# LIPOFUSCIN (AGING) PIGMENT GRANULES OF THE NEWBORN HUMAN LIVER

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ABSTRACT

We have observed pigmented cytoplasmic granules, with the characteristic staining properties of lipofuscin (ceroid, "wear-and-tear") pigment, in newborn human liver. The pigment is found at the periphery of the lobule in hepatocytes and some bile ductular cells. It is acid-fast, PAS-positive after diastase digestion, slightly argyophilic and sudanophilic, and markedly Schmorl's- and peroxidase positive in paraffin sections. Difficult to see in sections stained with hematoxylin and eosin, the pigment can be detected in unstained sections. The granules also resemble lipofuscin found in adult tissues, in their ultra-structural and enzymatic properties. They are polymorphic, contain granular material of moderate and high electron opacity, and are delimited by a single membrane. Acid phosphatase and β-glucuronidase activities are visualized in the newborn granules, identifying them as lysosomes. The granules also contain copper and, to a much lesser extent, iron. The accumulation of lipofuscin pigment in lysosomes in many tissues correlates well with aging, and this process has been interpreted as a reflection of cellular degeneration or wear-and-tear. However, the presence of lipofuscin granules as a constant component of neonatal liver suggests that they are not a measure of cellular senescence.

A correlation between the accumulation of lipofuscin ("wear-and-tear") pigment granules and the passage of time is established in many types of cells (Strehler et al., 1959; Reichel, 1968). Although the granule was originally considered to be a single pigment, histochemical (Pearse, 1960) and ultrastructural studies have made it apparent that the colored cytoplasmic granule is a conglomerate of substances that accumulate within lysosomes (Essner and Novikoff, 1960). These substances include a lipid that is insoluble in alcohol and xylene, reducing substances, and acid-fast, autofluorescent, argentophilic, and diastase-resistant, periodic acid-Schiff (PAS)–positive components. Studies of isolated pigment granules show that they contain melanin (Siebert et al., 1962) and hemes (Björkerud, 1964). In the hepatocyte, lipofuscin granules also exhibit a peroxidatic activity that persists in paraffin-embedded tissue (Goldfischer et. al. 1966).

In fetal and newborn human liver, copper has recently been localized to cytoplasmic organelles that resemble lipofuscin granules in their ultrastructure (Goldfischer and Sternlieb, 1968). The presence of an "aging" pigment in neonatal tissue bears on the concept that this material reflects (Strehler, 1967) or causes cellular senescence (Tappel, 1967). This study was undertaken to determine: (*1*) whether the newborn hepatic granules had the staining, enzymatic, and morphological characteristics of lipofuscin, and (*2*) whether such granules are regularly present in newborn human liver.

## MATERIALS AND METHODS

### *Light Microscopy*

Liver specimens, obtained from 12 randomly chosen necropsy examinations of newborns and infants performed at this center, were fixed in forma-

lin and embedded in paraffin. Sections were stained with the standard methods for identifying lipofuscin (Pearse, 1960): reducing substances with Schmorl's technique; acid-fast substances by the Ziehl-Neelsen procedure; alcohol-insoluble lipid with Sudan black; argentophilic melanin-like material by Fontana's silver impregnation technique; and periodic acid-Schiff (PAS)-positive material after diastase digestion. Copper was stained by a rubeanic acid procedure (Goldfischer and Sternlieb, 1968) and iron by Perls' technique.[1]

Paraffin sections were also rehydrated and incubated for 60–90 min at room temperature for heat-resistant peroxidase activity (Goldfischer et al., 1966) in an alkaline modification (Novikoff and Goldfischer, 1968) of Graham and Karnovsky's (1966) 3,3′ diaminobenzidine (DAB) medium. The medium was made up of DAB tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.), 20 mg; 0.5 M propanediol buffer, pH 10, 10 ml; and 1–3% $H_2O_2$, 0.2 ml.[2]

### *Enzyme Cytochemistry*

Four additional specimens, obtained 2–7 hr postmortem, were fixed in cold 3% glutaraldehyde (Biological Grade, Fisher Scientific Co., Pittsburgh, Pa.) in 0.1 M cacodylate buffer, pH 7.4 (Sabatini et. al., 1963) for 3 hr. In these specimens, lysosomes were visualized in freely floating frozen sections, 10 $\mu$ in thickness, by incubation at 37°C for acid phosphatase activity in a Gomori (1952) medium with $\beta$-glycerophosphate as substrate, and for $\beta$-glucuronidase activity in a pararosanilin medium with naphthol AS-BI glucuronide as substrate (Hayashi et al., 1964).

