The Significance of Variations in the Distribution of Copper in Liver Disease

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Biopsy and autopsy specimens of liver from patients with Wilson's disease in various stages, chronic cholestatic conditions (including primary biliary cirrhosis, extrahepatic biliary obstruction, sclerosing cholangitis, and biliary atresia), chronic active hepatitis, and Indian childhood cirrhosis, as well as normal neonates, were examined by means of histochemical techniques for copper and copper-associated protein. The intracellular localization of copper and the lobular distribution of the metal and its associated protein differed in these conditions. Periportal hepatocytes containing granules (lysosomes) that were reactive for copper and for copper-associated protein were characteristic of cholestasis and neonatal liver. However, in cholestasis extralysosomal copper was often present in the hepatocellular cytoplasm. In contrast, in Wilson's disease, despite very high concentrations of copper in the early stages, the metal was diffuse in the cytoplasm, and the histochemical reactions for granular copper and its associated protein were usually negative. Therefore, a failure to stain for copper does not exclude the diagnosis of Wilson's disease. In the late stages of Wilson's disease staining varied in different nodules. In Indian childhood cirrhosis copper was present throughout the parenchyma, with periportal predominance. Differences in the distribution of copper and the cellular changes associated with its deposition suggest that different pathogenetic mechanisms and possibly different intracellular targets are susceptible to the toxic effects of the metal. For diagnosis, staining for copper and for copper-associated protein may assist in the differentiation of primary biliary cirrhosis from chronic active hepatitis. (Am J Pathol 1980, 99:715-730)

COPPER may be identified in histologic sections by staining with rhodanine,[1,2] rubeanic acid,[3,4] or Timm's silver sulfide.[4,5] Although these simple and reproducible staining procedures are only semiquantitative, they make it possible to determine the intracellular and lobular distribution of copper. A complementary stain was recently discovered by Sipponen,[6] who found that orcein stained a poorly characterized "copper-associated protein," rich in sulfhydryl groups, within hepatocellular granules. The combined use of these various histochemical techniques has provided new information on the varying patterns of copper distribution in diseases associated with excessive accumulations of the

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metal in the liver.[6-11] We report the histologic and intracellular localization of copper and its associated protein in primary biliary cirrhosis (PBC), extrahepatic biliary obstruction, biliary atresia, cryptogenic cirrhosis with cholestasis, Wilson's disease, and Indian childhood cirrhosis (ICC), as well as in normal neonatal livers, all conditions known to be associated with high concentrations of hepatic copper.[8,12-20] Our goal was to compare and evaluate the diagnostic and pathogenetic implications of the topographic distribution of hepatic copper in these diseases.

Materials and Methods

This study is based on liver tissue, mainly paraffin blocks, on file at the Albert Einstein College of Medicine and the Mount Sinai School of Medicine and consists of diagnostic biopsy specimens and autopsy material. All specimens had been fixed in neutral formalin, embedded in paraffin, and stained by the common routine histologic methods. Copper was stained orange-red with 5(p-dimethylaminobenzylidene)rhodanine (Eastman No. 2748),[1,2] green-black with rubeanic acid (dithiooxamide) (Eastman),[4] and brown-black with the silver sulfide (Timm's) procedure.[4] Copper-associated protein was visualized with the Shikata orcein (British Drug House) technique [6] with the use of counterstaining with lithium green.

Hepatocellular lipofuscin granules were demonstrated by the periodic acid-Schiff procedure after diastase treatment and by Schmorl's ferro-ferricyanide reaction for reducing substances.[21]

For quantitative assays for copper, 42 dehydrated specimens weighing 1-15 mg were wet-washed and subjected to spectrophotometric analysis.[22,23] The paraffin was removed from embedded tissues with two changes of xylene, at 24-hour intervals, before dehydration in a vacuum oven. The normal adult liver contains less than 50 μg copper/g dry tissue. Normal liver removed from paraffin blocks for quantitative determination contains less than 86 μg copper/g dry tissue.[11] These results are summarized in Table 1.

Histochemical analyses were carried out on 18 patients with PBC in advanced stages, 7 with extrahepatic biliary obstruction, 7 with chronic active hepatitis associated with cholestasis, 3 with mixed micronodular-macronodular cirrhosis with jaundice, 2 with biliary atresia, 14 with Wilson's disease in various stages, 3 with ICC in terminal stages, and 4 premature infants with normal liver. In addition, 27 biopsy specimens in all stages of PBC and 7 from patients with sclerosing cholangitis were studied in which only rhodanine and Shikata's orcein stain were used.

