HEMOGLOBIN UPTAKE BY RAT HEPATOCYTES AND ITS BREAKDOWN WITHIN LYSOSOMES

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ABSTRACT

The peroxidatic activity of hemoglobin permitted visualization of its uptake by rat hepatocytes by means of the Graham-Karnovsky 3,3′-diaminobenzidine (DAB) procedure. Lysosomes were visualized by their acid phosphatase, β-glucuronidase, and glucosaminidase activities. When large doses of rat, cow, or human hemoglobin are intravenously injected, or when hemoglobinemia is induced by injection of distilled water, DAB-positive hemoglobin is engulfed by pinocytosis. Pinocytotic vacuoles become digestive vacuoles ("phagolysosomes") by fusion with lysosomes of the dense body type that have moved from their pericanalicular position. By 16–24 hr after even massive amounts of hemoglobin (400 mg/100 g), the protein is barely demonstrable in hepatocytes. At the lowest doses of injected hemoglobin (15 mg/100 g body weight), DAB-positive vacuoles are demonstrable only in the Kupffer cells.

The destruction of red blood cells in man is estimated to occur at a rate of 2.16×10^{11} cells/day, or about 9,000,000 cells/hr (Wintrobe, 1958). A major fraction of the hemoglobin derived from these erythrocytes is broken down to bile pigments. Although the role of the hepatocyte in hemoglobin breakdown has not been resolved, it is generally believed that the conversion of hemoglobin to bilirubin takes place in reticuloendothelial cells. According to this view, bilirubin is released into the circulation by these cells and is then taken up by hepatocytes, which conjugate and excrete it into the bile canaliculi. In contrast, other investigators have suggested that hemoglobin degradation into bilirubin occurs in the hepatocyte (see discussion in With, 1968).

This study was designed to visualize, by *in situ* staining techniques, the manner in which rat hepatocytes and Kupffer cells respond to large quantities of circulating hemoglobin. Because hemoglobin has peroxidatic activity, it is possible to localize the protein in aldehyde-fixed tissues, by incubation in Graham and Karnovsky's (1966) 3,3′-diaminobenzidine (DAB) medium. Hemoglobin from three mammalian sources was injected intravenously in varying amounts and visualized at intervals from 90 sec to 24 hr after injection. The rat's own hemoglobin, derived from lysis of circulating red blood cells induced by intravenous injection of distilled water, was also traced.

MATERIALS AND METHODS

Experimental Procedure

Young Sprague-Dawley rats (approximately 200 g) raised on a Rockland chow diet (Rockland Co., Winfield, Iowa) were injected intravenously with solutions of hemoglobin in normal saline. Hemoglobin, obtained from cows (Sigma Chemical Co., St. Louis, Mo.), rats (prepared according to Ericsson, 1964), or humans (sickle hemoglobin), was injected into the tail vein in concentrations ranging from

