael y col., 1972). En animales que se recuperan de la crisis hemolítica estos parámetros aumentan gradualmente; pero luego disminuyen, al desarrollarse nuevas

crisis. En frotis sanguíneos obtenidos durante o inmediatamente después de un episodio hemolítico, se ha observado fragmentación de glóbulos rojos, anisocitosis, poiquilocitosis, policromasia, cuerpos de Howell Jolly y ocasionalmente normoblastos. Los cuerpos de Heinz se detectan en hasta un 15% de los eritrocitos. La recuperación del episodio hemolítico está asociada con reticulocitosis (Ishmael y col., 1972).

Ishmael y col. (1972) informan que durante el período pre-hemolítico existen leves fluctuaciones en los valores leucocitarios; pero durante la hemólisis se ha detectado un incremento de 2-3 veces, junto a un aumento en el porcentaje de neutrófilos. Esto último puede deberse a un estímulo proveniente de las células hepáticas dañadas.

Experimentalmente se ha observado que al aumentar los niveles de cobre aumenta la actividad de enzimas séricas, tales como dehidrogenasa láctica, "transaminasa glutámico oxalacética" y enzimas hepáticas específicas, como arginasa, sorbitol dehidrogenasa y glutamato dehidrogenasa. Esto se ha observado varias semanas previas a la hemólisis. Inmediatamente antes y/o durante ella, además del aumento de actividad de las enzimas ya mencionadas, ocurre aumento de los niveles de transaminasa glutámico pirúvica (Todd y Thompson, 1963; Ishmael y col., 1972; Gopinath y Howell, 1975). Se ha informado que en aquellos animales que sobreviven a una crisis hemolítica, la actividad de las enzimas baja después de la hemólisis y aumenta nuevamente antes o durante la hemólisis subsecuente (Ishmael y col., 1972, Gopinath y Howell, 1975).

HALLAZGOS ANATOMOPATOLOGICOS

No se han observado lesiones macroscópicas en ovinos sacrificados durante el período pre-hemolítico; sin embargo, histológicamente esta fase se caracteriza por tumefacción turbia y necrosis de algunos hepatocitos, hipertrofia e hiperplasia de las células de Kupfer, cuya intensidad depende de la cantidad de cobre entregada en la ración (Ishmael y col., 1971).

En la crisis hemolítica se describe aumento de tamaño del hígado, el cual presenta consistencia friable y color que varía desde amarillo anaranjado a café amarillento. Los riñones se observan aumentados de tamaño, de color rojo oscuro o café rojizo (Adamson y Valks, 1969; Ishmael y col., 1971; Gopinath y col., 1974; Gopinath y Howell, 1975; Soli y Nafstad, 1976; Arora y col., 1971; Gooneratne y Howell, 1983). El bazo se encuentra aumentado de tamaño y su consistencia es friable (Ishmael y col., 1971; Arora y col., 1977).

Los principales cambios histopatológicos observados durante la fase hemolítica en el hígado son áreas focales de necrosis de las células hepáticas y acumulación de pigmento biliar en los canalículos biliares (Ishmael y col., 1971; Gopinath y Howell, 1975). En el riñón se ha informado aumento de la eosinofilia, degeneración, vacuolización, necrosis y descamación del epitelio tubular; sobre todo a nivel de los túbulos contorneados proximales. Las células epiteliales contienen gránulos eosinofílicos refráctiles de tamaño variable, los que se tiñen positivamente para cobre y fierro; algunos túbulos renales se observan marcadamente dilatados y contienen cilindros hialinos (Gopinath y col., 1974; Gopinath y Howell, 1975; Soli y Nafstad, 1976; Gooneratne y Howell, 1983). La degeneración y necrosis de las células de los túbulos renales se deberían a la combinación de hemoglobinemia e hipercupremia (Gopinath y col., 1974). En el bazo existe necrosis intrafolicular y se observan eritrocitos fragmentados y hemosiderina dentro de las células fagocíticas de la pulpa roja y áreas perifoliculares (Soli y Nafstad, 1976). A nivel de cerebro y médula espinal ocurre espongiosis (Ishmael y col., 1971; Morgan, 1973; Howell y col., 1974; Gopinath y Howell, 1975).

Durante la fase post-hemolítica se observa coloración pálida del hígado y ausencia de lesiones macroscópicas. Los riñones pueden aparecer normales o tumefactos, de color café oscuro o negros (Gopinath y Howell, 1975; Gooneratne y Howell, 1983).

En ovinos muertos durante la fase post-

hemolítica se encuentran grandes cantidades de pigmento biliar en las canalículos, aumento de la actividad fibroblástica en las tríadas portales y proliferación de ductos biliares (Ishmael y col., 1971; Gopinath y Howell, 1975). A nivel renal se detecta aumento del tejido fibroso intersticial, además de las lesiones observadas durante la fase hemolítica (Gopinath y col., 1974; Gopinath y Howell, 1975; Gooneratne y Howell, 1983). La función renal mejora con el tiempo, a pesar de que cuando cesa la hemólisis aún existen evidencias histológicas de daño renal, lo cual indica que los nefrones sanos o ligeramente dañados son capaces de suplir funcionalmente a aquellos irreversiblemente afectados (Gooneratne y Howell, 1983).

En cortes de hígado de ovinos intoxicados en forma crónica también se ha informado presencia de gránulos intracitoplasmáticos teñidos positivamente para cobre en los hepatocitos, células de Kupfer, macrófagos de las triadas portales y células epiteliales de túbulos renales. Mediante el empleo de técnicas histoquímicas para cobre, se ha demostrado que estos gránulos intracitoplasmáticos son lisosomas (Ishmael y col., 1971; Gopinath y col., 1974).

Existen pocos trabajos sobre cambios ultraestructurales y mediciones morfométricas en hígados de ovinos con intoxicación crónica por cobre. Gooneratne y col., (1980) encontraron que al aumentar la cantidad de cobre en la ración hay un aumento en el número de hepatocitos que sufren degeneración y necrosis; además de aumento de tamaño de los lisosomas dentro de los hepatocitos. Estos mismos autores observaron algunos cambios ultraestructurales en músculos, tales como tumefacción, vacuolización, fragmentación de cristas, formación de membranas mitocondriales, degeneración de las miofibrillas y separación entre los sarcomeros.

PREVENCION Y TRATAMIENTO

Para prevenir la intoxicación crónica en ovinos se ha recomendado el uso de molibdeno y SO_4 (Harker, 1976; Suttle, 1977). La incidencia de intoxicación crónica puede reducirse al incorporar 4 mgs de molibdeno y 4 grs de SO_4 por kilo de materia seca de alimentos concentrados (Suttle, 1977).