### *Electron Microscopy*

Portions of the glutaraldehyde-fixed specimens were also diced into small (less than 1 $mm^3$) cubes, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 for 2 hr, and embedded in Epon. Thin sections were mounted on copper grids and stained with both lead citrate (Reynolds, 1963) and uranyl acetate.

TABLE I

| Patient | Birth weight | Gestation | Age | Body length |
|---|---|---|---|---|
| | *g* | *wk* | | *cm* |
| 1 | 600 | 33 | 3 hr | 31 |
| 2 | 720 | — | 30 hr | 31 |
| 3 | 750 | — | 36 hr | 32 |
| 4 | 850 | 28 | 10½ hr | 37 |
| 5 | 940 | 26 | 14 hr | 32 |
| 6 | 1040 | 26 | 3 days | 34 |
| 7 | 1100 | 28 | 30 hr | 36 |
| 8 | 1100 | 28 | 1 day | 36 |
| 9 | 1500 | 30 | 55 hr | 40 |
| 10 | 1770 | 30 | 6 hr | 44 |
| 11 | 2320 | 37 | 7 hr | 47 |
| 12 | 2940 | 43 | Stillborn | 55 |
| 13 | 3650 | 40 | 7½ hr | 52 |
| 14 | 3580 | 38 | 1 month | — |
| 15 | 2353 | 40 | 1 month | — |
| 16 | 2210 | 38 | 2 months | |

### *Clinical Information*

The ages, birth weights, body lengths, and periods of gestation of the patients studied are listed in Table I.

## RESULTS

### *Light Microscopy*

Brown pigment granules were found in all specimens, mainly around portal triads, in hepatocytes and a few bile ductular cells (Fig. 1). They were usually small and less than 1 $\mu$ in size, but larger granules were also encountered. The granules were seen more readily in unstained slides than in hematoxylin-eosin preparations. Generally acid-fast (Fig. 2), the pigment contained PAS-positive material that resisted digestion with diastase. Argentophilic material (Fig. 3) and Sudan-positive lipid (Fig. 4) were present in some granules (Fig. 4). Occasionally, the pigment granules were iron-positive; however, iron was present mainly in nonparenchymal cells. Patterns of staining varied among the cases, indicating that the granules varied in composition. The largest number of granules were evident in preparations stained for reducing substances with the Schmorl technique (Fig. 5) and for peroxidase activity in an alkaline DAB medium (Fig. 6). Many of the granules stained for copper with rubeanic acid

[1] Lipofuscin granules are often autofluorescent; however, when they contain iron (Pearse, 1960) or copper (Barka et al., 1964) the fluorescence is quenched. The pigment granules in neonatal livers did not fluoresce in ultraviolet light, presumably because of their metallic contents.

[2] In the adult liver, microbodies (peroxisomes) are also stained in this medium when *frozen* sections of aldehyde-fixed tissue are incubated at 37°C (Novikoff and Goldfischer, 1968). When we tried this procedure with specimens of neonatal liver, microbody staining was not detectable in the light microscope but could be seen in the electron microscope (Dr. Edward Essner, personal communication).