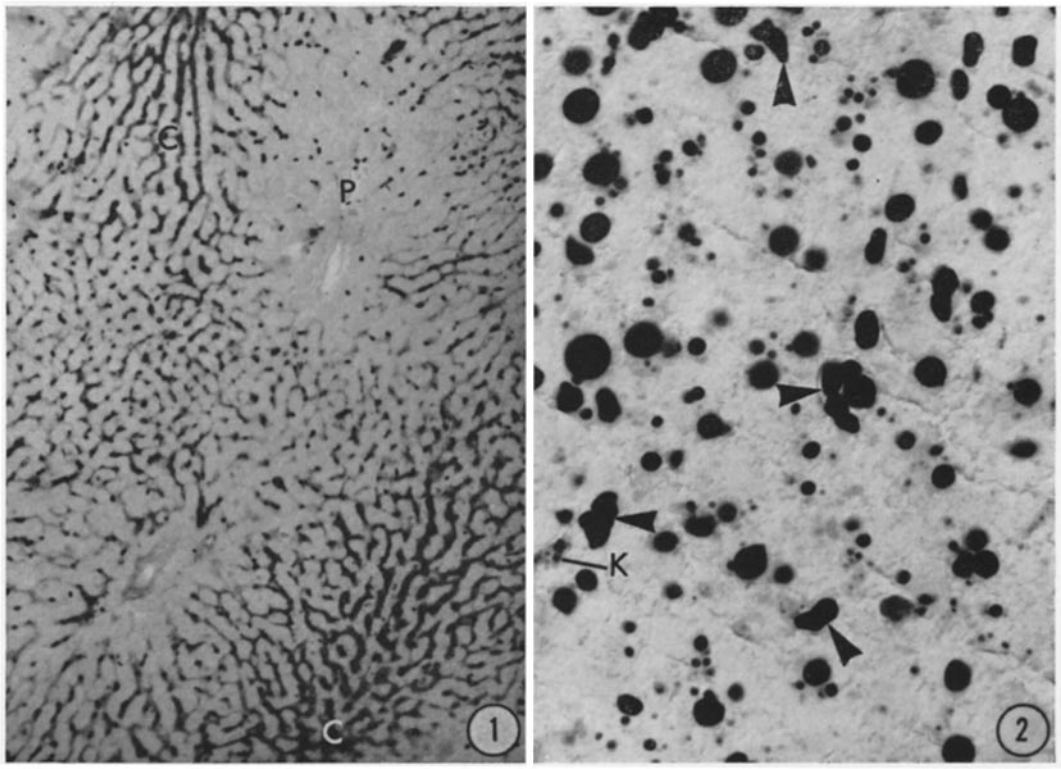


FIGURE 1 Frozen section of rat liver, immediately after completion of an injection of bovine hemoglobin (200 mg/100 g). Incubated for 60 min in a DAB medium to visualize hemoglobin. Diffuse staining of hemoglobin is present in the hepatic sinusoids, particularly in the centrilobular zones (*C*). In addition, red blood cells stain; they are seen more clearly in the portal areas (*P*). × 100.

FIGURE 2 Frozen section of rat liver, 60 min after injection of human sickle hemoglobin (400 mg/100 g). Incubated 45 min in the DAB medium. Numerous rounded, darkly stained hemoglobin droplets of varying sizes, from 15 μ to the lowest limit of light microscopy, are present in almost all hepatocytes. Clumps of red blood cells in sinusoids can be identified by their distorted shapes (arrows). Hemoglobin droplets are also seen in some Kupffer cells (*K*). × 500.

15–400 mg/100 g of rat body weight. The hemoglobin was dissolved in 3.5–7.0 ml of normal saline except for the lowest dose, 15 mg/100 g, which was dissolved in 1.5 ml of saline. Hemoglobinemia was also induced by intravenous injection of 7, 10, or 14 ml of distilled water.

The hemoglobin injection, under ether anesthesia, took about 90 sec. Rats were sacrificed at the completion of the injection or at intervals of 3, 6, 8, 13, 20, and 30 min and 1, 8, 16, 20, and 24 hr. Animals that had been injected only with saline under the same conditions and normal rats that had not been injected served as controls. After injection of hemoglobin the rats appeared to be in shock, and recovery from the anesthesia was delayed often as long as 30 min. However, rats injected with saline or distilled water recovered from the anesthesia promptly.

Fixation and Incubation

Thin slices (1–2 mm) of liver were fixed by immersion for 3–6 hr in cold (4°C) 3% glutaraldehyde-cacodylate, pH 7.4 (Sabatini, Bensch, and Barrnett, 1963) or overnight in cold 4% formaldehyde containing 1% calcium chloride (Baker, 1946). Similar staining results were obtained with either aldehyde, but structural preservation was superior with glutaraldehyde. Preservation of the very large vacuoles (to be described below) in frozen sections, especially when incubated at acid pH, required overnight fixation in glutaraldehyde.

For light microscopy, freely-floating 10-μ frozen sections were stained in the following manner. *Hemoglobin* was made visible by incubation at *room temperature* in Graham and Karnovsky's (1966) DAB