Gooneratne y col., (1981) describen la administración de tiomolibdato por vía endovenosa. Estos autores sostienen que dosis de 100 mg de tiomolibdato, dos veces a la semana, previenen el desarrollo de hemólisis y además la acumulación excesiva de cobre en el hígado. Más aún, señalan que la administración de 50 mg de tiomolibdato en el primer día de hemólisis, seguido de la misma dosis dos veces a la semana, sería efectiva en el tratamiento de crisis hemolítica y en la prevención de hemólisis posteriores.

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Evaluation of histochemical methods for the detection of copper overload in rat liver

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ABSTRACT – Histochemical methods have invariably shown a good correlation with copper analysis by absorption spectrophotometry in the identification of canine copper storage diseases. But, in Wilson's disease (WD) in humans no such correlation exists and similar discrepancies have also been observed in copper-loaded rats. This study attempts to quantify stainable copper in the livers of copper-loaded rats and relate this to the hepatic copper concentrations. Male rats fed a high copper diet (1500 ppm) for 16 weeks were killed at intervals. The livers were analysed for copper and graded according to stainable copper present in paraffin sections stained with rhodanine and rubeanic acid. Initially there was a good correlation between histochemically demonstrable copper and its total concentration, but subsequently, when high liver levels of the metal were present, copper staining was very variable. This unreliability has similarities with WD, in which the higher hepatic concentrations of presymptomatic patients are difficult to detect by conventional copper stains. The variation in the binding of copper and its intracellular localisation suggested by these results may have considerable significance in the pathogenesis of copper storage diseases.

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Copper toxicosis, associated with the accumulation of copper in the liver, has been reported in man (1), Bedlington terriers (2), West Highland White terriers (3), and in sheep (4). Elevated hepatic copper can be identified for diagnostic purposes by either histochemical means or atomic absorption spectrophotometry. Histochemical methods are faster and less costly (5), and rhodanine, which stains tissue-bound copper (6), has been shown to provide a semi-quantitative analysis when compared with atomic absorption values in Bedlington terriers (7, 8, 9). However, in Wilson's disease in young patients with very high liver copper concentrations copper staining is unreliable (10, 11). Also, in the copper-loaded rat, there is evidence that stainable copper does not always compare with total copper concentration measured by spectrophotometric analysis (12, 13).

The aim of this study was to quantify stainable copper in the livers of copper-loaded rats and to relate this to the hepatic copper concentrations.

Material and methods

Male weanling Wistar rats of uniform age and weight were caged and fed a pelleted diet (Labsur Animal Diet, RHM Agriculture South Ltd.) containing 1500 ppm copper (range 1300–1800 ppm) and killed in groups of four at 1, 2, 3, 4, 5, 6, 8, 12 and 16 weeks. Similar groups of control animals were fed the unsupplemented diet (Cu content 10–20 ppm) and killed at 1, 4, 8, 12 and 16 weeks. Food and water were freely available to all animals.

The livers were removed for histological preparation and copper analysis, samples being taken from the right median lobe for both purposes (14).

Histochemistry

Sections from formalin-fixed blocks of liver routinely processed and paraffin-embedded were cut at 5 μm and stained for copper. Tissue sections were incubated in rubeanic acid at room temperature for 72 h and counterstained alternatively with neutral red and metanil yellow. The modified method of Okamoto & Utamara was used for rhodanine counterstained with haematoxylin (15).

The copper-stained slides were graded by two pathologists (I.F. and S.H.), using a 1 to 4 point system based on the number and size of copper-containing granules within the cytoplasm of the hepatocytes, according to their zonal distribution.

0 = absence of granules
+ = few very small granules
++ = many small granules
+++ = numerous small to medium size granules
++++ = cells saturated with small to medium size granules
+++/0 = considerable individual variation, many cells being negative for copper.

Copper analysis

All glassware was soaked before use for 48 h in 0.1 mmol/l nitric acid, followed by three 24-h washes each in deionized water, rinsed, drained and dried in a hot-air oven at 70 C.

Triplicate 0.3 g samples of liver were removed with plastic knives, oven-dried at 70 C and digested in Aristar grade 70% nitric, 70% perchloric acid (2:1) in Pyrex glass test tubes using the following procedure: 70 C for 30 min, 150 C for 30 min, 250 C for 1 h and 300 C for 1 h in a heating block; each batch of 24 included three recoveries. The copper content was analysed in an IL Atomic Absorption Spectrophotometer (Instrumentation Laboratory Inc. USA). Precinorm "U" Q.C. serum (BCL) was used to monitor the performance of the spectrophotometer. Standardisation was with a solution containing 2 and 4 ppm copper in 0.1 mmol l HCl prepared from 15.7 mmol l cupric nitrate stock solution "Spectrosol" (BDH).

Liver copper concentrations are expressed as the mean and standard error of the mean, in μg g of liver on a dry weight basis.

Statistical analyses employed Student's *t*-test and the Pearson-Moment coefficient correlation.

Results

Histochemistry

As seen in Table 1, copper could be demonstrated initially after 2 weeks of copper-loading in the hepatocytes of all zones of the hepatic lobules. This sparse distribution of copper remained constant within the central zone throughout the trial period. Meanwhile copper staining increased in intensity in the two outer zones, achieving maximum intensity in the outer zones at 5 weeks (Fig. 1). The metal was identified only in copper-containing granules within the cytoplasm of the hepatocytes. Before this time there was a remarkable uniformity of staining between individual livers at any one sampling point. There were no differences between the sensitivity of rubeanic acid with the different counterstains and rhodanine.

At 5 and 6 weeks, differences appeared in the response; although six livers demonstrated maximum staining intensity, the other two displayed diminished copper-staining in the outer zones (Fig. 2). This loss of staining intensity was very

Table 1
Hepatic copper concentration and histochemical grading with rubeanic acid and rhodanine staining in copper-loaded rats

Time (weeks)	Copper concentration (μg g)†	Histochemical grade		
		Central zone	Midzone	Periportal zone
1	411.0±40.1	0	0	0
2	943.0±34.8	+	+	+
3	1478.25±59.5	+	++	++
4	1967.75±63.5	+	+++	+++
5*	2899.5±60.1	+	++++	+++
6*	2888.0±57.3	+	++++	+++
8	2957.54±134.1	+	+++/0	+++/0
12	2645.75±107.8	+	+++/0	+++/0
16	1928.25±90.9	+	++/0	++/0

* One rat had copper granules varying from +++ 0 in the outer zones.
† mean±SE of mean (n=4).

