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Orcein Positive Granules in the Hepatocytes in Chronic Intrahepatic Cholestasis

Morphological, Histochemical and Electron X-Ray Microanalytical Examination

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Summary. The morphological characteristics of orcein positive granules in hepatocytes from 11 patients with chronic intrahepatic cholestasis and from 2 newborn normal livers were studied. Histochemical investigations revealed their protein nature and many sulphydryl and/or disulphide groups. Copper was demonstrated in the granules by histochemical techniques and electron X-ray micronalysis. No difference was observed in the hepatic distribution and appearance of the granules between the livers of those with chronic cholestasis and the newborn. Ultrastructurally, a variety of electron dense granules were seen at the site of orcein positive granules in the hepatocytes of the patient with primary biliary cirrhosis. Some had a single-layered membrane and seem to be lysosomal derivatives. It is suggested that the copper in lysosomes seen in both chronic cholestasis and normal newborn livers, need not to be cytotoxic.

Key words: Chronic intrahepatic cholestasis – Primary biliary cirrhosis – Hepatocellular orcein positive granules – Copper hepatocytotoxicity.

Introduction

Orcein positive granules are frequently seen in the livers of patients with chronic intrahepatic cholestatic liver disease, Wilson's disease (cirrhotic stage) and in normal newborn livers (Nakanuma et al., 1978; Sipponen, 1976). They have recently been related to the accumulation of copper in hepatocytes, but differ in distribution and appearance from the deposition of HBsAg (Nakanuma et al., 1978; Shikata, 1974; Sipponen, 1976). The aim of this work is to obtain more knowledge about the morphologic and cytochemic characteristics of the orcein positive granules and the possible differences between chronic cholestatic and

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normal newborn livers. The granules were also studied by electron X-ray microanalyzer in the liver cells of a patient with primary biliary cirrhosis (PBC).

Material and Methods

Liver specimens consisted of 10 cases of PBC, one case of hepatic sarcoidosis with chronic intrahepatic cholestasis (Rudzki et al., 1975) for 2 and a half years, 2 of normal newborn livers and liver from a normal adult.

Ten PBC liver specimens (5 from autopsies and 5 wedge biopsy specimens) were kindly given to us from many institutes in Japan, including our medical clinics. The clinical and morphologic diagnosis of all patients was PBC. Jaundice was present in all, except one asymtomatic case, and extrahepatic bile duct obstruction was ruled out by laparotomy and/or autopsy in all patients. Mitochondrial antibodies (AMA) were present in 9 of 10 patients with PBC. The patient without AMA was diagnosed as PBC on the basis of the other clinical, laboratory and morphologic findings. In every instance the livers showed the characteristic histologic changes of PBC, namely, chronic nonsuppurative destructive cholangitis (CNSDC) (Rubin et al., 1965) and/or marked reduction of the number of the interlobular bile ducts (Baggenstoss et al., 1964). Two patients with PBC including the asymptomatic case exhibited CNSDC without periportal fibrosis, 5 cases CNSDC with a variable degree of periportal fibrosis and 3 cases, cirrhosis.

The liver specimens were fixed in 10% formalin, embedded in paraffin and cut into 5 μ section. Frozen sections from two PBC livers were used for histochemical methods.

One PBC liver showing CNSDC with periportal fibrosis, large amounts of orcein positive granules and a rubeanic acid reaction positive for copper, which contained 2,155 μg copper per g dry weight (normal hepatic copper content: 23.1–52.3 μg/g dry liver, Nakanuma and Ohta, 1978) was subjected to electron microscopy and electron X-ray microanalysis. The tissue was immediately fixed in phosphate-buffered 3% glutaraldehyde, postfixed in 1% osmium tetraoxide and dehydrated with alcohol and embedded in EPON 812. Thin sections were stained with uranylacetate and lead citrate for electron microscopy (JEM 100B). Sections, 0.5 μ in thickness, were mounted on gold grids with a supporting membrane and examined with a Hitachi kevex 5,100 X-ray microanalyzer with energy diversive system (accelerating voltage of 75 kV, resolving power of 165 eV) (Watanabe and Nagatani, 1974).