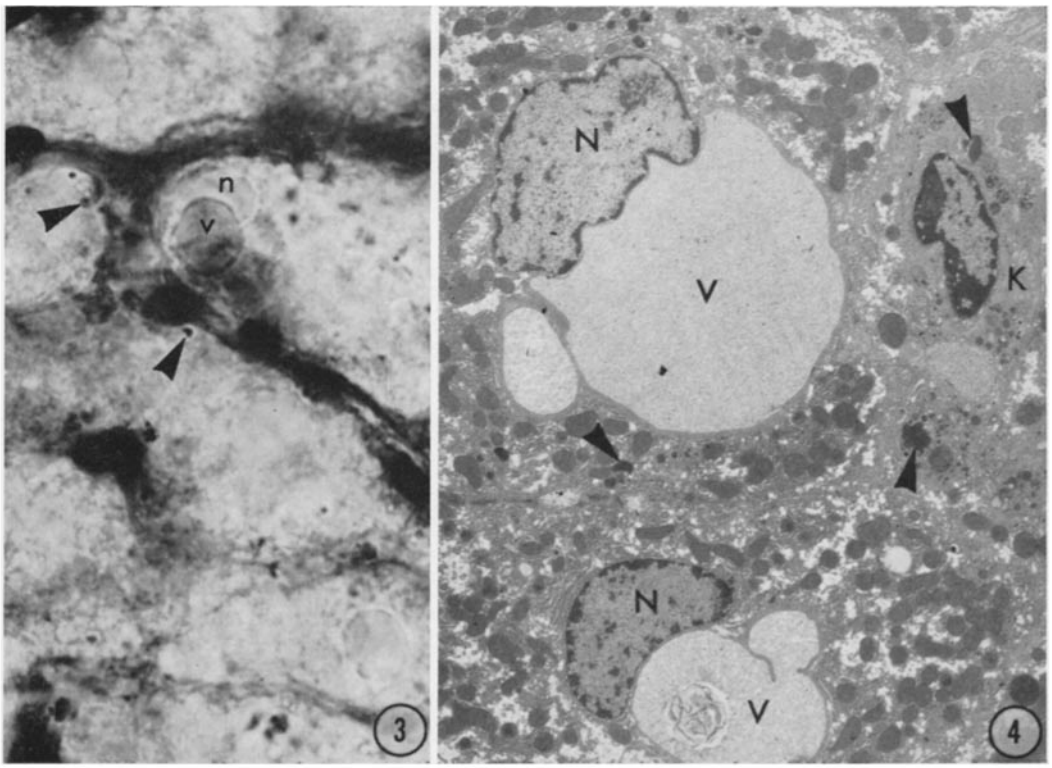


FIGURE 3 Frozen section of rat liver, 3 min after completion of injection of bovine hemoglobin (200 mg/100 g). 15-μ sections of a specimen fixed overnight in 3% glutaraldehyde were incubated for 45 min in the DAB medium. A huge hemoglobin vacuole (*v*) that is lightly stained encroaches upon the nucleus (*n*). Darkly staining hemoglobin is present in the sinusoids and in a few small, dark vacuoles (arrows) in the cytoplasm nearby. × 1400.

FIGURE 4 Frozen section of rat liver 8 min after injection of bovine hemoglobin (400 mg/100 g). Incubated in the DAB medium for 120 min. Thin section stained with uranium and lead. Two huge vacuoles (*V*) indent the nuclei (*N*) of parenchymal cells. Smaller vacuoles, darkly staining because of their high concentrations of hemoglobin, are also present (arrows) in the hepatocytes and the Kupffer cell (*K*) on the right. × 3000.

medium containing three times the original concentration of both DAB and H_2O_2 (Goldfischer, 1967). *Lysosomes* were made visible by their acid hydrolase activities. Sections were incubated at 37°C for the following activities: acid phosphatase in Gomori's medium (1952) with β-glycerophosphate as substrate, or with cytidine monophosphate (CMP) (Novikoff, 1963), and in Barka and Anderson's (1962) hexazonium pararosaniline medium with naphthol AS-TR phosphate as substrate; β-glucuronidase in the medium of Hayashi, Nakajima, and Fishman (1964); and *N*-acetyl-β-glucosaminidase in Hayashi's (1965) medium. Controls were incubated in substrate-free media.

Portions of each liver were embedded in paraffin. Sections of this material were stained in hematoxylin and eosin in the usual fashion and in the DAB medium to visualize hemoglobin, followed by counterstaining with hematoxylin.

For electron microscopy, portions of each specimen were also fixed at 4°C or room temperature for 2 hr in 1% OsO_4 in Veronal-acetate buffer, pH 7.4 (Palade, 1952) or phosphate buffer, pH 7.4 (Millonig, 1961), or in 3% glutaraldehyde-cacodylate for 3 hr and postfixed in OsO_4 in phosphate buffer for 1 hr. Tissues were rapidly dehydrated in graded alcohols and embedded in Epon (Luft, 1961).

For electron microscopic study of cytochemical preparations, 40-μ nonfrozen sections were prepared with the TC-2 Smith & Farquhar tissue sectioner (Ivan Sorvall Inc., Norwalk, Conn.) and were incubated for the following: (*a*) acid phosphatase activity in Gomori's medium with β-glycerophosphate or CMP as substrate, (*b*) hemoglobin, or (*c*) for acid