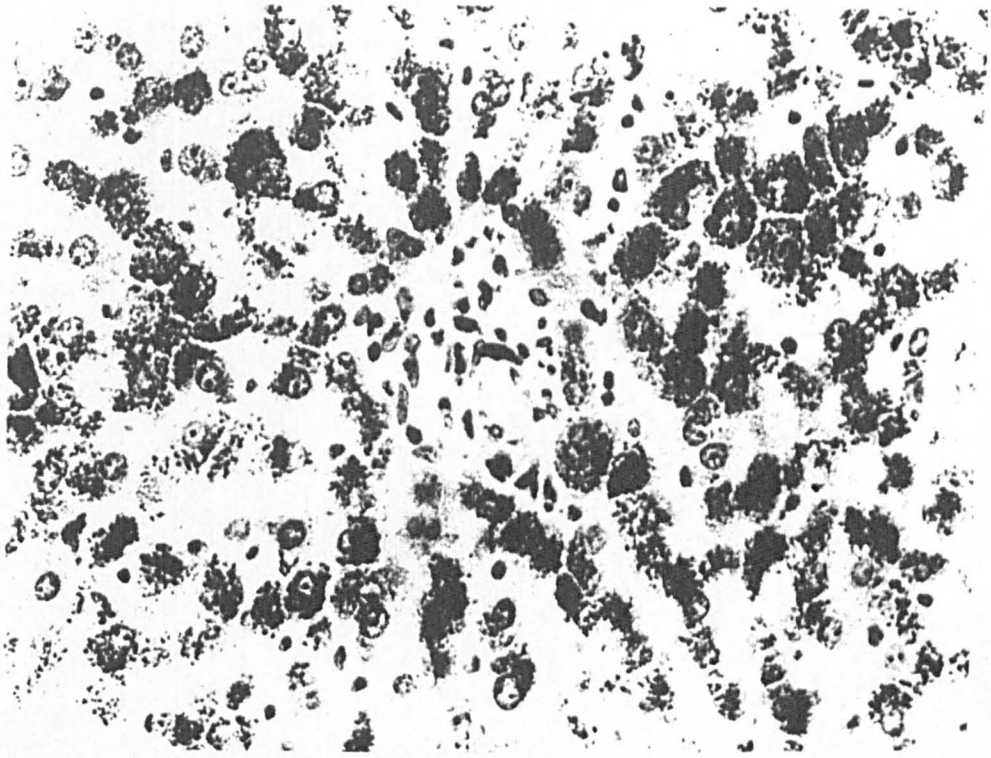

Fig. 1. Liver from a rat after 5 weeks of copper-loading (Cu concentration 2920 μg/g) showing the presence of numerous copper-positive granules in the periportal, midzonal hepatocytes. Rhodanine and haematoxylin (×435).

variable between individual cells, some of which apparently contained no copper whereas others remained saturated (Fig. 3). Subsequently the livers from all animals killed displayed a similar, progressively diminishing intensity of copper-staining.

No copper was detected in the livers of control animals.

Liver copper content

The liver copper content rose to 2899.50 ± 60.1 μg/g at 5 weeks and remained high until week 8 (2957.54 ± 134.1 μg/g), after which it began to fall, and at week 16 the liver copper concentration was significantly less than at week 8 ($p < 0.05$).

The liver copper content in control rats varied from 19 ± 0.39 to 28.75 ± 2.3 μg/g.

Up to weeks 5–6, there was a good correlation ($r = 0.78$) between quantitative copper determination and the light microscopic grading of copper-stained sections of liver tissue, and no differences were found between the two pathologists' scoring. However, from 8 to 16 weeks of copper-overload, despite the very marked loss of overall staining intensity and persistently high copper concentrations, no comparison could be made since the grading system no longer strictly applied in a quantitative fashion, and some subjective variation in scoring was noted.

Discussion

In the present study no differences were found between the sensitivity of rhodanine and rubeanic acid staining in the detection of copper. In canine copper storage diseases, rubeanic acid has been found to be superior to rhodanine staining for detecting threshold levels of copper in livers, and both methods have shown a good correlation with

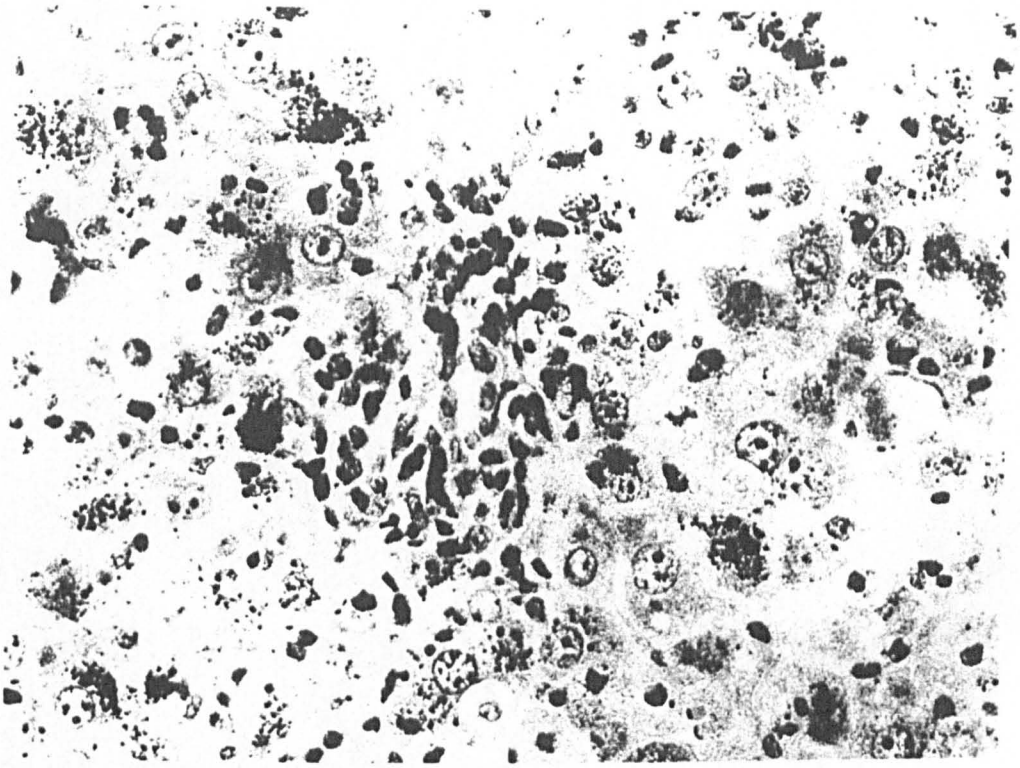

Fig. 2. Liver from a rat after 6 weeks of copper-loading (Cu concentration 3151 μg/g) showing the loss of overall copper staining intensity. Rhodanine and haematoxylin (×435).

copper content measured by atomic absorption spectrophotometry (7, 8).