Control specimens were also stained, including specimens from normal and diseased

Table 1—Hepatic Copper Concentration

	Number of patients	Range of Cu (μg/g dry wt)	Mean	SD
Primary biliary cirrhosis	10	37-714	356	262
Extra hepatic biliary obstruction	2	111-226	169	—
Cryptogenic cirrhosis	1	289	—	—
Wilson's disease	14	188-1422	728	374
Newborn	4	206-413	295	89
Indian childhood cirrhosis	3	1367-4788	2570	—
Chronic active hepatitis	1	42	—	—
Normal control subjects (6-86 years)	7	30-86	58	19

livers (Dubin–Johnson syndrome, hemochromatosis, hepatocellular tumors, and conditions with excess lipofuscin accumulation).

Results

Incidence and Distribution of Positive Copper Reactions: Granular Staining

Granular copper staining in hepatocytes was demonstrable with rhodanine, rubeanic acid, and Timm's procedure in 17 of 18 patients with advanced PBC (Figure 1), 2 of 2 infants with biliary atresia, 5 of 7 patients with extrahepatic biliary obstruction, 3 of 3 patients with postnecrotic cirrhosis with cholestasis, 9 of 14 patients with Wilson's disease (Figure 3), 3 of 3 children with advanced ICC (Figure 5), 1 of 7 patients with chronic active hepatitis, and all 4 newborns. No stainable copper was present in the control livers. A similar staining pattern for the copper-associated protein was obtained with orcein (Figures 2, 4, 6, and 7). However, more granules were stained with orcein than with the copper stains. This situation was notable in Wilson's disease, where 3 specimens gave a positive orcein reaction but did not stain with rhodanine or rubeanic acid. The granules were identified as lysosomes because in unstained sections they have the characteristic golden brown color of lipofuscin and they displayed both a positive Schmorl's staining reaction for lipofuscin and a periodic acid–Schiff (PAS) reaction that resisted diastase digestion (Figure 8).

Orcein, rhodanine, and rubeanic acid did not stain either the pigment present in the hepatocytes of patients with Dubin–Johnson syndrome or hemosiderin. Iron, however, gave a positive Timm's reaction.

Diffuse Cytoplasmic Staining

An orange–pink diffuse cytoplasmic staining of hepatocytes was occasionally observed with rhodanine. A similarly distributed brown stain was frequently seen with the aid of Timm's procedure. The diffuse reaction was present in 8 of 17 patients with PBC (Figure 9), in 2 of 3 patients with early Wilson's disease, and in 2 of 3 children with ICC.

Specific Disorders

Primary Biliary Cirrhosis

In addition to the 18 cases listed above, we examined 27 cases of PBC in which only orcein and rhodanine staining were compared. In PBC the presence of copper generally correlated with the stage of the disease. It was always absent in the early stages, in which only chronic nonsuppurative destructive cholangitis was present and an intact limiting he-

patocytic plate surrounded the large portal tracts.[24] In the second stage, which is characterized by proliferation and destruction of ductules, the presence of copper varied with the type of erosion of the periportal parenchyma. If this destruction was associated with a predominantly lymphocytic exudate, hepatocytic copper deposition was not encountered. Copper was noted when vacuolated macrophages were crowded between periportal hepatocytes. Such copper-containing hepatocytes often displayed a vacuolated cytoplasm even in the absence of demonstrable bile pigment. The number of copper granules increased when histologically demonstrable cholestasis was associated with significant portal and periportal fibrosis, reflecting a transition into the third, or scarring, stage of PBC. Progression to cirrhosis of the biliary type, which is characterized by preservation of hepatic vein tributaries, was regularly associated with a further increase in demonstrable copper, though in some forms of mixed micromacronodular cirrhosis it was absent. The destruction of bile ducts did not seem to affect the distribution of copper.

In late stages of PBC copper-containing granules were abundant in hepatocytes around portal tracts, particularly in those trapped in periportal zones undergoing fibrosis (Figure 1). However, around normal small portal tracts, which are encountered even in advanced stages of PBC, copper was not seen. Portal macrophages sometimes contained copper-stained granules. The intensity of the histologic staining correlated fairly well with quantitative assays for copper. Centrolobular cholestasis, relatively rare in primary biliary cirrhosis, was not associated with copper deposition. In some specimens lysosomes stained with orcein but not with rhodanine.

When, in addition to the particulate staining, diffuse cytoplasmic staining was demonstrated by rhodanine or Timm's silver sulfide stain, it was most conspicuous in hepatocytes located near altered portal tracts or connective septums and in isolated trapped hepatocytes. Mallory's hyalin, present in 6 cases, was rimmed by copper-containing granules and often assumed a reddish hue in rhodanine preparations.