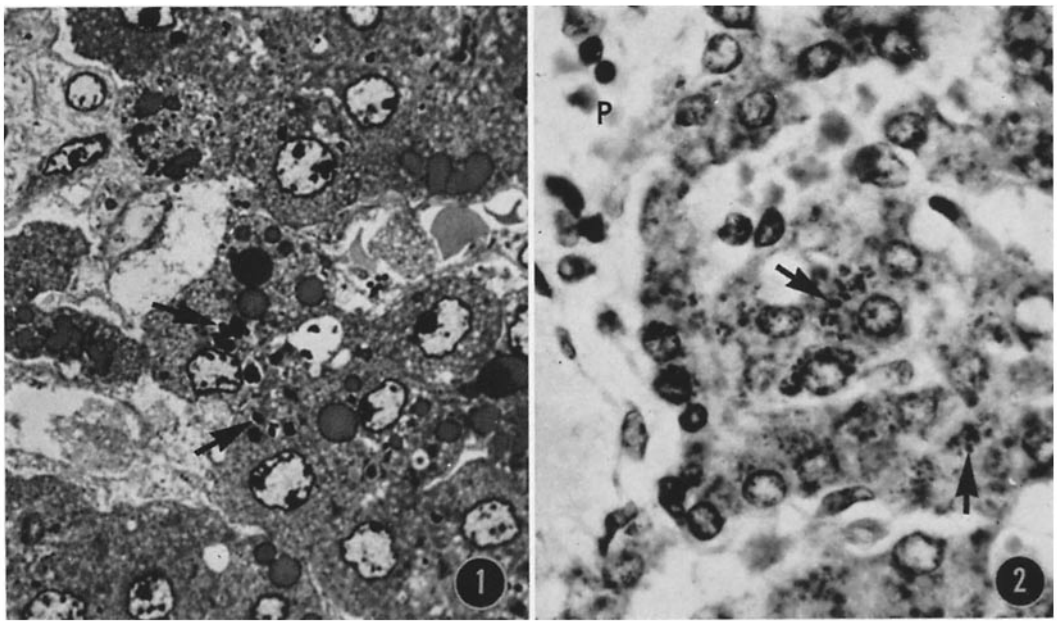



FIGURE 1 Section of epoxy-embedded newborn human liver, stained with toluidine blue. Cytoplasmic pigment granules (arrows) are found mainly at the periphery of the lobule around portal triads. Patient No. 13. × 600.

FIGURE 2 Acid-fast material is present in many of the pigment granules (arrows). *p*, portal zone. Paraffin section stained by the Ziehl-Neelsen procedure. Patient No. 1. × 600.

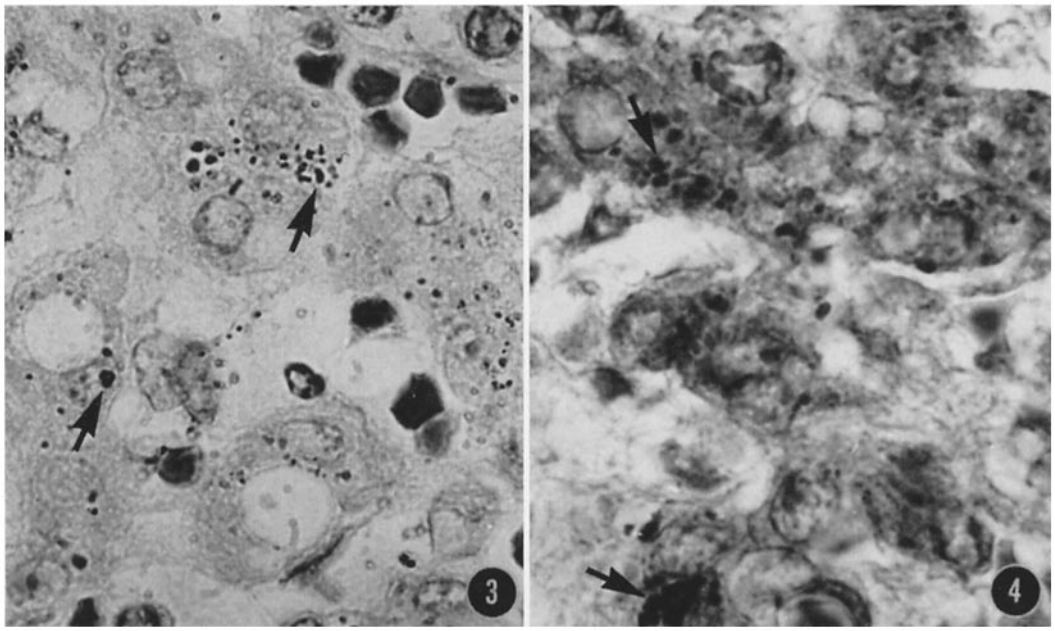



FIGURE 3 Argentophilic components of the pigment granules (arrows) are stained by the Fontana silver impregnation procedure. Paraffin section. Patient No. 1. × 1000.