The findings from this study confirm the original observations (12, 13) that copper stains are unreliable for detecting copper-overload in rats. They also contribute to a better understanding of canine copper toxicosis, in which histochemical methods have invariably shown a good correlation with analysis by absorption spectrophotometry (7–10), and Wilson's disease, where no such correlation exists (11).

The rat is an interesting species in that it is less susceptible to cumulative copper poisoning than certain species such as the sheep and the pig (1). A reason for this is that rats can adapt to tissue-damaging doses of copper (12, 16). An investigation into the mechanisms of this response (17) found that, following initial storage of copper in hepatic lysosomes, the nucleus appeared to take up excess copper; the saturation of this organelle coincided with the onset of severe cellular disturbance. Subsequently, liver copper levels fell; this was associated with renal excretion of excess copper; cellular recovery took place and the animals became tolerant (17).

It is thought that excess liver copper is excreted from the liver into the blood in a transportable form and removed by the kidney. The period of adaptation reflected by changes in liver copper from a stainable to a non-stainable form may represent a change from lysosomal storage of copper to a soluble and more transportable form.

The differences with respect to staining in copper storage diseases may be concerned with the intracellular localisation and binding of copper, and probably indicate a real difference in the pathogenesis of the human and canine forms.

Intralysosomal copper metallothionein (Lys-Cu-MT) has been demonstrated in considerable quantities in Bedlington terrier toxicosis (18),

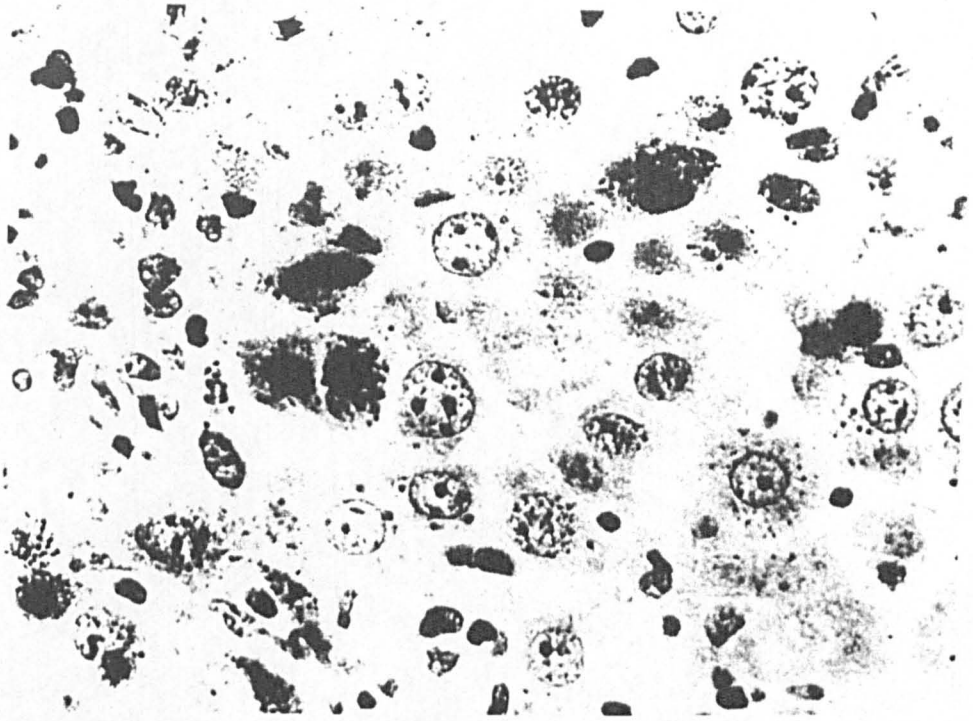

Fig. 3. Liver from a rat after 6 weeks of copper loading. Note the variability of copper-positive granules within individual hepatocytes. Rhodanine and haematoxylin (× 710).

whereas in Wilson's disease lysosomal-bound copper is not marked in the early stages of the disease despite high levels of the metal, although a diffuse distribution of copper has been identified (10, 11).

The discussion so far has concerned itself solely with demonstrable cytoplasmic copper but it is interesting to note that histochemical staining has not demonstrated copper in the nucleus of copper-loaded rats (16, 17), sheep (4, 19, 20) or man (6), despite considerable evidence of nuclear sequestration of the metal (21). It is clear that not all intracellular copper is in a stainable form, and that only particular bound forms take up copper stains. It can be concluded that although copper stains can be useful in certain defined circumstances, their value as a diagnostic aid unsupported by copper analysis is limited.

Acknowledgements

We wish to thank Mrs M. Harling for the histology and histochemical demonstration of copper, Mrs. P. Jenkins for the illustrations and Professor D. F. Kelly for help and advice. One of us (ICF) has a University of Liverpool Research Studentship and support from an Overseas Research Students Award.

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Variations in the Intralobular Distribution of Copper in the Livers of Copper-loaded Rats in Relation to the Pathogenesis of Copper Storage Diseases

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There are differences in the hepatic intralobular distribution of copper in copper storage related diseases which may be of pathogenetic significance. Male rats fed a high copper diet (1500 ppm) for 16 weeks were killed at intervals in an attempt to compare copper distribution in their livers with those in human, canine and ovine copper toxicosis. Copper was found to accumulate almost exclusively in the periportal and mid-zones of the rat liver lobules and was associated with progressive pathological changes which included focal and periportal degeneration and necrosis. This pattern of copper distribution contrasts markedly with the centrilobular retention reported in familial canine copper toxicosis and chronic copper poisoning in sheep which suggests that, in these conditions, a secretory deficiency may be less important than a metabolic zonal defect of intracellular copper metabolism. The pathological changes observed in copper-loaded rats have a different micro-anatomical localization from those in dogs and sheep, but show similarities to the early changes reported in the latter species and indicate the possibility of a similar cellular lesion.

Introduction

Copper toxicosis, associated with increased hepatic copper content, occurs as a familial disorder (Wilson's disease) in man (Underwood, 1977) and has been a well recognized problem in sheep for many years (Soli, 1980). More recently, inherited copper storage diseases have been reported in Bedlington Terriers (Hardy, Stevens and Stowe, 1975) and West Highland Whites (Thornburg, Shaw, Dolan, Raisbeck, Crawford, Dennis and Olwin, 1986).