Chronic Cholestasis

Copper granules were also found in biliary atresia, prolonged extrahepatic biliary obstruction, and sclerosing cholangitis and in one instance of mixed micromacronodular cirrhosis but were generally absent in chronic active hepatitis with cholestasis. Periportal cytoplasmic staining was seen in a patient with a 10-year history of extrahepatic biliary obstruction and in one with cholestatic, mixed micromacronodular cirrhosis.

Wilson's Disease

The intracellular and lobular distribution of stainable copper and copper-associated protein varied, depending on the stage of the disease.[25] In the earliest stage, which manifested diffuse steatosis, often with portal and intralobular fibrosis, granular copper and copper-binding protein could not be demonstrated histologically by any of the methods applied, despite concentrations of copper as high as 1123 μg/g dry tissue. However, in such specimens diffuse cytoplasmic staining was revealed by the Timm's silver sulfide procedure. At a later stage, when histologic features of chronic active hepatitis appeared, copper and the copper-associated protein were demonstrable in hepatocellular granules near portal tracts and septums, often extending into the lobule. The granular copper that was seen at this stage could be stained by all of the procedures employed. In some areas a diffuse cytoplasmic reaction for copper was also noted. In fully developed cirrhosis the copper reaction was usually restricted to hepatocellular lysosomes. The number of positive cells varied from a few at the periphery of a nodule to large numbers occupying a segment of a nodule or an entire nodule. Unreactive nodules were often adjacent to those with large amounts of copper (Figures 3 and 4). This variability may explain the absence of staining in 2 cirrhotic specimens.

Indian Childhood Cirrhosis

Markedly elevated hepatic copper concentrations were found in autopsy specimens from individuals in the terminal stages of ICC (Figures 5 and 6). One had the highest concentration of copper ever recorded in human liver, 4788 μg/g. Histochemical stains demonstrated widely distributed granular and diffuse cytoplasmic staining as well as an association of copper with Mallory bodies.[11] The intensity of the copper stain did not always correlate with the concentration of copper in the tissues, and in some hepatocytes and macrophages orcein demonstrated copper-associated protein in granules that failed to give a copper reaction. Tanner et al [26] have reported similar findings in ICC. The granules in macrophages were less distinctly stained than those in hepatocytes and were not deep black in color.

Discussion

In the normal human adult [23] the concentration of hepatic copper remains remarkably stable throughout life. This balance is achieved in spite of an abundance of dietary copper in relation to the body's needs,[20,23,28] mainly through the excretion of excess copper via the bile.[20,29] Con-

sequently an interference in this process either by a genetic defect or cholestasis may result in retention of copper by the liver. Increased concentrations of hepatic copper are also present in normal babies during the first months of life.[17,27]

The lobular and intracellular distribution of copper differs in various liver diseases. In cholestasis as well as in the liver of the normal neonates, copper and its associated protein are found mostly in hepatocellular lysosomal granules at the periphery of lobules or nodules.[6,8,10,27] In some specimens copper may also be diffuse in the cytoplasm. In the late stages of ICC staining is diffuse and granular, involving the entire parenchyma, with peripheral predominance. In advanced Wilson's disease only certain nodules or portions of nodules accept the stains.[4] However, in young asymptomatic patients with Wilson's disease whose chemically determined hepatic copper concentrations are higher,[4,25,31] there is no granular lysosomal copper demonstrable. In such livers, Timm's silver sulfide yields diffuse cytoplasmic staining that is generally uniform throughout the hepatic parenchyma.

For diagnostic purposes, it should be emphasized that a negative histochemical reaction for copper with rhodanine or rubeanic acid, or for copper-associated protein with orcein, does not exclude Wilson's disease.[4,8] In conditions other than Wilson's disease histochemical estimates generally correlate with quantitative determinations of total hepatic copper.[8] Histochemical preparations are particularly useful in detecting focal concentrations of copper affecting a few lysosomes, even though the tissue concentration is normal. The demonstration of copper is often helpful in distinguishing the late stages of PBC from chronic active hepatitis, where it is usually absent. This conclusion is consistent with that of Ludwig et al,[10] based on a study of North American patients, and of Sipponen et al,[30] who investigated Finnish patients. It differs, however, from that of Jain et al,[8] who reported positive stains in 7 of 16 British patients with chronic active hepatitis.

The orcein, rhodanine, and rubeanic acid reactions are all equally simple to use and consistently reproducible. More granules react with orcein than with the copper stains. These granules, which stain black with orcein, are easily distinguished from the brown cytoplasmic staining of the hepatitis B surface antigen present in "ground-glass" hepatocytes and from elastin fibers.[32] The most sensitive method for demonstrating metals is Timm's silver sulfide procedure, but it is also more complex and less specific than the rhodanine and rubeanic acid stains. The latter, therefore, are more suitable for routine diagnostic laboratory procedures.