FIGURE 4 Alcohol-insoluble lipids are visualized in some granules (arrows). Paraffin section stained with Sudan black. Patient No. 13. × 1200.

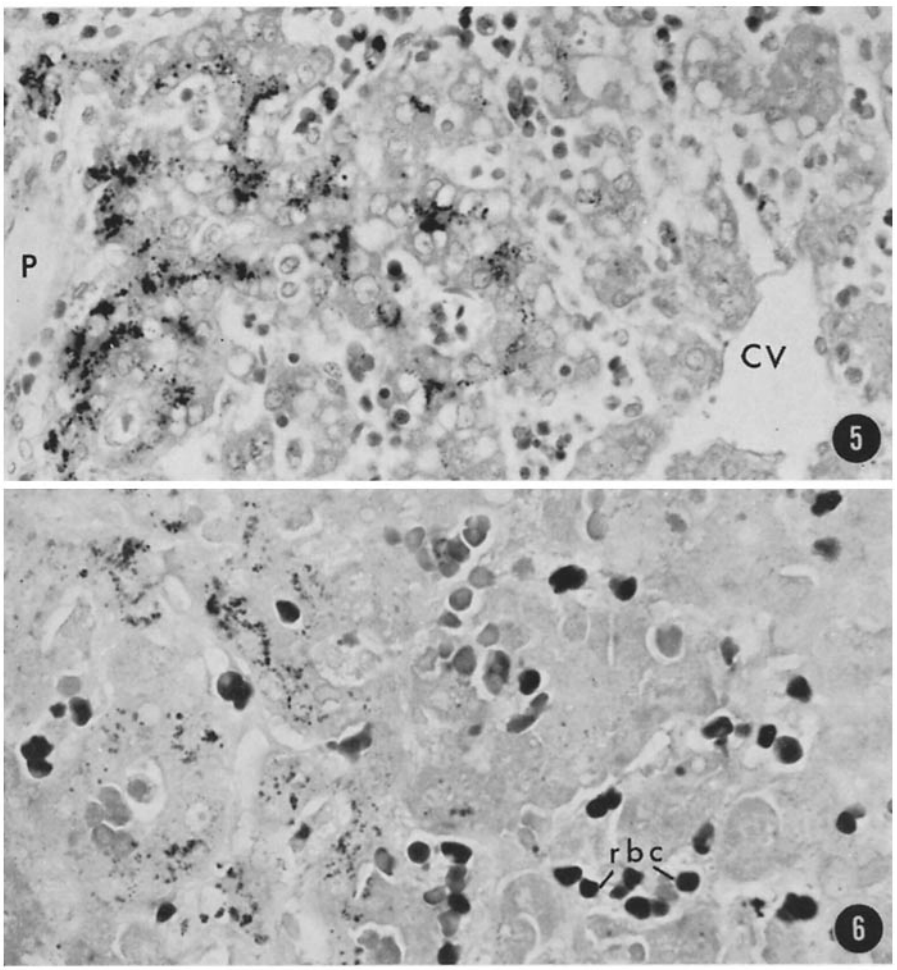



FIGURE 5 All granules are stained by Schmorl's procedure for demonstrating reducing substances. Cells around central veins (*cv*) are free of pigment. *p*, portal zone. Paraffin section. Patient No. 9. × 400.

FIGURE 6 Peroxidase activity that survives paraffin embedding is visualized in the pigment granules by incubation in a DAB medium. Erythrocytes (*rbc*) also are stained by this procedure. Patient No. 3. × 500.

(Fig. 7). The number of pigment granules decreased with increasing postnatal age, and very few granules were seen in a 2-month-old infant. In this specimen, the pigment was concentrated around central veins, and copper staining was barely evident.

In size and intracellular distribution, the pigment granules (Fig. 8) resembled lysosomes that were made visible in preparations incubated for acid phosphatase (Fig. 9) and β-glucuronidase activities. In sections incubated for hydrolase activities, unstained pigment granules were not evident, indicating that all the granules were lysosomes.