A study of the pattern of copper distribution in chronic liver disease in man showed that cholestatic disease such as primary biliary cirrhosis is characterized by a periportal granular (lysosomal) deposition of copper, whereas staining in Wilson's disease is more diffuse and without a particular lobular pattern (Goldfischer, Popper and Sternlieb, 1980). By way of contrast, in copper storage diseases in the Bedlington and West Highland White, copper has been identified almost exclusively in the centrilobular zones (Twedt, Sternlieb and Gilbertson, 1979; Ludwig Owen, Barham, McCall and Hardy, 1980; Erikkson, 1983; Thornburg *et al.*, 1986; Haywood and Fuentealba, 1987).

A similar distribution of copper has been reported in copper toxicosis in sheep (Ishmael, Gopinath and Howell, 1971; Gooneratne, Howell and Graw-

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thorne, 1976) although there is some disagreement on this (King and Bremner, 1979; Olsen, 1979).

The study of copper accumulation in the livers and kidneys of rats on high copper diets is proving of considerable value for the investigation of the mechanism of copper overload (Haywood, 1980, 1981, 1985; Haywood and Loughran, 1985; Haywood, Loughran and Batt, 1985). The purpose of the present study was to explore quantitatively the pattern of copper deposition and associated changes in the livers of copper-loaded rats. It was hoped thereby to establish a basis for the interpretation of the hepatic deposition of excess copper and help to provide an explanation for variations in copper build-up in the different copper associated diseases.

Material and Methods

Thirty six young male weanling Wistar rats of uniform age and weight were caged and fed a pelleted diet (Labsur Animal Diet, RHM Agriculture South Ltd) containing 1500 ppm copper (range 1300 to 1800). Twenty control animals were fed the unsupplemented diet (Cu content 10 to 20 ppm). Food and tap water were freely available to all animals. Groups of 4 rats on the supplemented diet were sampled at 1, 2, 3, 4, 5, 6, 8, 12 and 16 weeks. They were killed by exposure to CO_2 followed by cervical dislocation. Control rats were similarly treated at 1, 4, 8, 12 and 16 weeks. The livers were removed for histological preparation and copper analysis, samples being taken from the right median lobe for both purposes (Haywood, 1981).

Histology

Transverse sections were taken at approximately 0·5 mm from the ventral border of the right median lobe and the formalin-fixed blocks of liver were routinely processed, embedded in paraffin wax cut in 5 μm thick and stained with haematoxylin and eosin (HE).

From all HE sections the total area of the tissue was measured with a digitaser tablet linked to a VIDS III image analysis program running on an IBM compatible computer (COMCEN). The sections were examined with a 40 × objective and the cellular changes recorded as the number of hyperchromatic hypertrophic parenchymal cells per high power field and the number of inflammatory foci per mean total area of 69·6 ± 12·2 mm^2. Necrosis was identified in single cells and focal aggregates and the severity graded on the following scale: mild = +; moderate = + +; severe = + + +.

Histochemistry

The following stains were used for the demonstration of intracellular copper:

Rubeanic acid: tissue sections were incubated at room temperature for 72 h and counterstained alternatively with neutral red and metanil yellow (Uzman, 1956).

Rhodanine: the modified method of Okamoto and Utamara was used (Lindquist, 1969).

The stained slides were graded as described in a previous study (Fuentealba, Haywood and Trafford, 1987).

Copper analysis

Triplicate 0·3 g samples of the right median lobe of the liver were removed with plastic knives and analysed for copper (Fuentealba *et al.*, 1987).

Intralobular Copper

Liver copper concentrations are expressed as the mean and the standard error of the mean, in μg of copper per g of liver on a dry weight basis.

Results

Histological changes

The following changes are recorded in Table 1.

Week 1. Minimal changes were observed in the livers of the copper-supplemented groups and consisted of a few individual necrotic cells and mononuclear cells with no particular zonal distribution.

Week 2. Scattered foci of hypertrophied parenchymal cells with enlarged nuclei and homogeneous intensely stained cytoplasm appeared in the periportal zones, associated with minimal mononuclear cell response. A sparse distribution of particulate copper was present in the cytoplasm of the hepatocytes in all zones.

Week 3. Inflammatory foci were clearly established (Fig. 1), although limited mainly to the periportal zones. These lesions consisted of hypertrophied hyperchromatic parenchymal cells undergoing necrosis with an associated inflammatory response of polymorphonuclear neutrophilic leucocytes and mononuclear cells.

There were many granules of copper in the cytoplasm of the parenchymal cells of the outer (periportal and mid-zone) areas.

Week 4. Abundant hypertrophic hyperchromatic parenchymal cells occupied the outer zone (Fig. 2). Multiple foci of inflammatory cells were present. Copper deposition was very marked in the outer zones and was characterized

Table 1

Pathological changes, hepatic copper concentration and histochemical copper grading with rubeanic acid and rhodanine staining in copper-loaded and control rats

Time (weeks)	*No. hypertrophic hyperchromatic cells per high power field*	*Necrosis*	*No. inflammatory foci per slide**	*Copper concentration (μg per g)†*	*Histochemical grade*	
					Central (perianicinar) zone	*Outer (mid and periportal) zones*
1	1	+	10	411 ± 40	0	0
2	1 to 2	+	10 to 20	934 ± 35	+	+
3	1 to 3	+ +	20 to 30	1479 ± 60	+	+ +
4	3 to 6	+ + +	30 to 40	1968 ± 64	+	+ + +
5 to 6‡	6	+ + +	Diffuse	2900 ± 60	+	+ + + +
8	6	+ +	Diffuse	2888 ± 57	+	+ + + to 0
12	6	+ +	10 to 20	2958 ± 134	+	+ + + to 0
16	6	—	10	2646 ± 108	+	+ + to 0
Control§	1	—	10	20 ± 1	0	0

* Mean total area = 69·6 ± 12·2 mm².

† Mean ± S.E. of mean.

‡ Two rats had copper granules varying from + + + to 0 in the outer zones.

§ Sum of 5 groups of 4 rats each.

Evaluation is based on examination of livers from 4 treated rats at each time interval.