There are two copper-binding proteins in the cytosol: L-6-D, or

CuLP,[33] often confused with metallothionein, and hepatocuprein, or superoxide dismutase.[34] "Mitochondrocuprein," originally extracted from "mitochondrial" fractions of newborn human and calf liver,[35,36] is a lysosomal protein [27,37] that is probably identical with the copper-associated protein demonstrable with Shikata's orcein stain. This is evident from studies which indicate that the copper-containing granules are lysosomes that contain lipofuscin and PAS-positive, diastase-resistant material [38,39] as well as acid phosphatase, β-glucuronidase, and N-acetyl-β-D-glucosaminidase.[4,27,40,41]

From our histochemical observations of livers of patients with Wilson's disease and normal neonatal livers it appears that copper sequestered within lysosomes is innocuous, while that which is diffuse in the cytoplasm is cytotoxic. [4,25,40] Studies of cell fractions have also shown that in Wilson's disease [15,33] and in PBC [14] considerable amounts of copper are present in the cytosol as well as lysosomes. The proportion present in the cytosol varied from 26% to 85% of the total hepatic copper in 7 patients with Wilson's disease and from 45% to 75% in 4 patients with PBC. Extralysosomal copper has also been detected in Wilson's disease by electron microscopy of liver stained with silver sulfide and by microanalysis.[4,42] The mechanisms regulating the uptake and retention of copper by lysosomes are not known. The consistent staining of the lysosomal copper-associated protein (LCAP) in the livers of newborns and children with biliary atresia [43] and ICC constrasts sharply with the failure to demonstrate LCAP in young patients with Wilson's disease who have high concentrations of hepatic copper. A deficiency of LCAP in the early stages of Wilson's disease may account for the hepatocytes' inability to concentrate copper in lysosomes.

Recent reports suggest that the deposition of copper may be a primary pathogenetic event in ICC.[44] There is, however, no direct evidence that the secondarily retained copper in cholestasis is toxic, as it is in Wilson's disease. Both steatosis and the ultrastructural abnormalities associated with diffuse cytoplasmic copper seen in young patients with Wilson's disease [25,31] are absent in both PBC and ICC. These differences suggest that different ligands may bind the extralysosomal copper in the three conditions.

References

1. Lindquist RR: Studies on the pathogenesis of hepatolenticular degeneration: II. Cytochemical methods for the localization of copper. Arch Pathol 1969, 87:370–379
2. Irons RD, Schenk EA, Lee JCK: Cytochemical methods for copper. Arch Pathol Lab Med 1977, 101:298–301

3. Uzman LL: Histochemical localization of copper with rubeanic acid. Lab Invest 1956, 5:299–305
4. Goldfischer S, Sternlieb I: Changes in the distribution of hepatic copper in relation to the progression of Wilson's diseases (Hepatolenticular degeneration). Am J Pathol 1968, 53:883–901
5. Timm F: Histochemical demonstration of copper in the brain. Histochemie 1961, 2:332–341
6. Sipponen P: Orcein positive hepatocellular material in longstanding biliary diseases: I. Histochemical characteristics. Scand J Gastroenterol 1976, 11:545–552
7. Salaspuro M, Sipponen P: Demonstration of an intracellular copper-binding protein by orcein staining in long-standing cholestatic liver diseases. Gut 1976, 17:787–790
8. Jain S, Scheuer PJ, Archer B, Newman SP, Sherlock S: Histological demonstration of copper and copper-associated protein in chronic liver diseases. J Clin Pathol 1978, 31:784–790
9. Nakanuma Y, Ohta G: Orcein-positive granular substances in primary biliary cirrhosis: II. Occurrence of the these substances observed with progression of the stage of PBC. Acta Hepatol Jpn 1978, 19:43–49
10. Ludwig J, McDonald GSA, Dickson ER, Elveback LR, McCall JT: Copper stains and the syndrome of primary biliary cirrhosis: Evaluation of staining methods and their usefulness for diagnosis and trials of penicillamine treatment. Arch Pathol Lab Med 1979, 103:467–470
11. Popper H, Goldfischer S, Sternlieb I, Nayak NC, Madhavan TV: Cytoplasmic copper and its toxic effects: Studies in Indian childhood cirrhosis. Lancet 1979, 1:1205–1208
12. Hunt AH, Parr RM, Taylor DM, Trott NG: Relation between cirrhosis and trace metal content of liver: With special reference to primary biliary cirrhosis and copper. Br Med J 1963, 2:1498–1501
13. Fleming CR, Dickson ER, Baggenstoss AH, McCall JT: Copper and primary biliary cirrhosis. Gastroenterology 1979, 67:1182–1187
14. Owen CA Jr, Dickson ER, Goldstein NP, Baggenstoss AH, McCall JT: Hepatic subcellular distribution of copper in primary biliary cirrhosis. Comparison with other hyperhepatocupric states and review of the literature. May Clin Proc 1977, 52:73–80
15. Sternlieb I, van den Hamer CJA, Morell AG, Alpert S, Gregoriadis G, Scheinberg IH: Lysosomal defect of hepatic copper excretion in Wilson's disease (Hepatolenticular degeneration). Gastroenterology 1973, 64:99–105
16. Sternlieb I, Harris RC, Scheinberg IH: Le cuivre dans la cirrhoses biliaire de l'enfant. Rev Int Hepat 1966, 16:1105–1110
17. Brückmann G, Zondek SG: Iron, copper and manganese in human organs at various ages. Biochem 1939, 33:1845–1857
18. Portmann B, Tanner MS, Mowat AP, Williams R: Orcein-positive liver deposits in Indian childhood cirrhosis. Lancet 1978, 1:1338–1340
19. Smallwood RA: Other liver diseases associated with increased liver copper concentration, Metals and the Liver. Edited by LW Powell. New York, Marcel Dekker, 1978, pp 313–330
20. Sternlieb I: Copper and the liver. Gastroenterology (In press)
21. Pearse AGE: Histochemistry, Theoretical and Applied. Boston, Little, Brown and Co, 1960
22. Morell AG, Windsor J, Sternlieb I, Scheinberg IH: Spectroscopic determination of microgram quantities of copper in biological materials. Laboratory Diagnosis of Liver Diseases. Edited by FW Sunderman, FW Sunderman, Jr. St. Louis, W.H. Green, 1968, pp 196–198
23. Committee on Medical and Biologic Effects of Environmental Pollutants, Copper.

