Age-Related Accumulation of Ceroid-Like Pigment in Mice With Chediak-Higashi Syndrome

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The Chediak-Higashi Syndrome (CHS) is a rare inherited disorder occurring in man and several animal homologs including the beige mouse; it is characterized by pigmentary dilution, susceptibility to pyogenic infections, and the presence of enlarged lysosomes in many cell types. Beige mice 6 months of age and older were found to have darkened livers, kidneys, and spleens, accompanied by splenomegaly. A fluorescence microscopic survey of tissues from beige mice revealed marked accumulations of a yellow autofluorescent pigment in hepatocytes, renal proximal tubule cells, and splenic macrophages. Additionally, large amounts of hemosiderin were present in the spleen. In beige mice, the pigment was noted in animals as young as 1 to 2 weeks of age and gradually increased in amount as the animals aged. A histochemical investigation of the pigment showed that it was ceroid-like in nature and contained in lysosomes. Ultrastructurally, the pigment was composed of lipid-like droplets embedded in a dense matrix and surrounded by a limiting membrane. The accumulation of ceroid-like material in beige mice may be a reflection of the metabolic disturbance which underlies CHS. (Am J Pathol 84:225–238, 1976)

THE CHEDIAK-HIGASHI SYNDROME (CHS) is a rare inherited disorder occurring in man, Aleutian mink,[1] partially albino Hereford cows,[2] beige mice,[3] and killer whales.[4] The syndrome is characterized by pigmentary dilution, recurrent pyogenic infections, and the presence of enlarged granules (considered to be lysosomes) in many cell types.[5-8] In the course of our previous investigations on the beige mouse, it was noted that the older animals tended to have enlarged spleens, and that their liver, kidneys, and spleen were darker in color than the corresponding organs of control animals. A study was then undertaken to investigate the nature of these alterations and to determine if other tissues in the beige mouse were similarly affected.

Materials and Methods

A total of 32 age- and sex-matched beige (*bg bg*) and C57BL mice (Jackson Laboratories, Bar Harbor, Me.) between 1 day and 14 months of age and weighing up to 30 g were used in this study. Animals were sacrificed by cervical dislocation.

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For fluorescence microscopy 4- to 5-μ sections of fresh-frozen tissue were cut on an Ames cryostat, collected on glass slides, and coverslips mounted with 50% glycerin in phosphate-buffered saline. The tissues were examined with a Leitz Orthomat microscope equipped with an HBO 200-W ultraviolet light source. Paraffin sections of tissue fixed in 10% neutral buffered formalin were stained for the presence of a) polysaccharides—periodic acid-Schiff, before and after diastase digestion;[9] b) phospholipids—copper phthalocyanin in alcohol;[9] c) sphingomyelin—copper phthalocyanin in chloroform;[9] d) ferric iron—Perl's method;[9] e) hemosiderin iron—hemotoxylin-Lake method;[9] f) bilirubin—Hall's method;[10] g) lipofuscins—Schmorls' method,[9] Ziehl-Neelson method,[9] Nile blue sulfate,[9] indophenol;[9] h) reducing substances—methenamine silver;[11] i) acid-fast substances—carbol fuchsin.[11] Paraffin sections of tissue fixed in Carnoy's fixative were stained for the presence of nucleic acids with both the Feulgen method[9] and methyl green-pyronin Y[9]

Frozen sections of tissue fixed in 4% formaldehyde and 1% calcium chloride[12] were stained for a) neutral lipids—oil red O and Sudan black in 60% triethylphosphate;[9] b) phospholipids—copper phthalocyanin in alcohol;[9] c) sphingomyelin—copper phthalocyanin in chloroform,[9] d) lipofuscin—diaminobenzidine;[13] e) lipid peroxides—Dam method.[9]

For the demonstration of acid phosphatase activity, 10-μ frozen sections of tissue fixed in 4% formaldehyde–1% calcium chloride were incubated in modified Gomori lead medium containing cytidine-5'-monophosphate as substrate.[14]