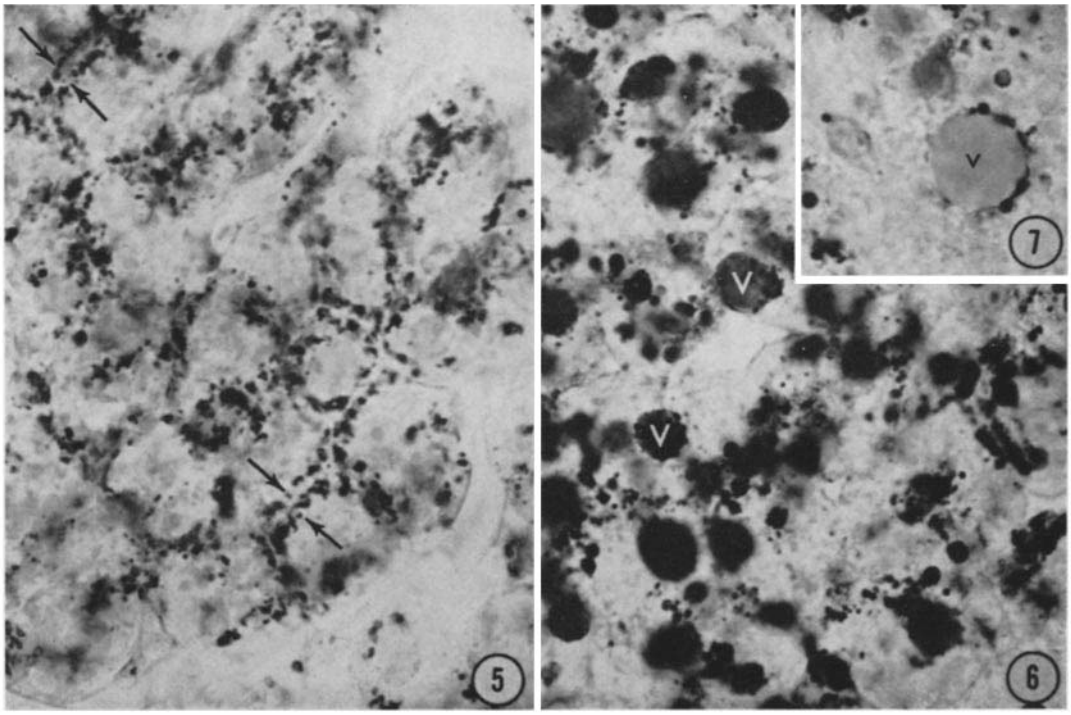


FIGURE 5 Frozen section of normal, uninjected rat liver. Incubated for 50 min in the CMP medium for acid phosphatase activity in lysosomes. Note the pericanalicular localization of lysosomes in the hepatocytes (arrows). × 760.

FIGURE 6 Frozen section of rat liver 30 min after injection of bovine hemoglobin (400 mg/100 g). Incubated for 30 min in the CMP medium for acid phosphatase activity. The lysosomes are greatly increased in size and decreased in number in most hepatocytes. Some small, darkly stained lysosomes are still found alongside bile canaliculi but many now ring the hemoglobin vacuoles (*v*), which have acquired varying amounts of acid phosphatase reaction product. × 760.

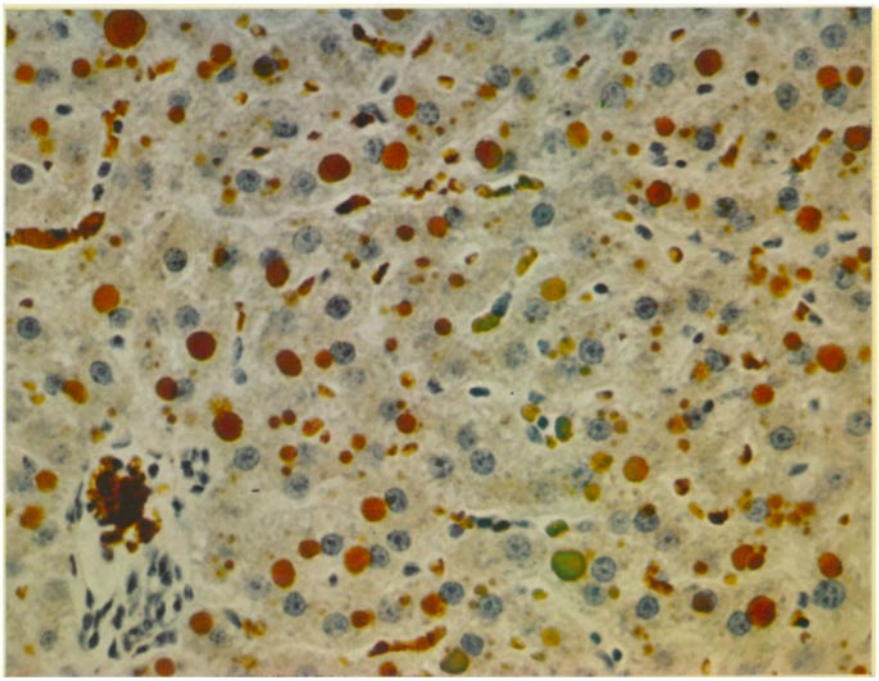
FIGURE 7 Frozen section of rat liver 60 min after injection of human sickle hemoglobin (400 mg/100 g). In this high power (oil immersion) micrograph, the lysosomes that ring a hemoglobin vacuoles (*v*) are clearly seen. × 1500.

phosphatase activity followed by buffer rinse and incubation for hemoglobin. After incubation the sections were rinsed in cacodylate buffer, postfixed for 1 hr in 1% OsO_4 in phosphate buffer, and embedded in Epon. Thin sections were stained with lead citrate (Reynolds, 1963) alone, or with uranyl acetate (Watson, 1958) followed by lead. In some experiments tissue blocks were stained with uranyl acetate before dehydration (Farquhar and Palade, 1965).