Publication 0-309-02536-2. Washington, DC, National Academy of Sciences, 1977, pp 71–72
24. Popper H: The problem of histologic evaluation of primary biliary cirrhosis. Virchows Arch [Path Anat] 1978, 379:99–102
25. Sternlieb I: Evolution of the hepatic lesion in Wilson's disease (Hepatolenticular degeneration), Progress in Liver Diseases. Vol 4. Edited by H Popper, F Schaffner. New York, Grune and Stratton, 1972, pp 511–525
26. Tanner MS, Portmann B, Mowat AP, Williams R, Pandit AN, Mills CF, Bremner I: Increased hepatic copper concentration in Indian childhood cirrhosis. Lancet 1979, 1:1203–1205
27. Goldfischer S, Bernstein J: Lipofuscin (aging) pigment granules of the newborn human liver. J Cell Biol 1969, 42:253–261
28. Scheinberg IH, Sternlieb I: Copper metabolism. Pharmacol Rev 1960, 12:355–381
29. Frommer DJ: Defective biliary excretion of copper in Wilson's disease. Gut 1974, 15:125–129
30. Sipponen P, Salaspuro MP, Makkonen H: Histological characteristics of chronic hepatides and primary biliary cirrhosis with special reference to orcein positive hepatocellular accumulations. Ann Clin Res 1976, 8:200–205
31. Sternlieb I, Scheinberg IH: Prevention of Wilson's disease in asymptomatic patients. N Engl J Med 1968, 278:352–359
32. Shikata T, Uzawa T, Yoshiwara N, Akatsuka T, Yamazaki S: Staining methods of Australia antigen in paraffin section: Detection of cytoplasmic inclusion bodies. Jpn J Exp Med 1974, 44:25–36
33. Morell AG, Shapiro JR, Scheinberg IH: Copper binding protein from human liver, Wilson's Disease: Some Current Concepts. Edited by JM Walshe, JN Cumings. Oxford, Blackwell, 1961, pp 36–42
34. Carrico RJ, Deutsch HF: Isolation of human hepatocuprein and cerebrocuprein. Their identity with erythrocuprein. J Biol Chem 1969, 244:6087–6093
35. Porter H: Neonatal hepatic mitochondrocuprein: III. Solubilization of the copper and protein from mitochondria of newborn liver by reduction with mercaptoethanol. Biochim Biophys Acta 1968, 154:236–238
36. Rydén L, Deutsch HF: Preparation and properties of the major copper-binding component in human fetal liver. J Biol Chem 1978, 253:519–524
37. Porter H: Copper proteins in brain and liver in normal subjects and in cases of Wilson's disease, Wilson's Disease. Birth Defects Original Article Series. Vol 4, No. 2. Edited by D Bergsma.
38. Novikoff AB, Essner E: The liver cell: Some new approaches to its study. Am J Med 1960, 29:102–131
39. Goldfischer S, Villaverde H, Forschirm R: The demonstration of acid hydrolase, thermostable reduced diphosphopyridine nucleotide tetrazolium reductase and peroxidase activities in human lipofuscin pigment granules. J Histochem Cytochem 1966, 14:641–652
40. Sternlieb I, Goldfischer S: Heavy metals and lysosomes. Lysosomes in Biology and Pathology. Vol 5. Edited by JT Dingle, RT Dean. New York, Elsevier, 1976, pp 185–200
41. Goldfischer S, Moskal J: Electron probe microanalysis of liver in Wilson's disease. Simultaneous assay for copper and for lead deposited by acid phosphatase activity in lysosomes. Am J Pathol 1966, 48:305–315
42. Wiesner RH, Barham SS, Dickson ER: X-ray microanalysis: A new technique to measure hepatic copper and iron in Wilson's disease and hemochromatosis. Gastroenterology 1979, 77:A47
43. Reed GB, Butt EM, Landing BH: Copper in childhood liver disease: A histologic, histochemical, and chemical survey. Arch Pathol 1972, 93:249–255