For electron microscopy, small blocks of tissue were fixed for 3 hours at 25 C in a modified Karnovsky fixative containing 1.9% formaldehyde (Ladd) and 1.4% glutaraldehyde (Ladd) in 0.2 M cacodylate buffer (pH 7.4) containing 0.025% $CaCl_2$. After fixation, the tissue was stored overnight at 4 C in 0.2 M cacodylate buffer containing 7% sucrose, fixed in 1% buffered osmium tetroxide, stained *en bloc* for 2 hours in 0.5% uranyl acetate in Michaelis buffer at pH 5.0, dehydrated through a graded series of ethanol and propylene oxide, and embedded in either Epon 812 or Spurr's resin.[15] Thin sections were cut on a Porter-Blum MT-1 microtome, mounted on bare copper grids, lightly stained with Reynolds' lead citrate,[16] and examined in either a Siemens Elmiskop 101 or a JEM 100-B electron microscope.

Results

Gross dissection of beige and C57BL mice of 6 months of age or older, revealed that the liver, kidneys, and spleen of beige mice were considerably darkened and that the liver and kidneys had an olive green cast. In addition, the spleens of the beige mice were markedly enlarged (Figure 1). Routine hematoxylin and eosin preparations showed an accumulation of a yellow to light brown pigment in hepatocytes, renal proximal tubule cells, and splenic macrophages in the beige mouse. The pigment displayed a yellow autofluorescence, which was employed as a marker in order to determine its presence in other tissues. Table 1 summarizes the results of this survey. Marked accumulations of pigment were found only in liver, kidneys, and spleen of beige mice (Figures 2–7). Additionally, in the red pulp of spleens from beige and control mice there were accumulations of a pigment with the characteristic red fluorescence of hemosiderin. These accumulations were considerably larger in the spleens from the beige mice.

Fresh frozen sections of liver, kidney, and spleen from younger animals

Table 1—Distribution of Pigment in Tissues of Older C57BL and Beige Mice as Detected by Fluorescence Microscopy

Tissue	C57BL Mouse	Beige mouse
Liver	+	+++
Kidney	+	+++
Spleen	+	+++
Heart	++	++
Cerebellum	+	+
Cerebrum	+	+
Spinal cord	+	+
Lung	+	+
Jejunum	−	−
Stomach	−	−
Pancreas	−	−

were also examined. Tissues from neonatal mice showed no accumulations of fluorescent material. However, small amounts of fluorescent material were seen in tissues from beige mice as young as 2 weeks. By 3 to 4 months the amount of pigment had increased, but it was not as extensive as that seen in the older animals.

The liver, kidney, and spleen of older beige mice were then examined further in an attempt to characterize the pigment. In all three tissues, the pigment was positive when incubated for acid phosphatase activity (Figures 8–10), thus indicating its accumulation within lysosomes.

The staining characteristics of the pigment are summarized in Table 2. From the results of the various procedures, the pigment appears to be ceroid-like (partially oxidized lipofuscin) in nature. In all of the tissues there was a considerable heterogeneity in the staining of the pigment with not all of it staining with any given stain. The methenamine silver stained the majority of pigment present (Figures 11–13), and its distribution most closely paralleled that of the acid phosphatase. The PAS reaction gave a variable reaction, staining a small amount of pigment in the liver (Figure 14) and a majority in kidney and spleen (Figures 15 and 16). The oil red O stained a very small amount of pigment in liver and spleen, but large amounts in the kidney. The carbol fuschin staining was very weak in all tissues and a large amount of the pigment, probably hemosiderin, was negative in the spleen.

In addition to the heterogeneity already noted, in the kidney, a portion of pigment stained lightly only with oil red O (Figure 18). Additionally, some of the pigment in the proximal convoluted tubule was found to contain ferric iron (Figure 15), but did not react when stained for hemosiderin. In the spleen, the majority of the pigment in the red pulp areas stained positively for iron. This pigment also stained for hemosiderin.

Ultrastructurally (Figures 20–23), the pigment often occupied a large

portion of the cell. It was very heterogeneous, consisting of fibrillar and particulate material embedded in an electron-dense matrix and surrounded by a single unit membrane. Additionally, in liver and kidney (Figures 21 and 22), there were numerous lipid droplets in the pigment. However, in the spleen there was little lipid present, and much of the pigment was composed of a fine granular material which may correspond to hemosiderin (Figure 23).