### *Electron Microscopy*

The pigment granules, seen in hepatocytes and preductular cells (Jézéquel et al., 1965), were polymorphic and osmiophilic (Figs. 10 and 11). They were concentrated along bile canaliculi in liver cells (Fig. 10). A single limiting membrane was occasionally preserved in these postmortem specimens. Larger granules appeared to contain aggregates of very dense material, whereas smaller granules were more heterogeneous.

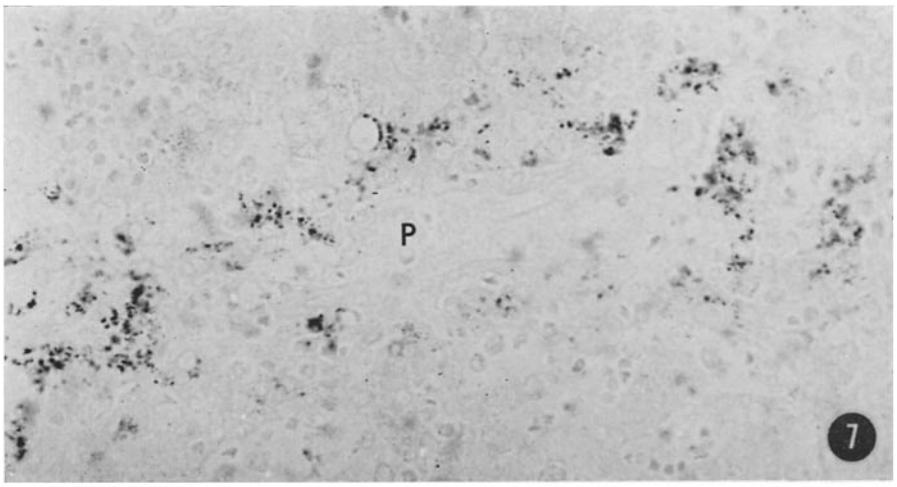
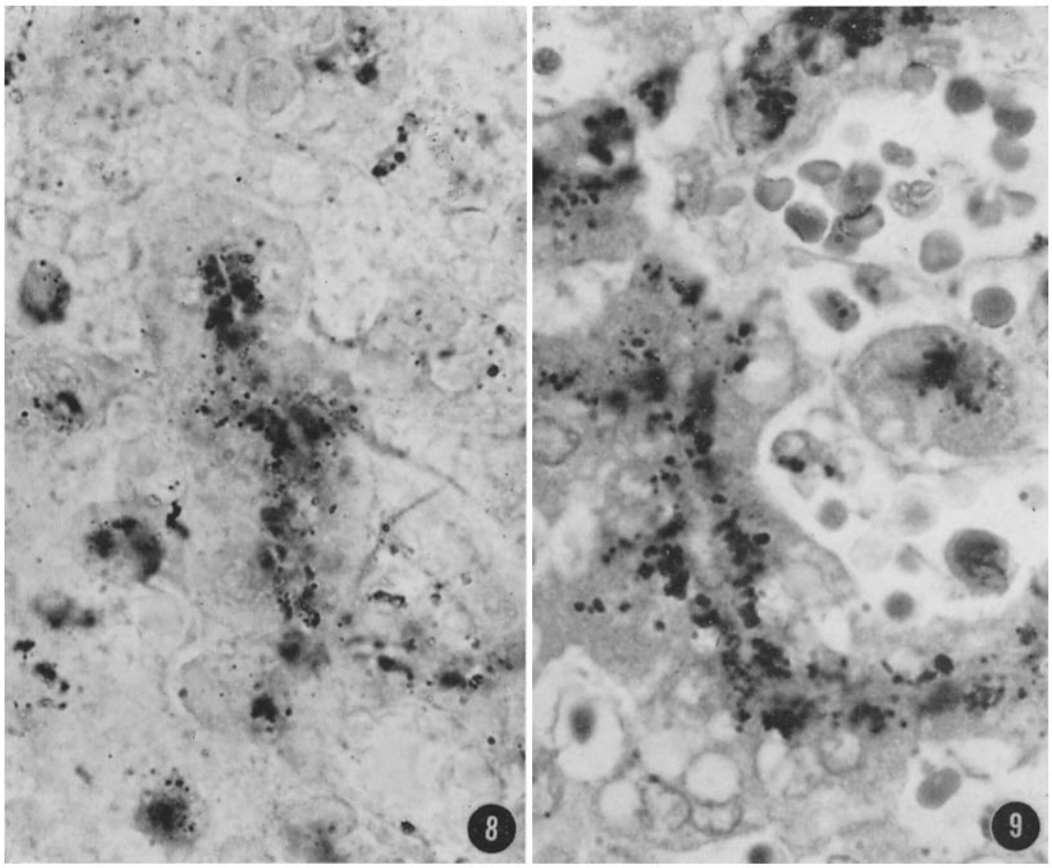