The histochemical methods applied were: orcein staining, performed by the method of Shikata (Shikata, 1974), the rubeanic acid and d-dimethyl-aminobenzylidine rhodanine staining for copper (Sano, 1976) Ziehl-Neelsen reaction, Schmorl's ferric ferricyanide method, congo red reaction for amyloid, Gmelin and Stein reaction for bile pigment, Perls reaction for iron, Sudan black B, oil red 0, Sudan III and Luxol fast blue for lipid. Alcian blue (pH 0.2, 1.0, 2.5), Gomori's aldehyde-fuchsin, mucicarmine and toluidine blue (pH 2.5, 4.1, 7.0) stains were also used. Sulphydryl and disulphide groups were stained by alcian blue with performic acid oxidation, Schmorl's ferric-ferricyanide with or without 0.1 M-N-ethylmaleimide blocking, dehydroxyl-dinaphthyl-disulphide (DDD) method and mercury orange reaction.

The oxidation of the tissue was performed in 0.3% potassium permanganate followed by decolarization in 1.5% oxalic acid. Deparaffinized sections were used in the study of autofluorescence under UV light. Hepatic copper concentration was measured by atomic absorption spectrophotometry (Nakanuma and Ohta, 1978).

Results

1. Histochemical Staining

Orcein positive granules were seen in the hepatocytes of the livers of all patients with PBC, with hepatic sarcoidosis and in newborns, when tissue sections were oxidized before staining (Fig. 1). Normal adult liver did not contain such gran-

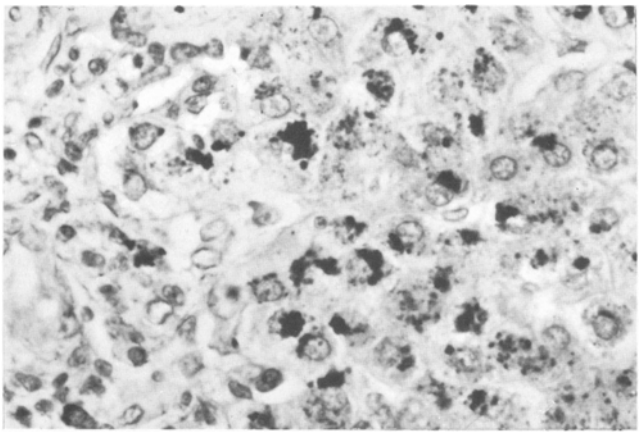

Fig. 1. Orcein positive granules accumulated in the perilobular hepatocytes of PBC liver (wedge biopsy). Orcein stain following oxidation. × 520

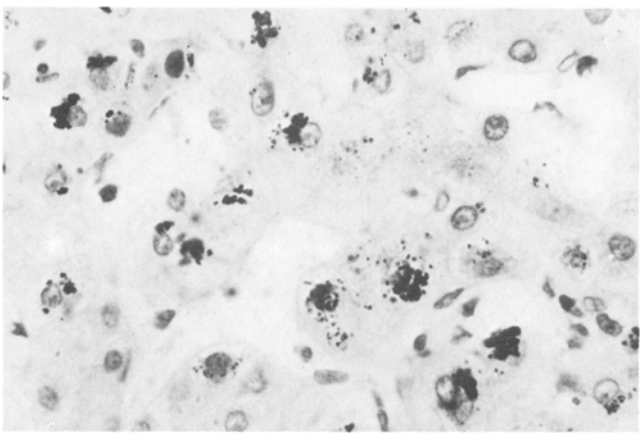

Fig. 2. Copper granules present in the perilobular hepatocytes of PBC liver (wedge biopsy). Rhodanine stain for copper. × 520

ules. The granules were seen mainly seen in the peripheral hepatocytes of either hepatic lobules or of regenerative nodules, except 2 cases of PBC in which a few granules were also seen in the midzonal and centrozonal areas of the lobules. These coarse granules tended to appear in the perinuclear portions of the cells. The staining pattern and intracellular distribution were the same in all parts of the specimens examined. There were no differences in the hepatic distribution and appearance of the granules between PBC, hepatic sarcoidosis and newborn livers.

Copper staining: hepatocellular copper was demonstrated by rubeanic acid and d-dimethyl-aminobenzylidine rhodanine (Fig. 2) in a location similar to that of the orcein positive granules in all patients. There was one exception, a liver with PBC which had been fixed in formalin for 2 years; the hepatic copper value was 323 μg/g dry liver. Oxidation of tissue sections completely abolished the copper reaction.