Table 2—Staining Characteristics of Pigment in Liver, Kidney, and Spleen of Older Beige Mice

	Liver		Kidney		Spleen	
	C57BL	Beige	C57BL	Beige	C57BL	Beige
Acid-fast substances						
Carbol fuchsin	−	+*	−	+*	−	+*
Polysaccharides						
Periodic acid-Schiff before and after diastase digestion	−	+	−	+	−	+
Neutral lipids						
Oil red O	−	+	+	+	±	+
Sudan black	−	+	±	+	±	+
Phospholipids						
Copper phthalocyanin (frozen sections)	−	+	−	+	−	+
Reducing substances						
Methenamine sliver	−	+	+*	+	+*	+
Lipofuscin						
Schmorl's method	−	+	+*	+	−	+
Nile blue sulfate	−	+*	−	+	−	+*
Diaminobenzidine oxidation	−	+	−	+	−	+
Lipid peroxides						
Dam method	−	+	−	+	−	+
Ferric iron						
Perl's method	−	−	+	+	+	+
Hemosiderin iron						
Hematoxylin-lake method	−	−	−	−	+	+
Phospholipids						
Copper phathalocyanin (paraffin sections)	−	−	−	−	−	−
Sphingomyelin						
Copper phthalocyanin (frozen and paraffin sections)	−	−	−	−	−	−
Bilirubin						
Hall's method	−	−	−	−	−	−
Lipofuscin	−	−	−	−	−	−
Ziehl-Neelson method	−	−	−	−	−	−
Indophenol	−	−	−	−	−	−
DNA						
Feulgen reaction	−	−	−	−	−	−
RNA						
Methyl green-pyronin Y	−	−	−	−	−	−

*Poorly stained.

Discussion

A fluorescence microscopic survey of tissues from beige mice revealed an accumulation of pigment in hepatocytes, kidney proximal tubule cells, and splenic macrophages. Histochemical examination showed that the material was similar to ceroid described by Endicott and Lillie [17] in that it had a characteristic yellow autofluorescence and exhibited many of the same staining properties. Characteristically, the ceroid, or partially oxidized lipofuscin,[9] was found to be contained within lysosomes.[18-20] In addition the material seen in the beige mouse kidney and spleen contained ferric iron. However, iron has been reported previously in lipofuscin in mice [21-24] and humans.[20]

The pigment seen in the beige mouse appeared to be incompletely oxidized and did not exhibit all of the staining characteristics of the lipofuscin which accumulates as a normal process of aging. Additionally, initial accumulations of ceroid were seen in beige mice as young as 1 to 2 weeks. It continued to accumulate, and in adult animals was present in greatly increased amounts.

Experimentally, ceroid has been produced in a number of ways: iron overloading; [25] vitamin E deficiency; [26-29] choline deficiency; [30,31] hormone treatment; [32] and feeding of butter yellow,[33] carbon tetrachloride,[34] or unsaturated fatty acids.[35] For a more complete discussion of experimentally induced ceroid see Porta and Hartroft.[36] Common to all of these is a metabolic disturbance which leads to the accumulation of lipid. *In vitro* studies by Casselman [37] suggest that ceroid is formed *in vivo* by the accumulation of unsaturated fatty acids in such a manner that there is a relative lack of biologic antioxidants which results in autooxidation of the fats and their conversion to ceroid. Additionally, he showed that the presence of hemoglobin may catalyze this oxidation, as well as account for the iron sometimes seen in ceroid.

A possible disturbance in lipid metabolism associated with CHS has been considered previously.[5,8,38-44] Although studies of serum lipid levels have proved inconclusive, lipid-like inclusions have been observed in a variety of tissues from both beige mice and Aleutian mink.[6,8,43,45-47] Additionally, the abnormal formation of lysosomes in leukocytes from beige mice [44] and Aleutian mink,[48,49] delayed fusion of lysosomes with phagosomes in beige mice [50] and humans with CHS,[51] and abnormal lysosomal enzyme secretion in beige mouse kidneys [52] may be explained by a defect in the lipid component of the limiting membrane.