RESULTS

Light Microscopy

When hemoglobin is injected intravenously (Fig. 1), hepatocytes engulf the protein by endocytosis (Figs. 2, 3, and 8). Vacuoles containing hemoglobin, some of which measure up to 15 μ in diameter and often indent the nucleus, are detectable immediately after injection of the protein (150–400 mg/100 g body weight) (Figs. 3, 4, and 13). By 6–8 min after injection, hemoglobin vacuoles of various sizes rapidly become more numerous and stain darkly in the DAB medium. With the highest doses, at 1 hr hemoglobin droplets in hepatocytes are present over the entire section (Figs. 2 and 8). This occurs with cow, human, and rat hemoglobin, and it is also seen when the rat's own hemoglobin is released into the circulation by distilled water injection.



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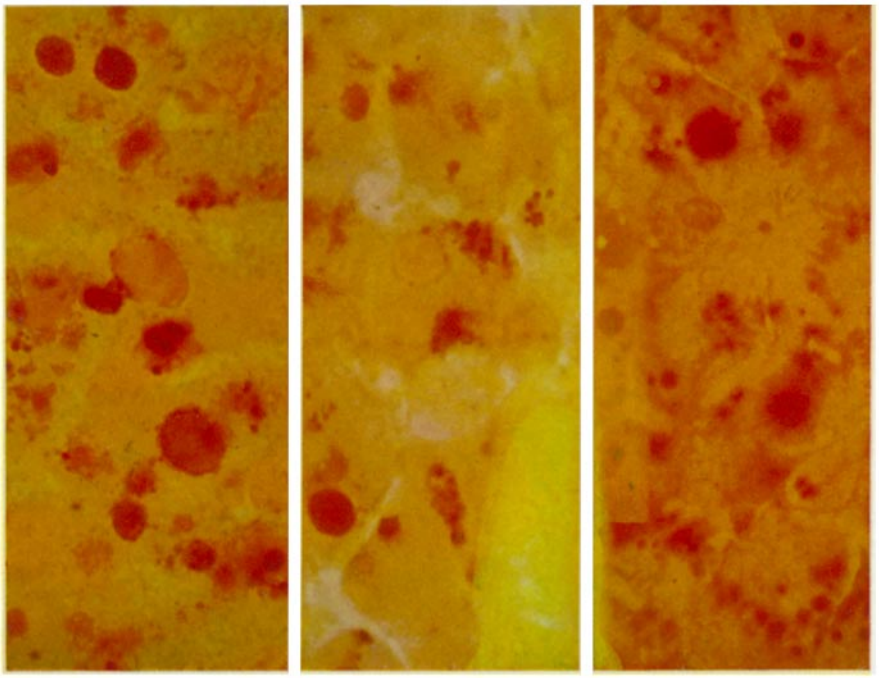

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FIGURES 8–11 Rat liver 60 min after injection of human sickle hemoglobin (400 mg/100 g). Fig. 8 shows a paraffin section of formalin-fixed tissue incubated for 60 min in the DAB medium and then counterstained with hematoxylin to demonstrate the nuclei. Hemoglobin droplets of varying sizes are present in the cytoplasm of hepatocytes. They are similar in color to the red blood cells seen in sinusoids and portal vein at lower left. × 400. Figs. 9–11 demonstrate frozen sections incubated for 60 min in azo dye media for acid phosphatase (Fig. 9), β-glucuronidase (Fig. 10), and glucosaminidase (Fig. 11) activities. Enzyme reaction product (red) is seen in lysosomes arranged along bile canaliculi and hemoglobin droplets. Note the dark staining for acid hydrolase activity in the large hemoglobin vacuoles. × 1000.