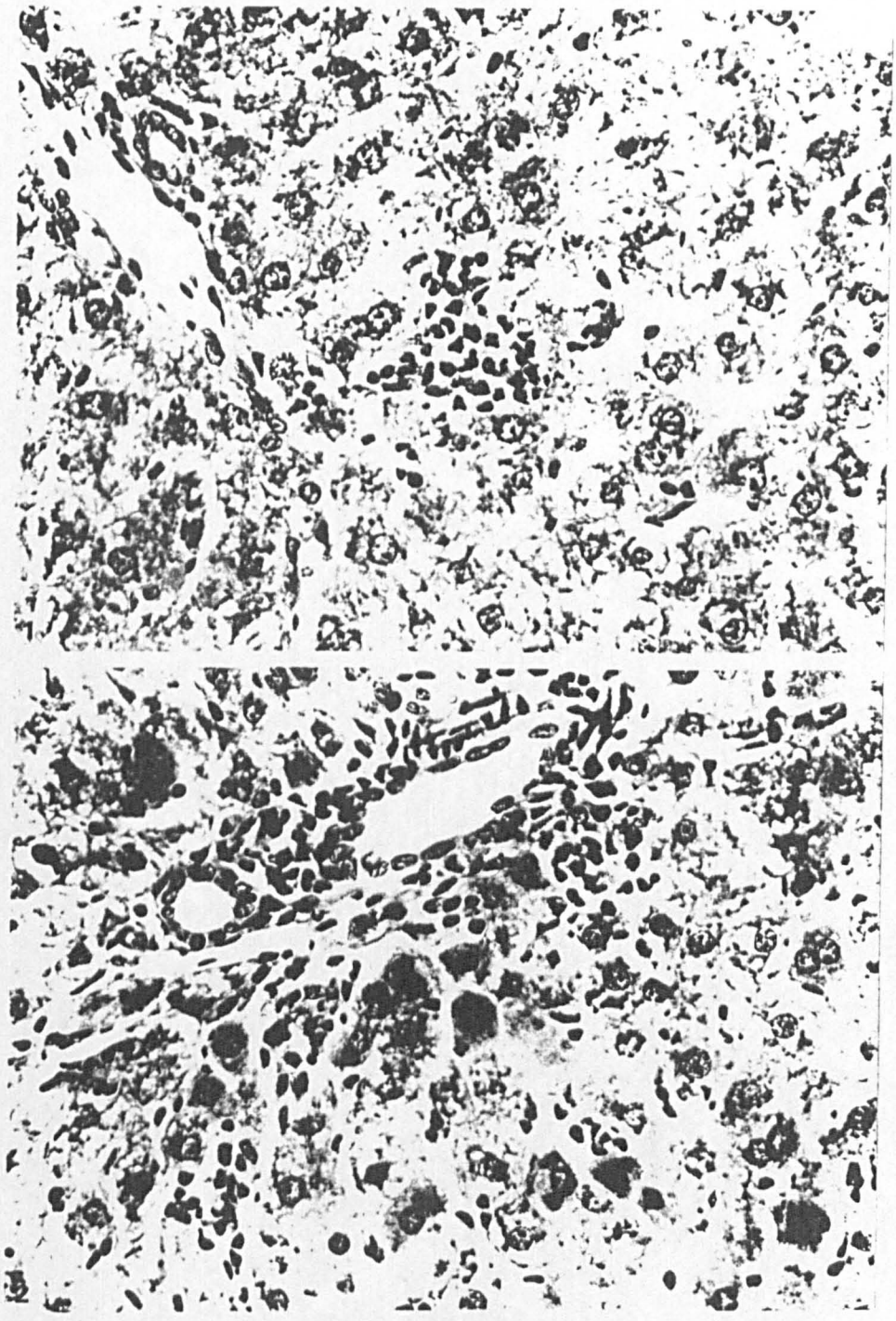
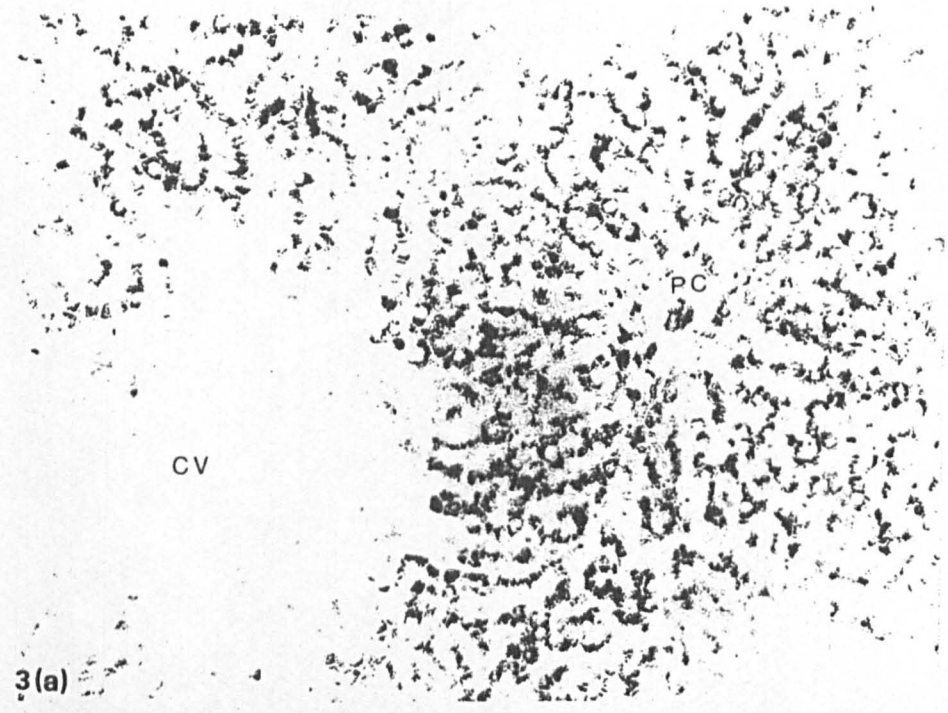


Fig. 1. Liver from a rat copper-loaded for 3 weeks showing periportal inflammatory focus. HE × 594.

Fig. 2. Liver from a rat copper-loaded for 4 weeks showing numerous hypertrophic hyperchromatic hepatocytes in the periportal zone. HE × 396.