The present finding of an accumulation of ceroid in tissues from beige mice provides further evidence for disturbances in lipid metabolism, and

stresses the need for additional investigations in order to elucidate the underlying defect in CHS.

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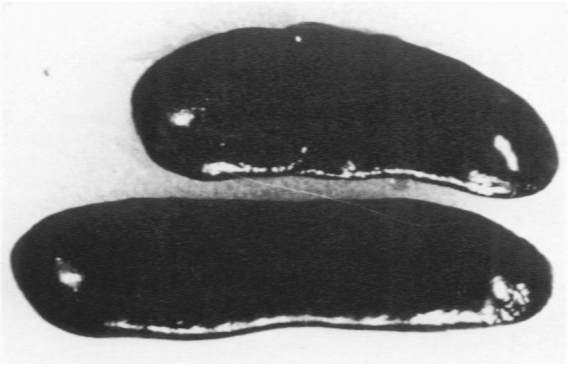
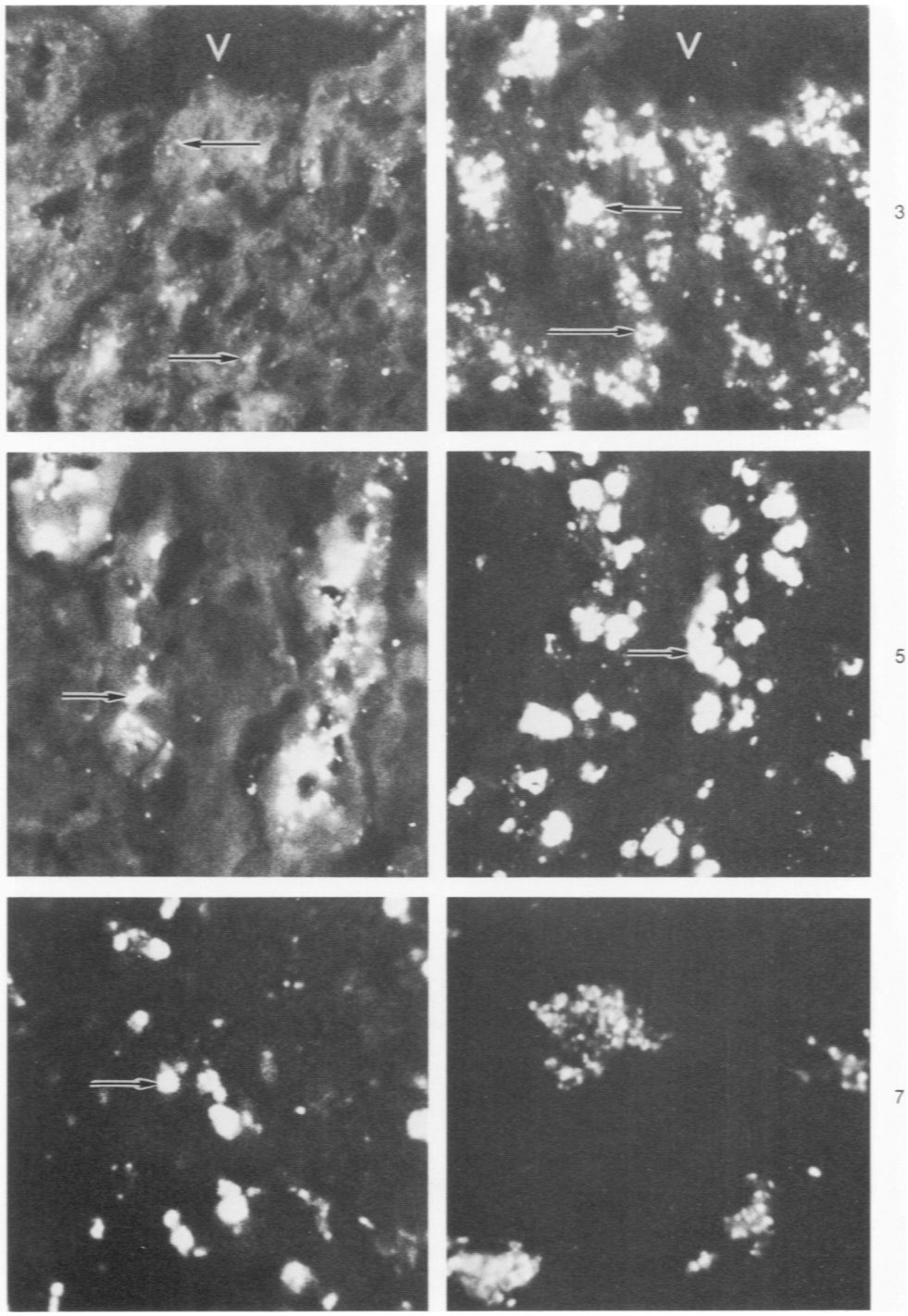