44. Portmann B, Tanner MS, Mowatt AP, Mills CF, Williams R: Evidence for a major role of hepatic copper deposition in Indian childhood cirrhosis. Proc Eur Assoc Study Liver 1979 (Falk Rapid Literature Review, September 1979, p 259)

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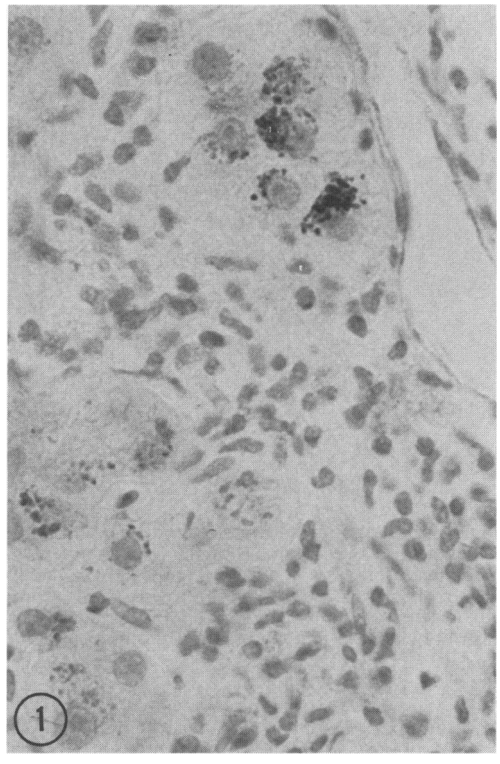
[*Illustrations follow*]

Figures 1 and 2—Biopsy specimen from a patient with primary biliary cirrhosis, stained for copper with rhodanine **(Figure 1)** and for the copper-associated protein with orcein **(Figure 2).** Granules with copper and its associated protein are abundant in hepatocytes at the periphery of the nodule and trapped in periportal tissue. (×600)