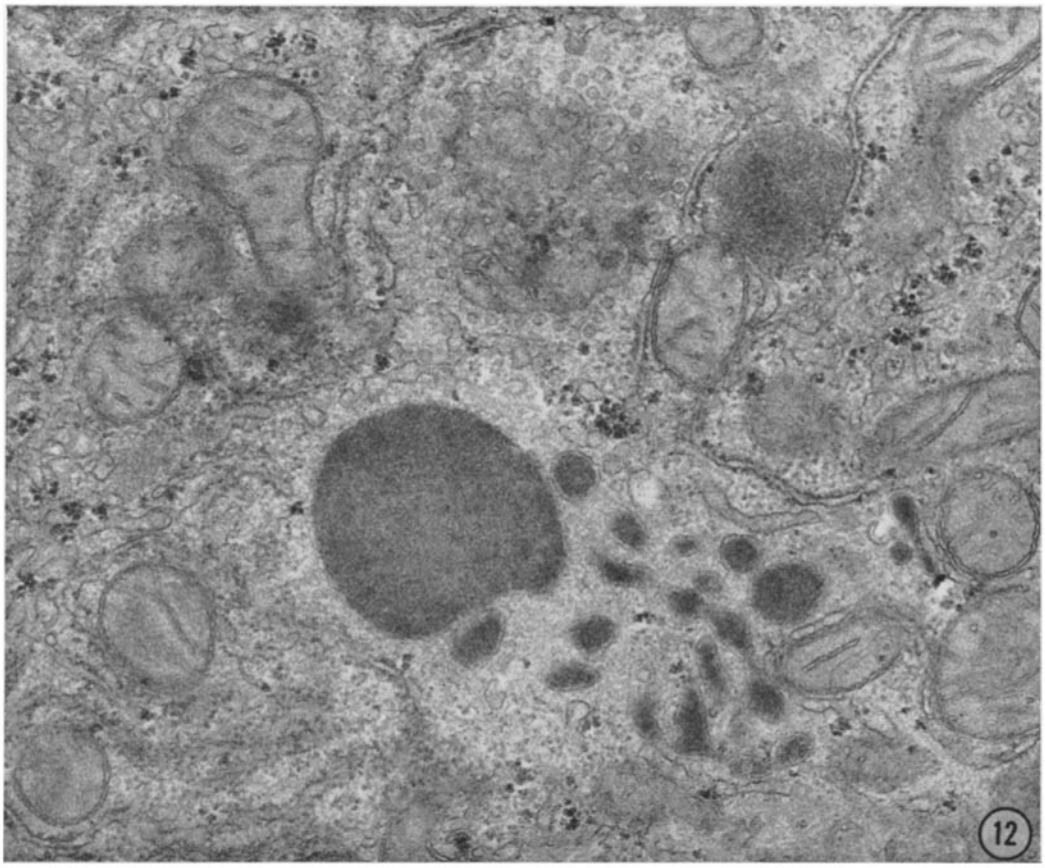


FIGURE 12 Rat liver 8 min after injection of bovine hemoglobin (75 mg/100 g). Incubated for 60 min in the DAB medium. Thin section stained with uranium and lead. The visualized hemoglobin is seen in tubular structures bounded by a single membrane and in vacuoles which appear to converge upon a small hemoglobin droplet. × 33,000.

The lysosomes, in the first few minutes after injection, are essentially similar to those of uninjected control animals (Fig. 5) in size, number, and pericanalicular distribution. By 8–13 min after injection the pericanalicular concentration of lysosomes is no longer evident, and the number of lysosomes is reduced in many hepatocytes. Small lysosomes ring the hemoglobin vacuoles, and some hemoglobin droplets have acquired acid hydrolase activity. By 30 min numerous hemoglobin vacuoles that have acquired acid phosphatase (Figs. 6, 7, and 9), β-glucuronidase (Fig. 10), and glucosaminidase (Fig. 11) activities are found throughout the lobule.

Following injection of even the highest doses of hemoglobin, the parenchymal cells return to their normal appearance; with doses of 400 mg/100 g, this occurs by 16–24 hr. Relatively few large hemoglobin droplets are seen in hepatocytes at this time (Fig. 27), and lysosomes again show a pericanalicular orientation (Fig. 28).

The incorporation of hemoglobin into lysosomes occurs in a similar fashion in animals injected with more moderate amounts (150 mg/100 g) of the protein. However, the disappearance of stainable hemoglobin from the hepatocytes takes place more rapidly and may occur as early as 1 hr after injection. As the dose of injected hemoglobin is reduced, still further differences in staining patterns are observed. With 40–80 mg/100 g hemoglobin, vacuoles within hepatocytes are difficult to detect by light microscopy but are seen by electron microscopy (Fig. 12). In rats injected with the lowest dose (15 mg/100