Table 1. Histochemic characteristics of the orcein positive granule

Staining methods	Section	Staining results	
		Without oxidation	With $KMnO_4$ oxidation
H.E. stain	para.	−	
Orcein stain	para.	−	+
Copper stain			
Rubeanic acid	para.	+ ~ −	−
Rhodanine	para.	+ ~ −	−
PAS after diastase digestion	para.	±	±
Long Ziehl-Neelsen	para.	+	+
Ferric-Ferricyanide	para.	+	−
Bile pigment			
Gmelin	para.	−	
Stain	para.	−	
Congo red for amyloid	para.	−	
Fe-stain	para.	−	
Lipid stains			
Sudan III	fro.	−	
Sudan black B	fro.	−	
Oil red O	fro.	−	
L.F.B.	para.	−	−
Alcian blue			
pH 0.2	para.	−	+
pH 1.0	para.	−	+
pH 2.5	para.	−	+
Aldehyde fuchsin	para.	−	+
Mucicarmine	para.	−	+
Toluidine blue			
pH 2.5	para.	−	slight metachromasia
pH 4.1	para.	−	slight metachromasia
pH 7.0	para.	−	slight metachromasia
Autorfluorescence	para.	−	

para. = paraffin block; fro. = frozen section; + = typical positive staining reaction; ± = questionable staining reaction; − = negative staining reaction; + ~ − = positive in some cases and negative in other cases.

Additional histochemical features are see in Table 1: the granules showed a slightly positive reaction with PAS after diastase digestion and a strong positive reaction with Ziehl-Neelsen stain with or without pre-oxidation. They revealed a strong reducing capacity when tested with Schmorl's ferric-ferricyanide method. The stains for bile pigments, amyloid and iron gave negative results. Lipids could not be demonstrated in the granules, using several stains.

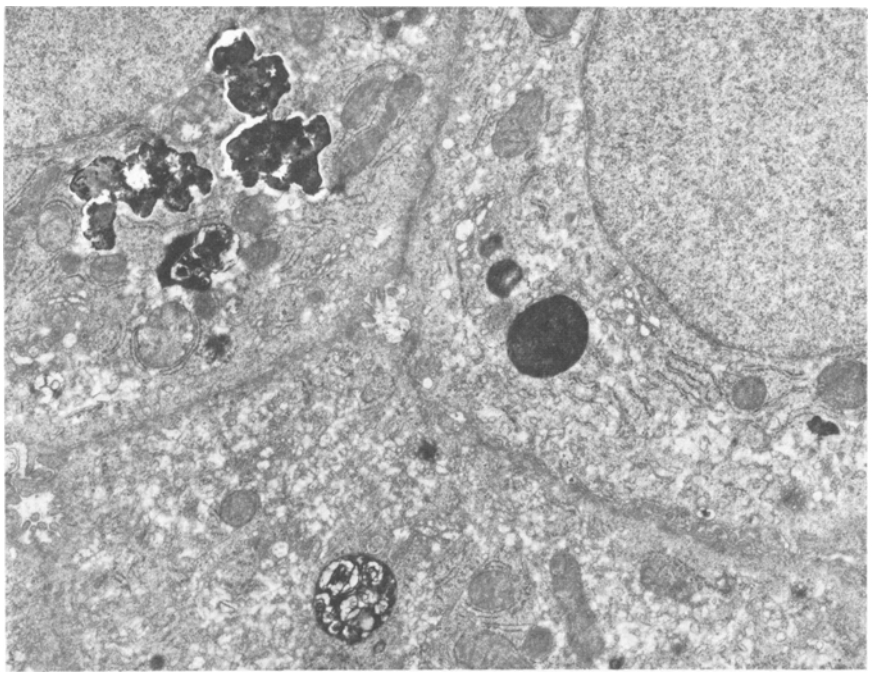

Fig. 3. Electron dense granules of various shape and size are seen in peripheral hepatocytes of a liver from primary biliary cirrhosis. ×11,700