Figure 1—Unfixed C57BL (*top*) and beige mouse (*bottom*) spleens from 1-year-old animals.

Figures 2-7—Fluorescence micrographs of unfixed frozen sections (× 350). **2**—C57BL mouse liver. A small amount of pigment (*arrows*) may be seen in hepatocytes. *V* = central vein. **3**—Beige mouse liver. Large accumulations of pigment (*arrows*) are present in hepatocytes. **4**—C57BL mouse kidney cortex. Some pigment (*arrow*) is present in proximal tubule cells. **5**—Beige mouse kidney. Proximal tubule cells contain large amounts of pigment (*arrow*). **6**—C57BL mouse spleen. Macrophages in red pulp area contain pigment (*arrow*). Fluorescence is a mixture of red (hemosiderin) and yellow (ceroid). **7**—Beige mouse spleen. Macrophages in red pulp area contain large amounts of fluorescent pigment. The pigment contained a mixture of red (hemosiderin) and yellow (ceroid) fluorescent substances.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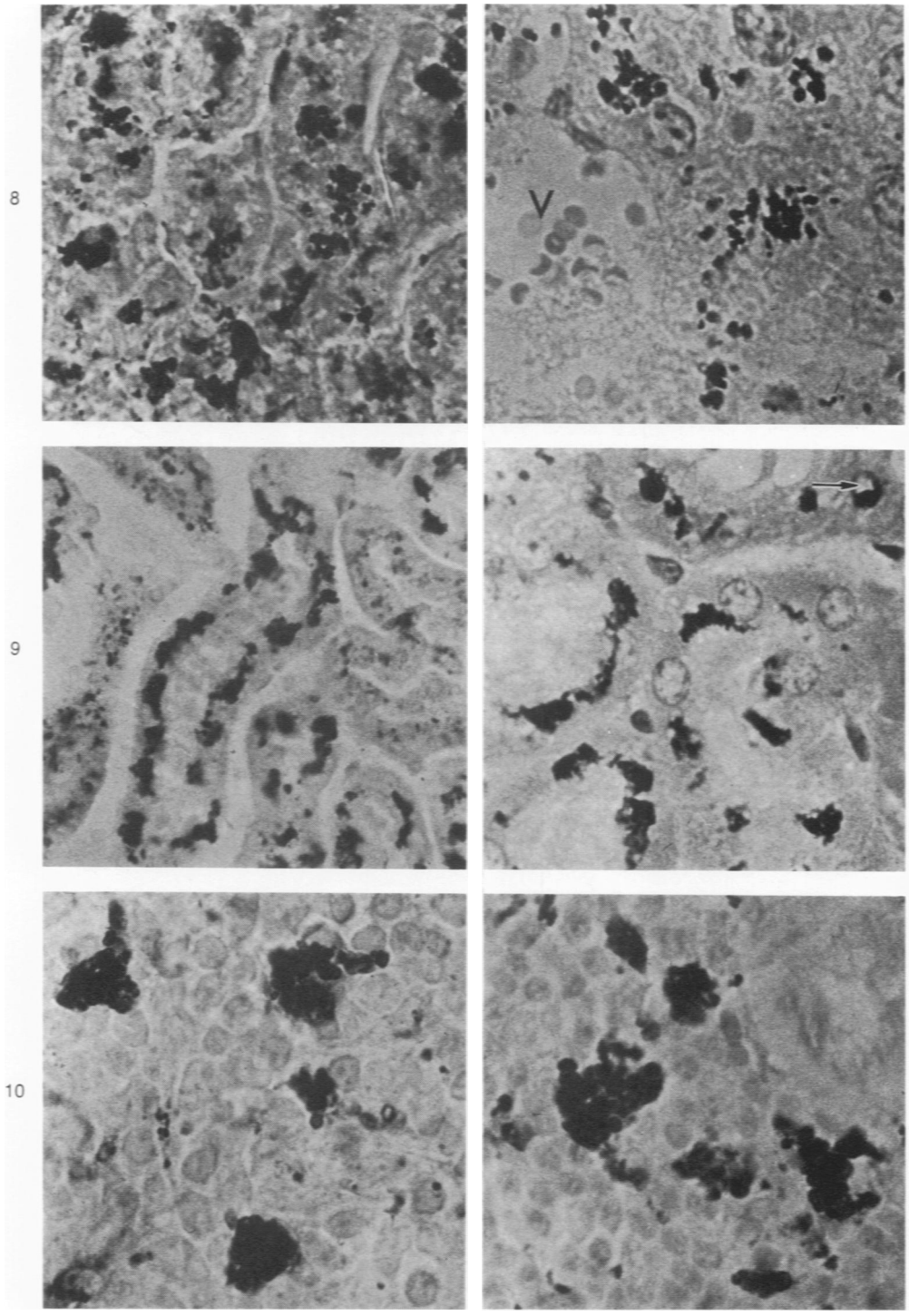
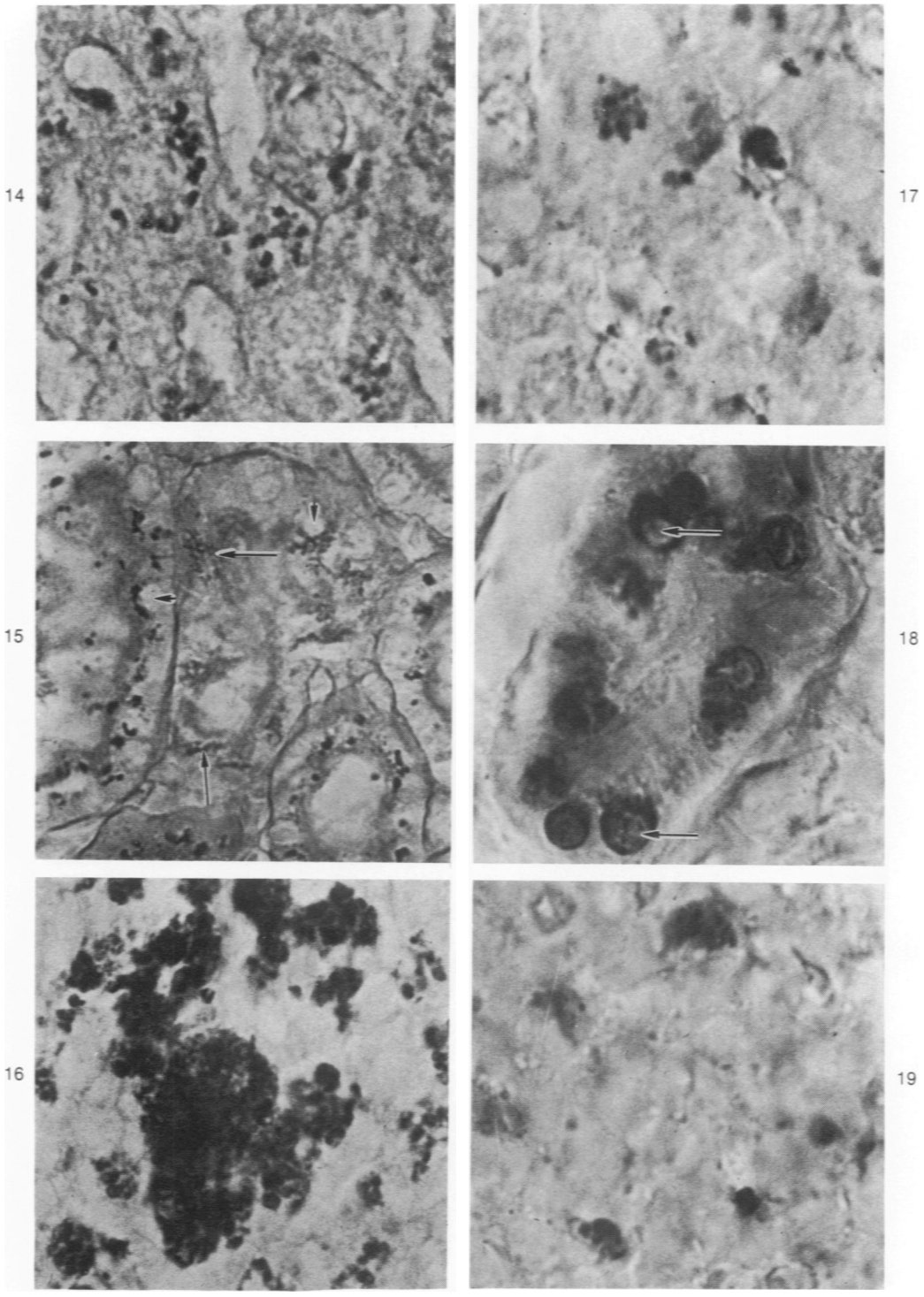
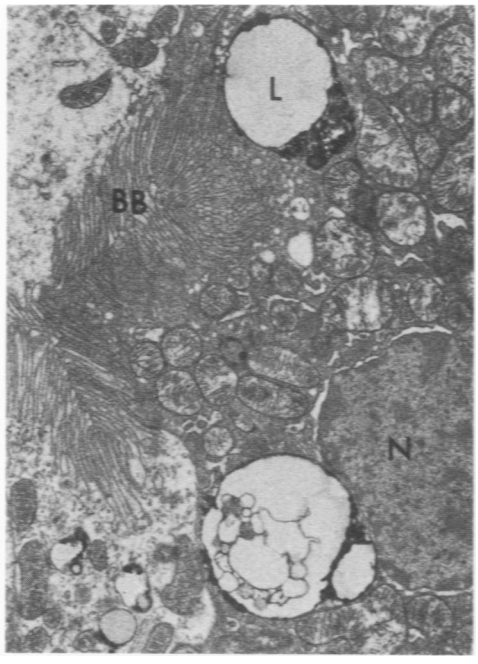