FIGURE 7 Copper is localized to the pigment granules in paraffin sections stained with rubeanic acid. The granules are seen in cells clustered around a partal vessel (*p*). Patient No. 1. × 350.




FIGURES 8 AND 9 Lysosomes, made visible in a frozen section incubated for acid phosphatase activity (Fig. 8; Patient No. 3), are similar in size and distribution to the pigment granules stained with Schmorl's technique, on a paraffin section (Fig. 9; Patient No. 9). The pericanalicular concentration of the granules is evident in both preparations. × 1000.

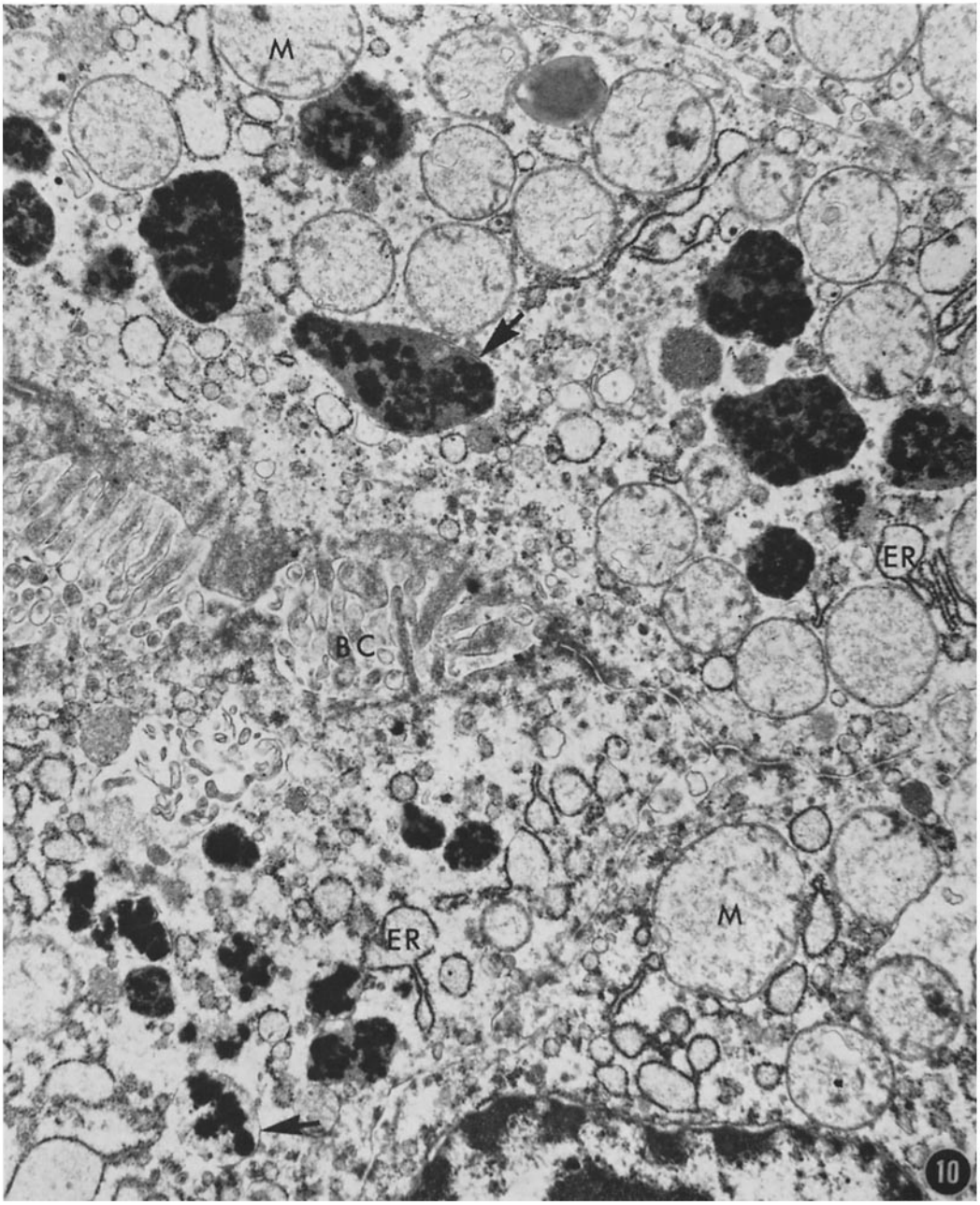