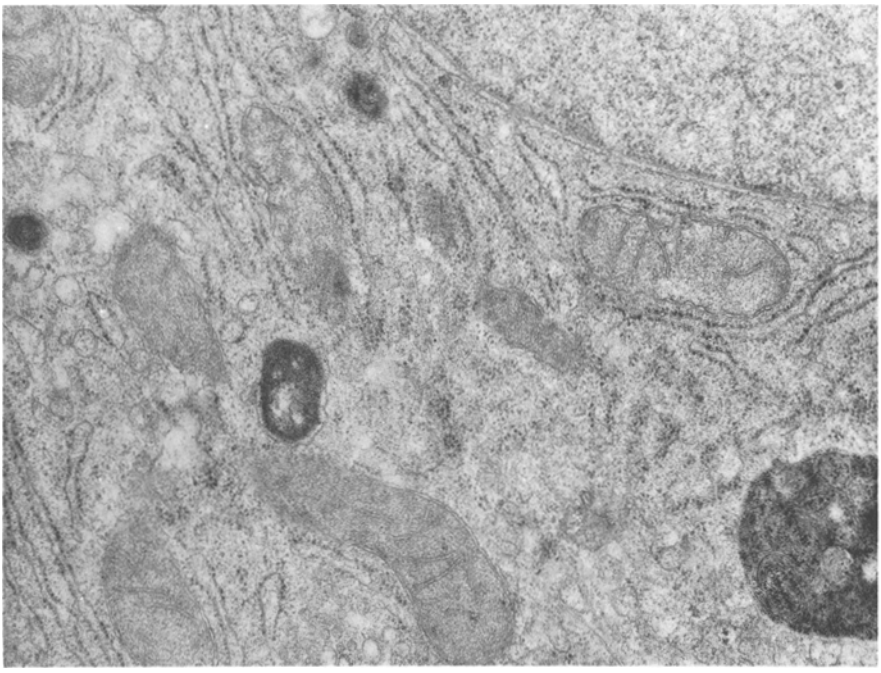

Fig. 4. Some of electron dense granules in hepatocytes are surrounded by a single layered membrane. ×23,400

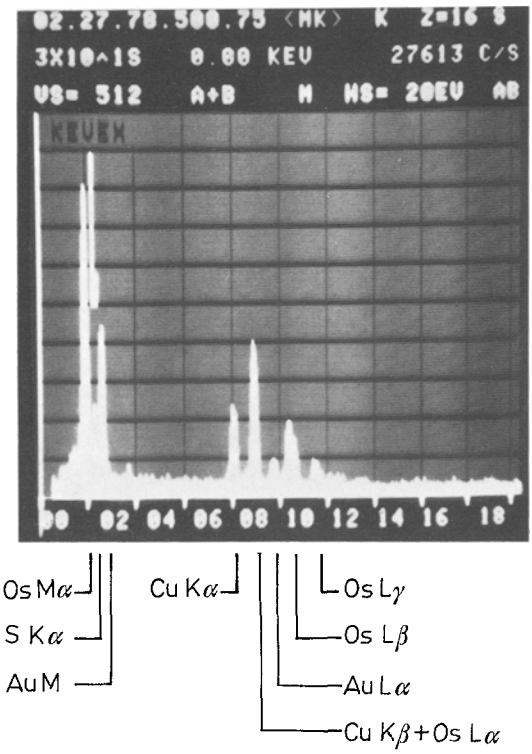

Fig. 5. Display of characteristic X-ray images in a electron dense granule in the hepatocyte of a patient with PBC. White bars denote characteristic X-ray images of sulphur

After pre-oxidation, alcian blue at pH as low as 0.2, Gomori's aldehyde-fuchsin and mucicarmine stains gave a positive reaction, all in the same portion of the hepatocytes and in the same distribution in the livers examined. The granules revealed a slight metachromasia with toluidine blue at a pH as low as 2.5.

The granules were not auto-fluorescent under UV light.

Alcian blue (pH 0.2) stain following performic acid oxidation, for the demonstration of sulphydryl and/or disulphide groups, revealed positive results. So did Schmorl's ferric-ferricyanide reaction, but this positivity could be prevented by n-ethylmaleimide pretreatment. These findings suggest the presence of sulphydryl and/or disulphide groups in the granules. However, the DDD method and mercury orange reaction gave negative results, probably because of the formalin fixation.

There was no difference in histochemical staining of the hepatocellular granules in PBC, hepatic sarcoidosis or normal newborns.

2. Electron Microscopy and Electron X-Ray Microanalysis

Toluidine blue (pH 7.2), with which the semithin sections from one PBC liver were stained, showed an affinity for hepatocellular granules in a location identi-