Figures 8-10—Formal-calcium-fixed frozen sections incubated for acid phosphatase activity. **8**—Beige mouse liver. The pigment is contained in acid phosphatase-positive lysosomes in hepatocytes. (× 320) **9**—Beige mouse kidney cortex. The pigment is contained in acid phosphatase-positive lysosomes in proximal convoluted tubule cells. (× 775) **10**—Beige mouse spleen. The pigment is contained in acid phosphatase-positive lysosomes in macrophages. (× 775) **Figures 11-13**—Paraffin sections stained for reducing substances with methenamine silver (× 775). **11**—Beige mouse liver. Pigment in hepatocytes is stained. *V* = central vein. **12**—Beige mouse kidney. Pigment in proximal tubules is stained. Occasional clear vacuoles in the pigment (*arrow*) may be seen. **13**—Beige mouse spleen. Pigment in red pulp area is stained.



Figures 14–16—Paraffin sections stained with PAS after diastase digestion (× 775). **14**—Beige mouse liver. The pigment in the hepatocytes is lightly stained. **15**—Beige mouse kidney. Section was also stained for ferric iron by Perl's method. The heterogeneous nature of the pigment may be seen. Some pigment stains only with PAS (*arrows*), while some stains with both PAS and Perl's. Vacuoles (*arrowheads*) ringed with pigment may also be seen. **16**—Beige mouse spleen. Pigment stains intensely with PAS. **Figures 17–19**—Frozen sections stained for neutral lipids with oil red O (× 1300). **17**—Beige mouse liver. Pigment stains lightly with oil red O. **18**—Beige mouse kidney. Darkly stained pigment may be seen surrounding unstained or lightly stained vacuoles (*arrows*). **19**—Beige mouse spleen. Pigment stains very weakly with oil red O.