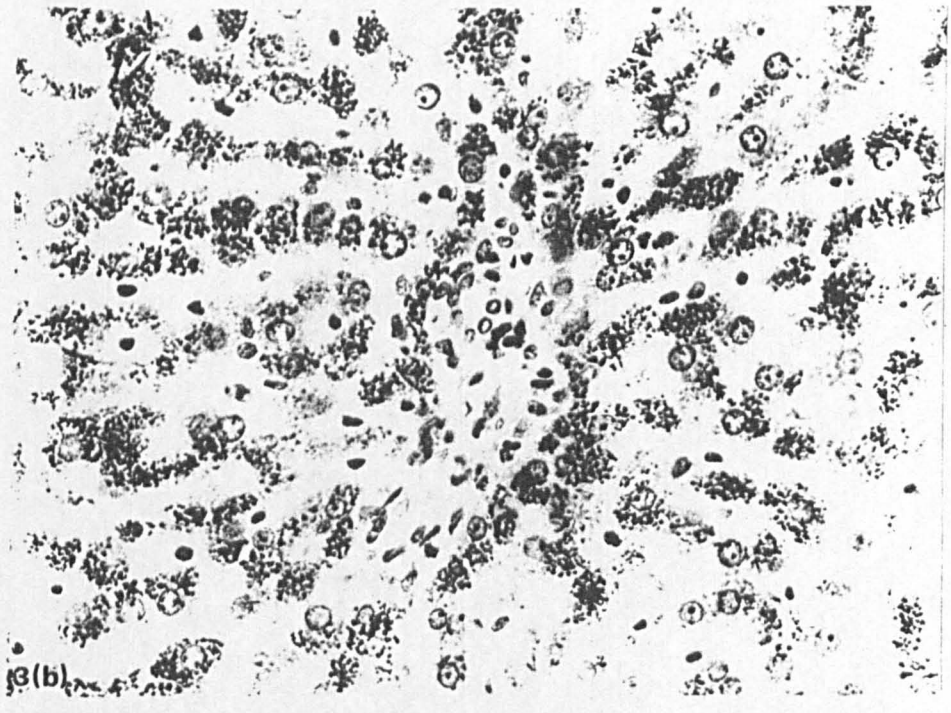


Fig. 3. (a) Liver from a rat copper-loaded for 5 weeks showing heavy deposition of copper in the periportal and mid-zones in contrast to the central zones. CV, central vein; PC, portal area. Rubeanic acid and metanil yellow. × 157·5.

Fig. 3. (b) Liver from a rat copper-loaded for 5 weeks showing the heavy granular deposition of copper positive stain in the periportal and mid-zonal hepatocytes. Rhodanine and haematoxylin. × 396.

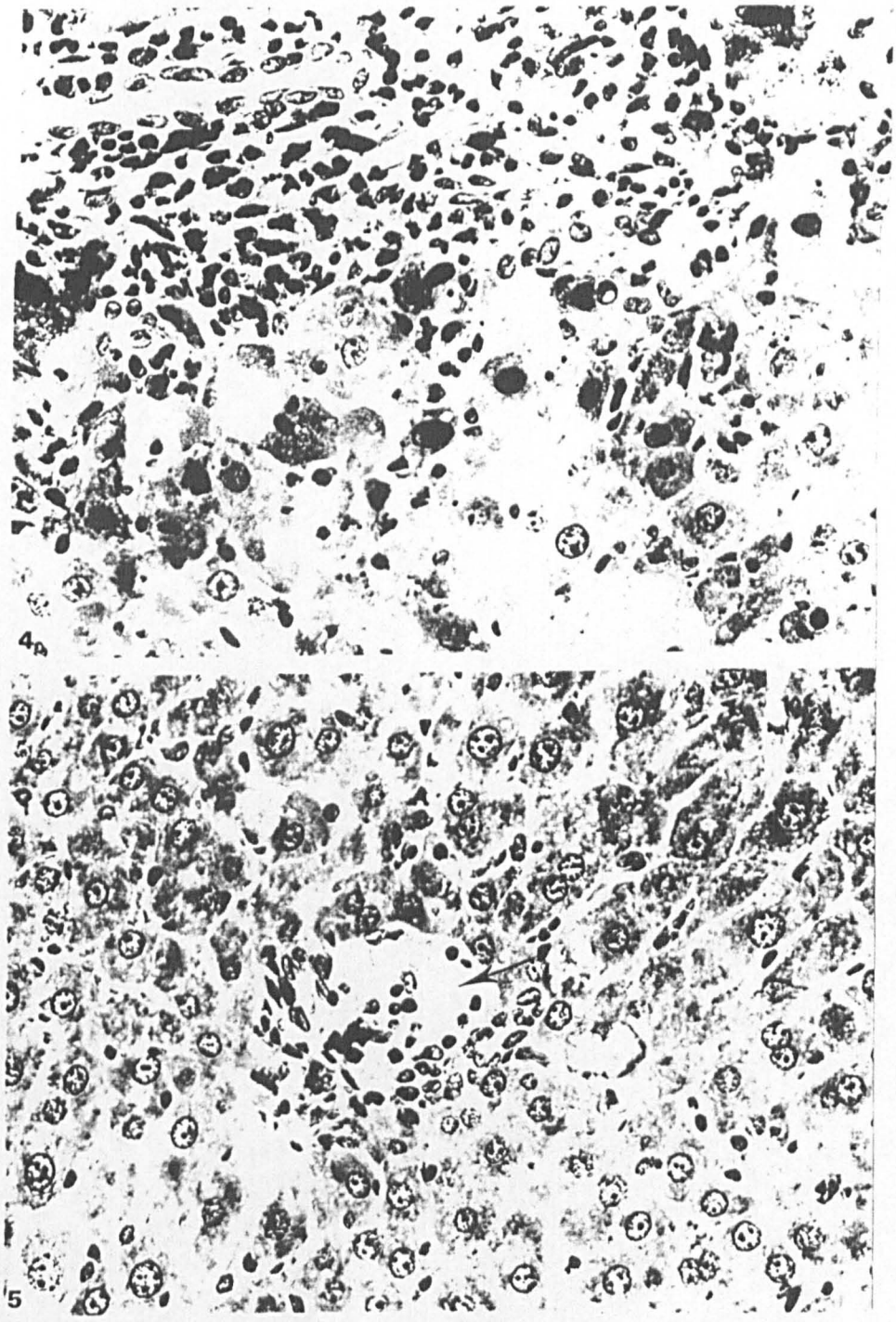


Fig. 4. Liver from a rat copper-loaded for 6 weeks showing extensive necrosis and inflammatory changes in adjacent portal tracts. HE × 396.

Fig. 5. Liver from a rat copper-loaded for 16 weeks showing the persistence of hyaline bodies in the portal tracts (arrow). Note recovery of hepatic parenchyma and the absence of inflammatory changes. × 396.

by the presence of numerous small to medium size granules in the cytoplasm of the hepatic cells.

Weeks 5 and 6. Hyperchromatic, hypertrophic cells now occupied the outer zones; their nuclei were often darkly stained and appeared degenerate. Inflammatory foci were numerous and tended to coalesce. The outer zones, and in particular the mid-zone, appeared to be saturated with small to medium size granules of copper, whereas the central zone still contained very little copper [Figs 3(a) and 3(b)].