Figures 3 and 4—Serial sections of a liver biopsy specimen from a patient with advanced Wilson's disease stained for copper with rhodanine **(Figure 3)** and for the copper-associated protein with orcein **(Figure 4).** Despite the high concentration of copper in this liver, 974 μg/g dry tissue, there is a striking variability in its distribution. Almost all hepatocytes in one nodule are reactive for granular copper and its associated protein, while adjacent nodules are unstained. (×60)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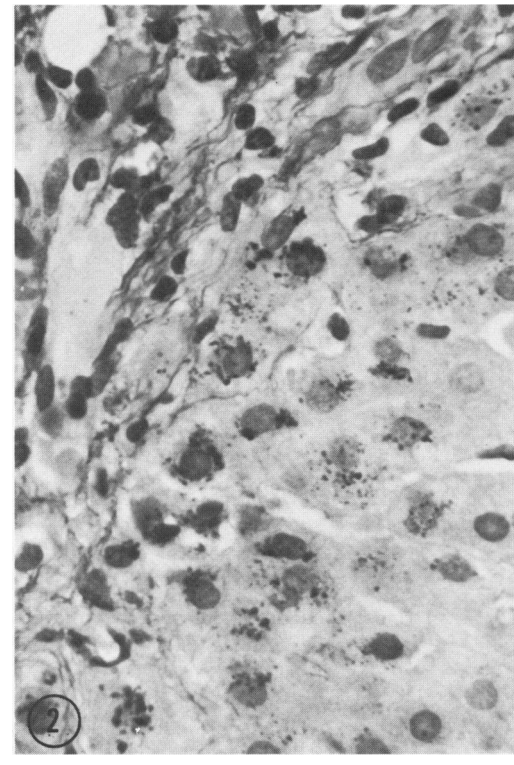
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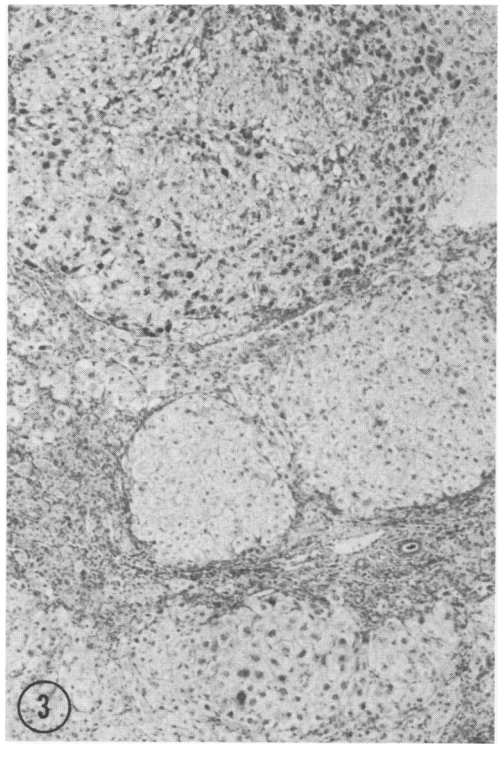
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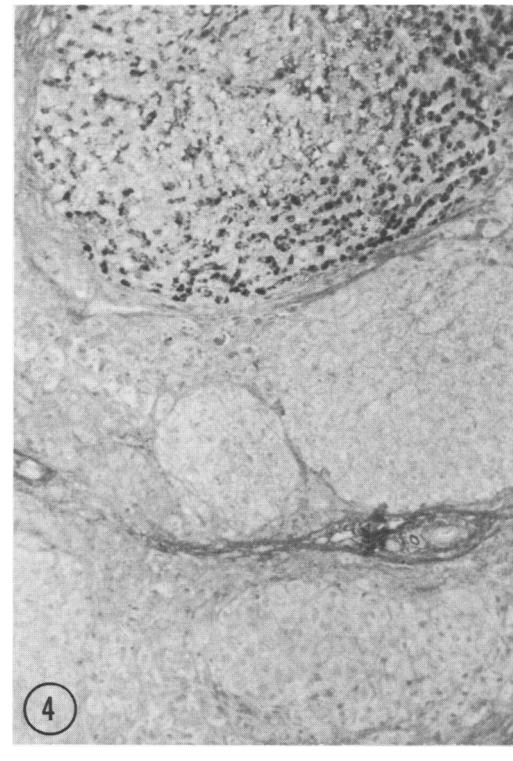
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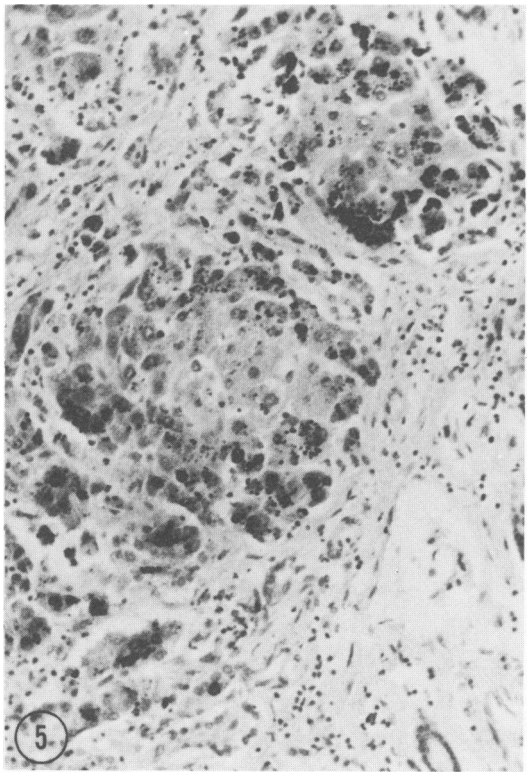
Figures 5 and 6—Serial sections of liver obtained at autopsy from a patient with Indian childhood cirrhosis. The liver contained 4788 μg copper/g dry tissue. Stained for copper with rhodanine **(Figure 5)** and for the copper-associated protein with orcein **(Figure 6).** Granules with copper and its associated protein are present in most hepatocytes: however, they are most abundant at the periphery of the lobule. The hepatocellular cytoplasm in this liver was also stained with rhodanine, but this staining is not evident in this black and white photograph. (×150)

Figure 7—Premature neonatal liver obtained at autopsy. The liver, which contained 310 μg copper/g dry tissue was stained with orcein. The copper-associated protein is present in granules within hepatocytes at the periphery of the lobule. (×600)