FIGURE 10 Electron micrograph of newborn human liver. Polymorphic pigment granules containing clumps of highly electron-opaque material are seen in two hepatocytes along the bile canaliculus (*BC*). A single limiting membrane (arrow) is sometimes preserved. Mitochondria (*M*) are swollen in this specimen fixed 7 hr postmortem. (*ER*) endoplasmic reticulum. Patient No. 2. × 20,000.

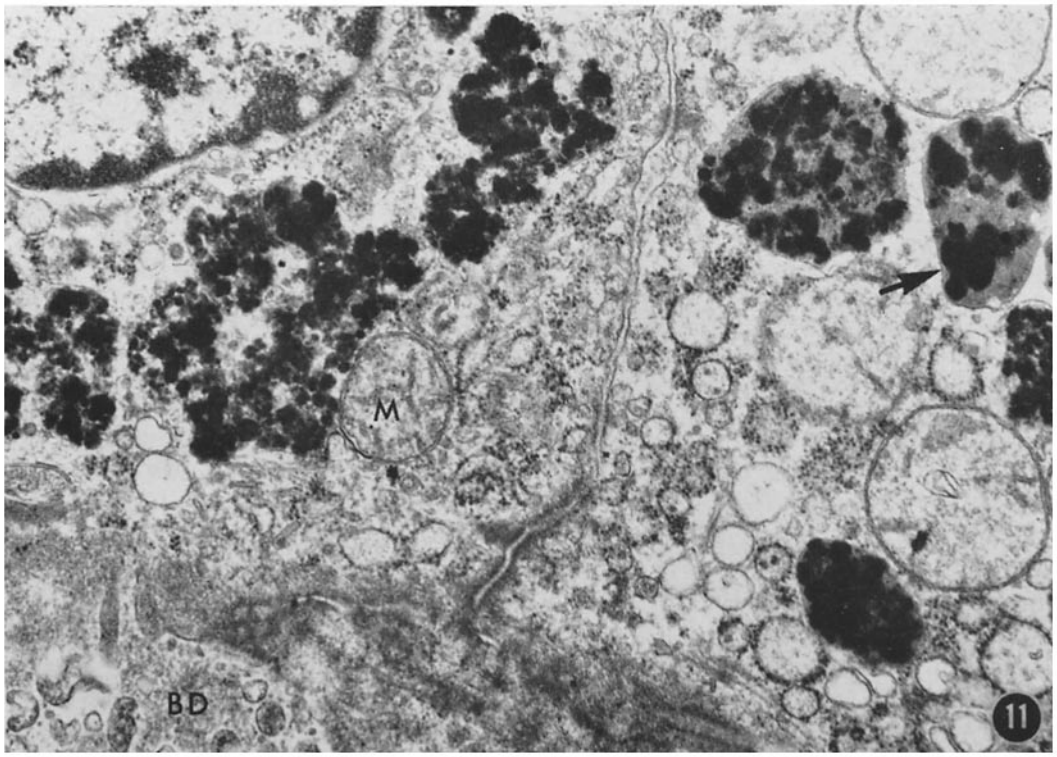



FIGURE 11 Pigment granules containing large clumps of dense material are also seen in two bile ductule or preductule (Duct of Hering) cells. The arrow points to a limiting membrane. Patient No. 2. (*BD*) bile ductule, (*M*) mitochondrion. × 25,000.