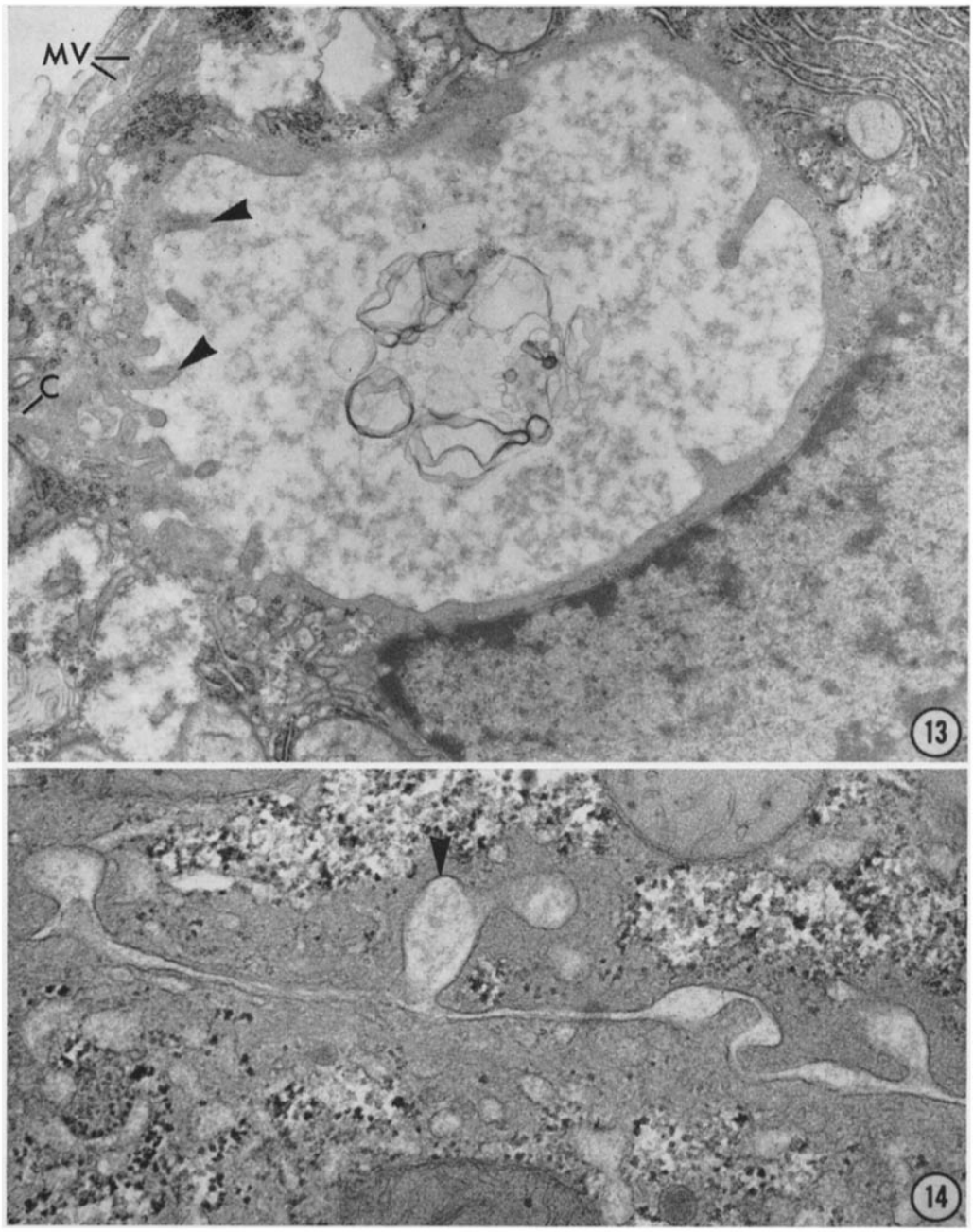
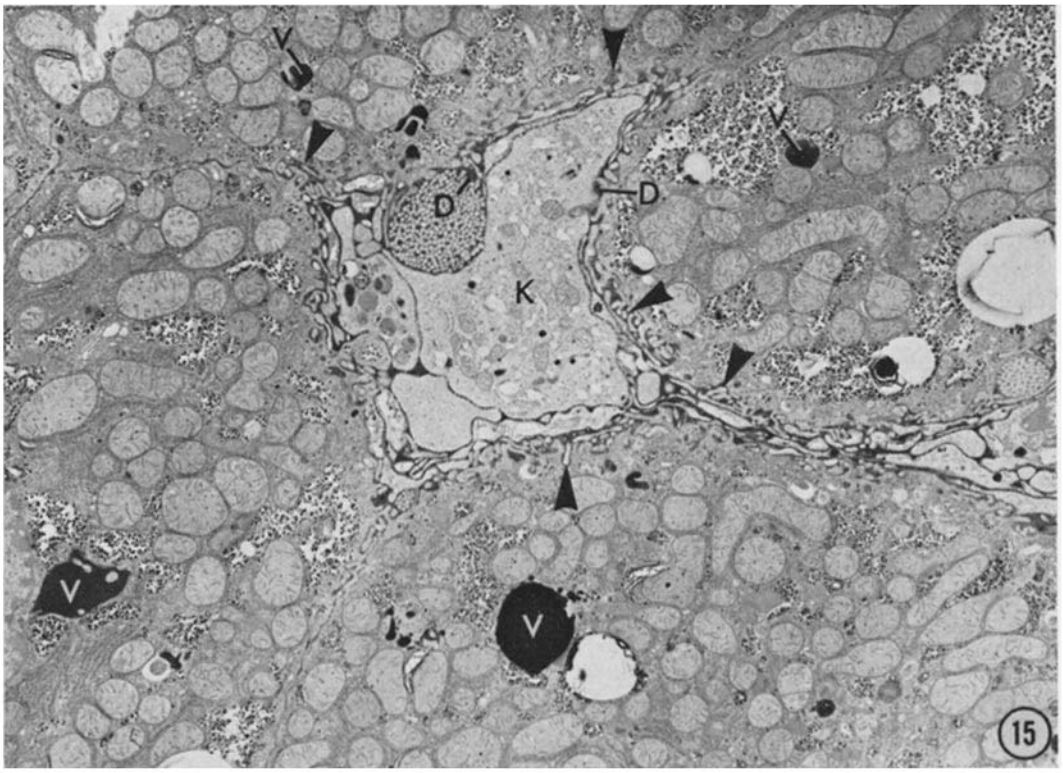