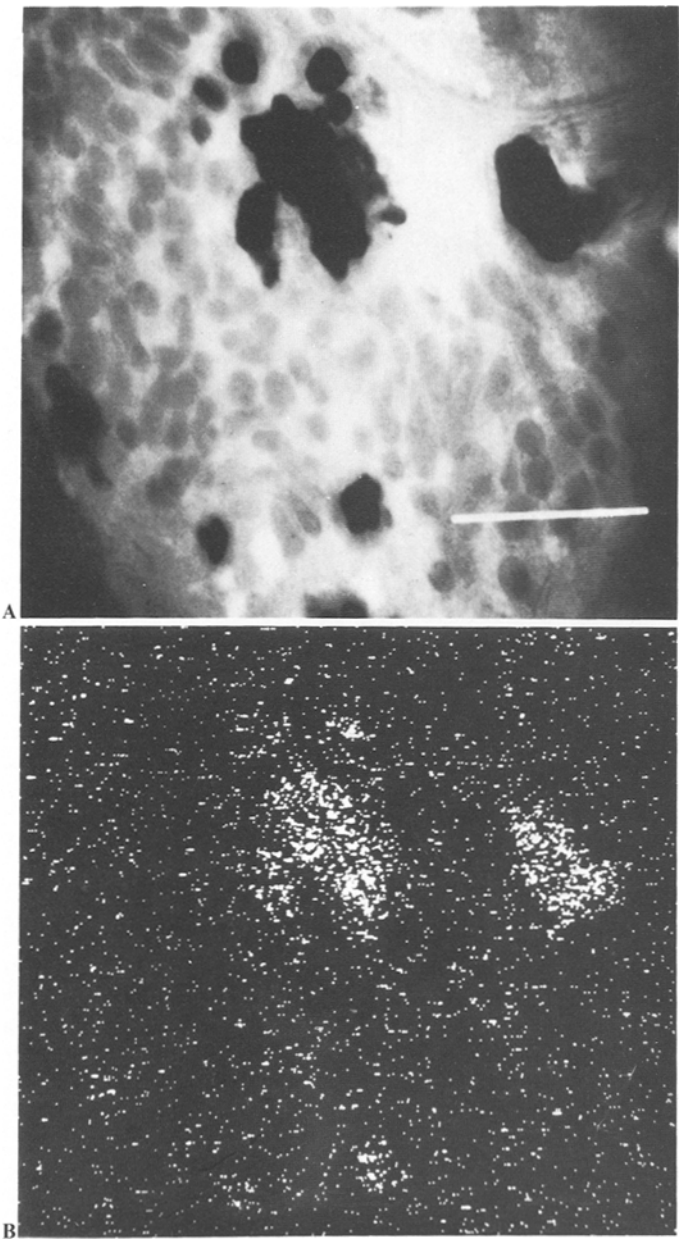

Fig. 6. A A number of electron dense granules are seen in the cytoplasm of hepatocyte of a patient with PBC. × 5,000. **B** Image produced by Cu Kα (7.98–8.10 keV) emission of the same hepatocyte of PBC demonstrated in Fig.6–A. electromicroscopically. High copper concentration is shown in the electron dense granules. × 5,000

cal to that of the orcein positive granules in paraffin sections. Ultrastructurally, most of the hepatocytes in the lobular periphery contained round or irregular shaped particles (ad 0.5–3 μ) of varying electron density (Fig. 3). Some particles consisted of irregular clusters of electron dense smaller material and some showed vacuolar or lamellated structures or uniform electron density. The particles were mainly in the perinuclear portions. Some of them were surrounded by a single-layered membrane (Fig. 4). In general, the morphological features of these particles were similar to autophagic vacuoles or secondary lysosomes (Goldfischer and Sternlieb, 1968; Hayashi et al., 1977; Sipponen, 1976).

Electron X-ray microanalysis revealed the characteristic X-ray picture of Cu kα (8.074 keV) and S Kα (2.307 keV) within electron dense granules in the liver specimen (Fig. 5). This suggests that the orcein positive granules are rich in copper and sulphur. However, the amounts of copper and sulphur estimated by the X-ray microanalysis varied, depending on the granules and even in different parts of the same granule. The X-ray image peaks specific for copper were much higher in strongly electron dense portions of the particles than in weakly electron dense portions. The X-ray image of copper was not detected in the nucleus and cytoplasm other than in the electron dense granules. Simultaneous detection of characteristic X-ray images of Os (Mα, Lα, Lβ, Lγ) and Au (M, Lα) were due to an atrifact of fixation of the liver tissue in osmium tetraoxide and the gold grids.

The X-ray image produced by Cu Kα (7.98–8.10 keV) emission under the microanalytical electron microscope (Fig. 6A) and observation of the same portion of the same liver cell by transmission electron microscopy (Fig. 6B) revealed that the copper was present in the electron dense granules.