## DISCUSSION

The finding of pigment granules in all 16 specimens, obtained from infants varying in gestational age from 26 to 43 wk and in birth weight from 600 to 3580 g and in age up to 2 months, indicates that they are constant components of fetal and newborn liver. The pigment granules were Schmorl's-, PAS- and peroxidase-positive, and they were to a lesser extent acid-fast, sudanophilic, and argyrophilic. They were identified as lipofuscin by these staining reactions. Electron microscopy showed the newborn pigment to resemble lipofuscin pigment granules of adult tissues (see, for example, Fig. 25 in Biava, 1965, and Fig. 2 in Hartroft and Porta, 1965). However, they contained few of the rounded, moderately dense, lipid-like inclusions that are common in the adult granules. Pigment granules, similar to those we have identified, are illustrated in an ultrastructural study of bile ducts and hepatocytes of infants with biliary atresia (Hollander and Schaffner, 1968). In very small fetuses (5–150 g in weight and 4–21 cm in length), Picardi, Gardiol, and Gautier (1968) have noted the presence of dense cytoplasmic granules in hepatocytes, ductular, and intermediate cells at the periphery of the lobule.

Essner and Novikoff (1960), in a study of the liver, showed that lipofuscin granules are lysosomes, and this has since been confirmed in neurons (Samorajski et al., 1964), heart muscle (Jamieson and Palade, 1964; Goldfischer et al., 1966), mammary gland (Miyawaki, 1965), interstitial cells of testis (Frank and Christensen, 1968), and liver (Goldfischer et al., 1966). The presence of acid phosphatase and $\beta$-glucuronidase also characterized the neonatal pigment granules as lysosomes. The contents of the lipofuscin granules are believed to be mainly the undigested residue of lysosomal hydrolysis (for discussion, see de Duve and Wattiaux, 1966; Goldfischer et al., 1966; Frank and Christensen, 1968).

Pearse (1960) has stressed that lipofuscin, ceroid

(Hartroft and Porta, 1965), and hemofuscin (Jayne, 1950) are essentially the same, encompassing similar lipid and nonlipid components that differ only in the degree to which they are oxidized. The oxidation of pigment precursors is probably catalyzed by metals or metalloproteins that are contained within lysosomes (Hartroft and Porta, 1965; Goldfischer et al., 1966). Administration of copper to rats results in the formation of hepatic lipofuscin granules that contain the metal (Barka et al., 1964; Goldfischer, 1967). Lipofuscin granules are abundant in the livers of patients with advanced Wilson's disease, a disorder of copper metabolism (Sternlieb and Scheinberg, 1963), and in these individuals the metal is also present in the pigment granules (Goldfischer and Sternlieb, 1968). In fetal and newborn livers, copper concentrations are 10 times higher than in adults (Brückmann and Zondek, 1939), probably reflecting the elevated levels of copper in maternal blood (Scheinberg et al., 1954). Serum copper increases continuously during pregnancy, reaching its highest level during the last month of gestation (Dokumov, 1968). Hepatic copper levels in infants remain elevated for several months (Brückmann and Zondek, 1939; Dokumov, 1968); how long the pigment granules persist has not yet been studied.

A correlation between age and the accumulation of lipofuscin has been demonstrated in heart muscle (Strehler et al., 1959; Munnell and Getty, 1968), brain (Reichel et al., 1968), adrenal cortex (Samorajski and Ordy, 1967; Reichel, 1968), kidney, testis, ovarian macrophages (Reichel, 1968), and liver (Bachmann, 1953). However, Ehrlich et al. (1960) have noted the occurrence of the pigment in livers of adolescents and point out that this is not consistent with the wear-and-tear concept. The presence of lipofuscin and copper in neonatal hepatic lysosomes suggests that the accumulation of lipofuscin is a function of the metabolites that are sequestered within lysosomes and not the result of biological "aging," an ill defined phenomenon. In the absence of any evidence that the pigment is harmful to the cell, it would be preferable not to refer to lipofuscin as wear-and-tear pigment since this designation may imply cellular degeneration. Although "womb-to-tomb" pigment is correct in a temporal sense, "sequestered-and-undigested" better describes the nature of lipofuscin.

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