In 2 individuals there was more extensive necrosis and a correspondingly marked cellular inflammatory reaction, mainly in the outer zones (Fig. 4). Bile duct hyperplasia and mitotic figures in the hepatic parenchyma were noticeable. Copper staining was much less in these livers and was very variable between individual cells.

Weeks 8 to 12. The hyperchromatic hypertrophic cells were still numerous although degenerative and inflammatory changes had lessened. Copper staining was now less intense than the preceding weeks and still limited to the outer zones and showed the same variation between individual cells.

Week 16. The inflammatory changes had regressed to control values, although the livers of copper-supplemented rats showed the persistence of enlarged deeply staining hepatocytes, the presence of hyaline bodies in the periportal areas (Fig. 5) and bile duct hyperplasia. Copper staining demonstrated the least copper deposition in the hepatic cells compared with livers examined from weeks 5 onwards.

Control livers contained small areas of non-specific periportal inflammation. Copper was not detected in sections treated with any of the stains used.

Discussion

This study has shown that copper-loaded rats store the metal predominantly in the periportal and mid-zones of the liver lobule. Storage is paralleled by pathological changes in both zones, unequivocally associated with the progressive accumulation of copper. Subsequently there is a reduction in stainable copper, followed by a decline in liver total copper concentrations and accompanied by recovery. The decline in stainable copper, its relationship to total copper concentration and to intracellular events, and the fall in copper concentration and adaptation that takes place, have been discussed elsewhere (Haywood, 1985; Haywood and Loughran, 1985; Fuentealba *et al.*, 1987).

The novel findings of this study lie in the pattern of copper deposition and the associated changes. This differential distribution might be attributed to the physiological specialization which exists within lobules, whereby the outer (mid and periportal) zones alone, due to haemodynamic factors, have the oxidative capacity to assimilate, store and excrete macromolecules such as iron (Rappaport, 1979). If follows that, should there be an excess of metal, either through increased input (absorption) or decreased output (cholestasis), it will normally be stored within the parenchymal cells of the outer zones. This is seen in the swan in which increased exposure to the metal (and therefore absorption) has occurred in contaminated waters (Molnar, 1983). A periportal accumulation of copper is apparent in human neonates, and the neonates of

many other species before the biliary secretory route matures (Goldfischer and Berstein, 1969). A similar distribution of copper in cholestatic diseases in man has been referred to earlier (Goldfischer *et al.*, 1980). Periportal copper accumulation also occurs in chronic active hepatitis, particularly in Doberman Pinschers, which is probably also associated with disruption of biliary excretion (Crawford, Schall, Jensen and Tasker, 1985).

The periportal accumulation of copper observed in the rat contrasts strikingly with the centrilobular retention reported in cases of familial copper-storage diseases in dogs (Tweldt *et al.*, 1979; Ludwig *et al.*, 1980; Erikkson, 1983; Thornburg *et al.*, 1986; Haywood and Fuentealba, 1987) and chronic copper poisoning in sheep (Ishmael *et al.*, 1971; Gooneratne *et al.*, 1979; Kumaratilake, 1984), which differences may reflect the abnormal copper metabolism inherent in these dogs and sheep. It has been suggested that defective biliary excretion of copper is responsible for abnormal copper retention in sheep (Underwood, 1977) and in the Bedlington Terrier (Su, Owen, Zollan, and Hardy, 1982; Owen and McCall, 1983). However, the centrilobular accumulation recorded in these species does not support this hypothesis. Perhaps it indicates a metabolic defect in the handling of copper peculiar to the hepatocytes of this zone.

The concept of metabolic zonation has developed from an increasing recognition of the microheterogeneity of liver function whereby hepatocytes in periportal (afferent) and perivenous (efferent) zones differ in their enzymic and subcellular structures (Thurman, Kauffman and Jugermann, 1986). It is thought that many toxic lesions with a specific zonal pattern may be explained by this functional difference.

Pathological changes in copper-loaded sheep occur initially within the central zone and consist of focal degeneration and necrosis associated with aggregates of polymorphonuclear neutrophilic leucocytes and accumulation of bile pigment in the canaliculi. Later, in the post-haemolytic phase, fibroblast proliferation in the portal areas and increase in bile ducts and lymphocytic infiltration occur (Ishmael *et al.*, 1971; Gopinath and Howell, 1975).

In Bedlington Terriers, there are 4 grades of abnormality in the liver as the disease progresses: pigment granules without any further hepatic tissue changes, mild focal hepatitis, a chronic active hepatitis and finally cirrhosis (Twedt *et al.*, 1979; Ludwig *et al.*, 1980; Kelly, Haywood and Bennett, 1984). As with the sheep (Ishmael *et al.*, 1971; Gooneratne *et al.*, 1979; Kumaratilake, 1984) these changes occur initially in the central zone (Twedt *et al.*, 1979; Ludwig *et al.*, 1980; Thornburg *et al.*, 1986).

Hyaline bodies present in the periportal zone in the present study have been observed previously in copper-overload in the rat (Haywood, 1985) and may be similar to the Mallory's hyaline present in primary biliary cirrhosis, long standing biliary obstruction, Wilson's disease and Indian childhood cirrhosis (MacSween, Anthony and Scheuer, 1979) but not in copper toxicosis in Bedlington Terriers (Ludwig *et al.*, 1980). The early pathological changes consisting of focal degeneration and necrosis which occur as a result of copper accumulation in the rat, although having a different micro-anatomical localization, are similar to those described in sheep and dogs and indicate the possibility of a similar cellular lesion.

The cytotoxic effect of copper has not been fully clarified but there is evidence to suggest that nuclear destabilization rather than lysosomal disruption may be the initiating factor (Haywood *et al.*, 1985). Nuclear changes observed in this study may reflect these events. Karyomegaly has also been recorded in sheep (Ishmael *et al.*, 1971; Gopinath and Howell, 1975; King and Bremner, 1979; Kumaratilake, 1984; Seaman, 1985). In Wilson's disease, diffuse degenerative changes occur throughout the lobule consisting of vacuolar and fatty change, without reliable copper staining (Goldfischer *et al.*, 1980; Goldfischer and Sternlieb, 1968). It seems that the primary metabolic defect occurs at a different locus from that in Bedlington Terriers and sheep.

In conclusion, the distinctive patterns of copper distribution which occur in copper-associated diseases indicate different pathological mechanisms and suggest that copper accumulation is not always the result of a simple overload associated with increased intake or reduced biliary excretion but may be related more to aberrations in intracellular events which have a particular distribution.

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