Figure 8—Liver biopsy specimen from a patient with primary biliary cirrhosis that contained 667 μg copper/g dry tissue. Stained by the PAS-positive, diastase resistant reaction that demonstrates hepatocellular lysosomes **(A)** and stained for copper with rhodanine **(B).** The stained granules have a similar pericanalicular distribution. (×900)

Figure 9—Biopsy specimen from a patient with primary biliary cirrhosis that contained 301 μg copper/g dry tissue. The specimen is stained with Timm's silver sulfide. This specimen had no stainable iron, and therefore the silver sulfide reaction represents copper. The diffuse cytoplasmic staining at the periphery of the nodule increases in intensity toward the limiting plate. (×100)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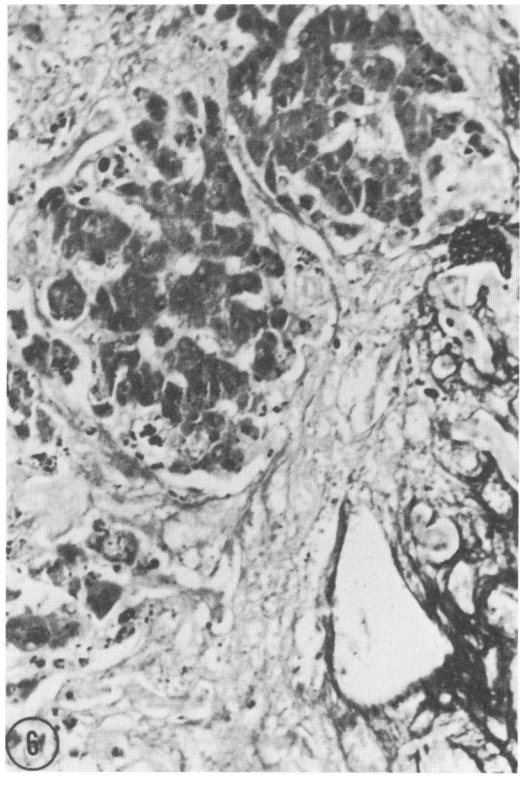
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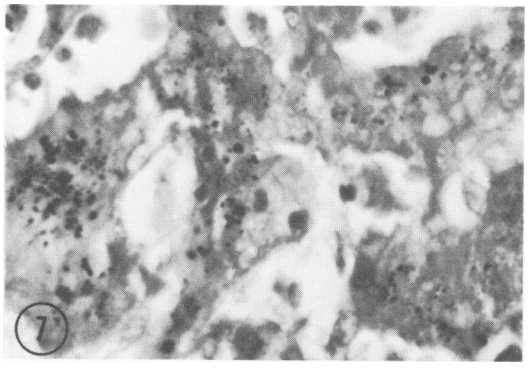
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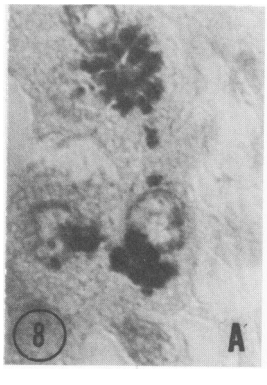
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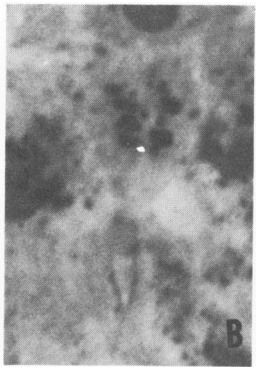
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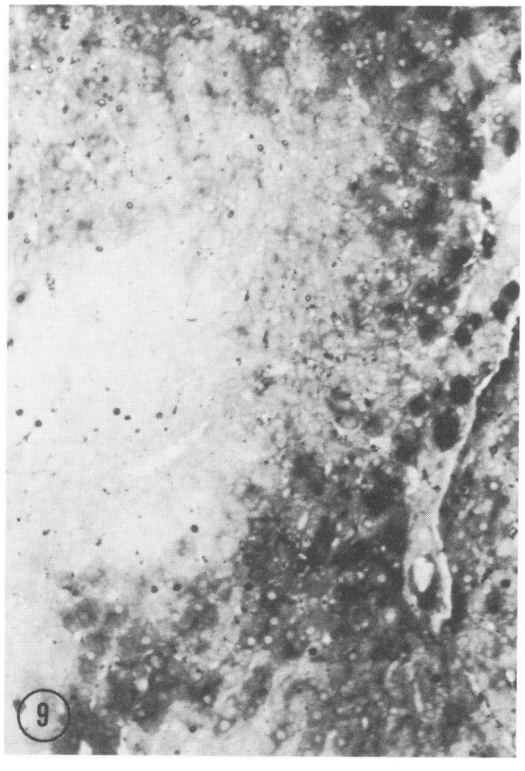
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