Discussion

The liver is regarded as the most important organ in copper metabolism. Abnormalities of metabolism have been reported in some liver diseases, thus accumulation of copper in hepatocytes of patients with Wilson disease is assumed to be the result of an inborn error of copper metabolism, while an icreased copper in chronic cholestatic liver diseases is interpretated as the results of diminished copper excretion via the biliary tree into the intestine (Deering et al., 1977; Flemming et al., 1974; Goldfischer and Sternlieb, 1968; Hunt et al., 1963; Jains et al., 1977; Sherlock, 1968).

Sipponen (Sipponen, 1976) first reported an accumulation of orcein positive granules in the hepatocytes of patients with chronic cholestatic liver diseases, especially in primary biliary cirrhosis (PBC). He considered them to be intracellular copper-binding protein. A previous paper (Nakanuma and Ohta, 1978) reported that the granules were to be found in the hepatocytes of neonates and in patients with chronic cholestatic liver diseases, in particular PBC. The present study indicates that the granules show a strong positive reaction with alcian blue, aldehyde-fuchsin and mucicarmine, and a slight metachromasia with toluidine blue after previous oxidation of the tissue sections. Because the sulphydryl and/or disulphide groups are directly demonstrated in the granules by some of the special stains for these groups, the sulphonic acid residue

produced in the granules by oxidation of sulphydryl and/or disulphide groups are held to be responsible for the basophilia and probably for the staining reactions similar to those of acid mucopolysaccharides. In addition, the staining characteristics indicate the protein nature of the granules. Copper was demonstrated in the granules by special cytochemic methods. These findings are consistent with the data obtained by Sipponen (Sipponen, 1976) and suggest that the granules are associated with copper in the hepatocytes and confirm the presence of copperprotein complexes. Ultrastructural studies and electron X-ray microanalysis reveal that the granules are secondary lysosomes and contain copper and sulphur.

Recent biochemical investigations (Evans, 1973; Kimura, 1975; Porter, 1974) have shown that copper is stored or accumulates in the form of copper-apoprotein, metallothionein. This copper rich form of metallothionein is known to be localized in a distinct population of heavy lysosomes and cupric ion is bound to the apoprotein with two or three cytine molecules. The morphologic features of the granules shown in the present study suggest that the granules are metallothionein or cuprothionein *per se* and stored in a form of lysosomal particles. The granules also show a positive reaction with Ziehl-Neelsen stain and a slight positive reaction with PAS before or after preoxidation of the tissue sections. Possibly the granules have staining characteristics similar to lipofuscin, a known lysosomal derivative.

Copper inhibits many enzyme reactions, and deposition of copper in the tissues is held responsible for the pathologic and functional changes in Wilson disease (Flemming, 1974; Goldfischer and Sternlieb, 1968; Hunt, 1963; Walshe, 1975). Striking differences in the intracellular distribution of copper in the hepatocytes of patients with different stages of Wilsons disease have been reported (Goldfisher and Sternlieb, 1968). In asymptomatic patients, copper distribution in the hepatocytes is diffuse but in patients with advanced disease, copper is found exclusively in lysosomes. Cytopathological changes in copper bearing hepatocytes are more pronounced in asymptomatic patients than in advanced disease. In normal newborns in whom copper is present in excess but not associated with hepatic injury, copper was also found exclusively in lysosomes. The authors suggest that intracytoplasmic sequestration and concentration of copper within the lysosomes might protect the hepatocytes from the toxic effect of the metal to some degree (Goldfischer and Sternlieb, 1968).

Although a causal relationship between copper deposition in the liver and the hepatocytic lesion in PBC has been accepted by some investigators, it remains a topic of vigorous and continuing debate (Deering et al., 1977; Flemming et al., 1974; Scott, 1978). Recent reports (Deering et al., 1977; Jains et al., 1977; Sherlock, 1978) have cited the therapeutic efficacy of the copper chelating agent, d-penicillamine in PBC. Recently Popper (Popper, 1978) suggested that d-penicillamine had another effect, similar to steroids, namely to reduce the inflammatory reaction in the liver of PBC. This effect was not necessarily a result of the copper chelating action; the copper granules in PBC livers need not then have cytotoxic effect on the hepatocytes. This concept is supported by the present studies, which failed to show a difference in the appearance of copper accumulating in the lysosomal granules of the hepatocytes in PBC, hepatic sarcoidosis with chronic intrahepatic cholestasis or in the normal newborn. Moreover,

electron X-ray microanalysis of PBC livers showed copper in no other organelles than electron dense granules.

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