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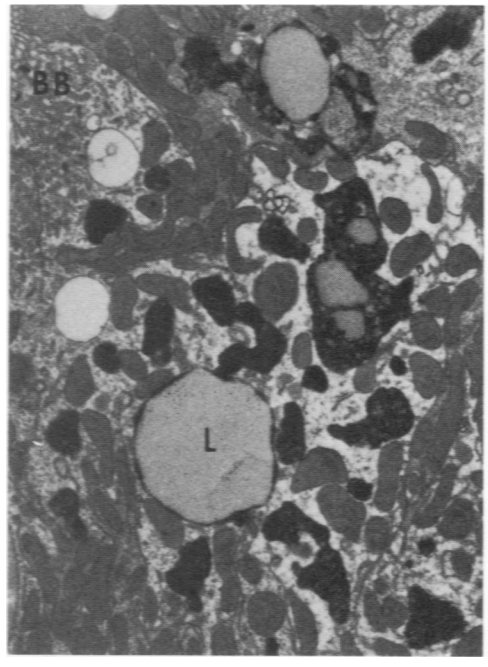

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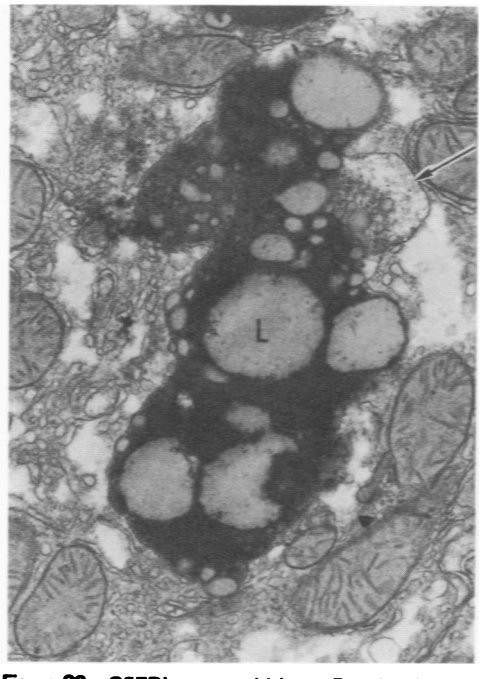

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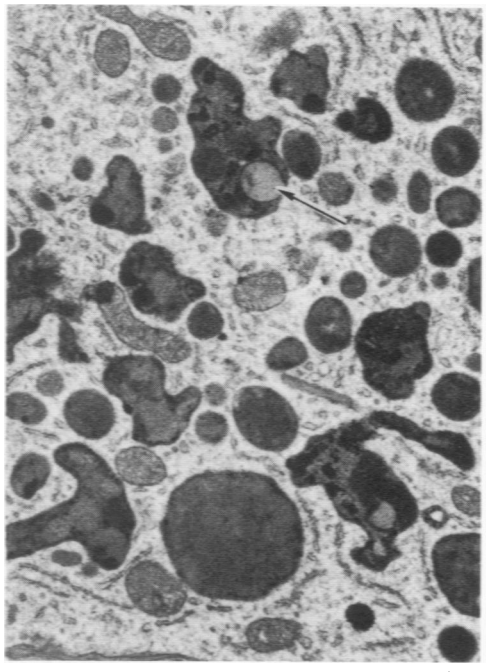

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Figure 20—C57BL mouse kidney. Proximal tubule cells contain pigment deposits surrounding lipid droplets (*L*). *BB* = brush border, *N* = nucleus (× 6000) **Figure 21**—Beige mouse kidney. Dense pigment granules fill much of proximal tubule cell. Large lipid droplets (*L*) are associated with the pigment. *BB* = brush border. (× 6000) **Figure 22**—Beige mouse liver. Portion of hepatocyte showing pigment granule composed of lipid droplets (*L*) embedded in a dense matrix and surrounded by a single membrane (*arrow*). (× 25,000) **Figure 23**—Beige mouse spleen. Portion of a macrophage showing dense granular nature of pigment with few lipid droplets (*arrow*). (× 12,000)