FIGURE 13 Rat liver immediately after completing injection of bovine hemoglobin (200 mg/100 g). Thin section stained with uranium and lead. Arrows point to projections into this large hemoglobin vacuole that resemble sinusoidal microvilli (*MV*). The cytoplasm that rings the vacuole appears homogeneous and is free of organelles and glycogen. A channel (*C*) that approaches the space of Disse is seen on the left. The irregular membranous structure in the center of the vacuole is probably an artifact of glutaraldehyde fixation. × 17,000.

FIGURE 14 Rat liver immediately after injection of bovine hemoglobin (75 mg/100 g). Thin section stained with uranium and lead. An invagination of the cell membrane (arrow) is evident. Note that the cytoplasm surrounding the infolding is free of organelles and resembles the cytoplasm enclosing the vacuole in Fig. 13. × 40,000.



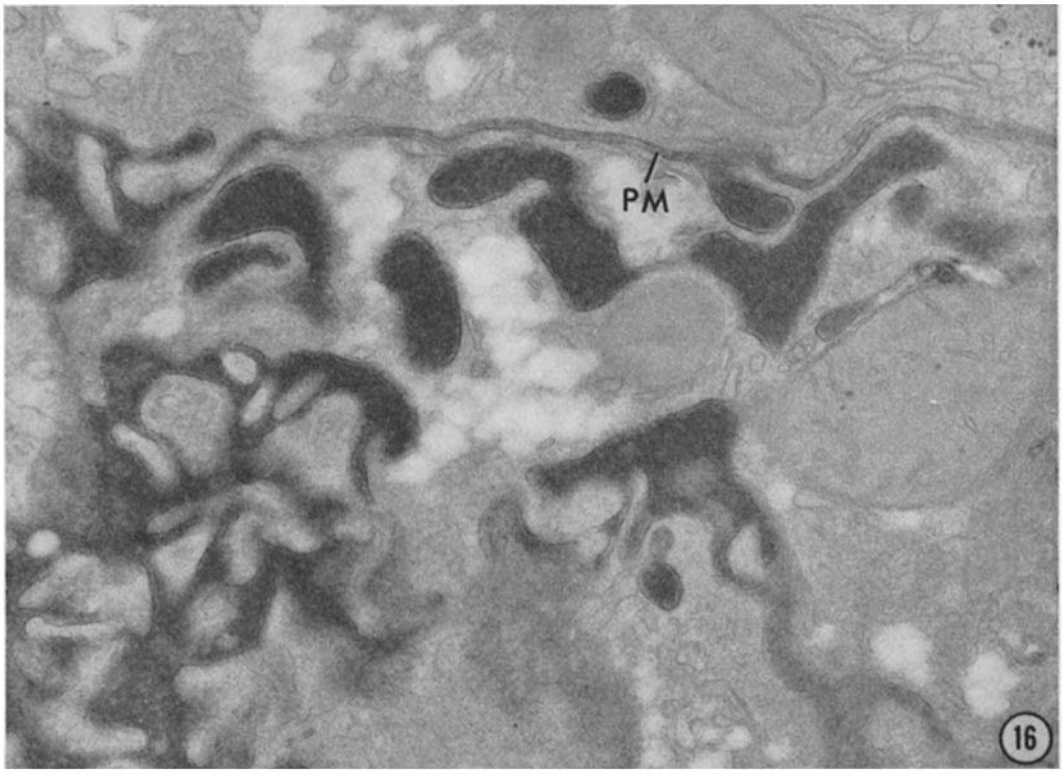


FIGURE 15 Rat liver 8 min after injection of bovine hemoglobin (400 mg/100 g). Incubated for 2 hr in the DAB medium. Thin section stained with lead. Hemoglobin reaction product is seen within the space of Disse (*D*), between cells in complex, trenchlike and tubular invaginations of the plasma membrane (arrows), in vacuoles (*V*) within the hepatocytes, and in vesicles within Kupffer cells (*K*). × 4,000.

FIGURE 16 Rat liver 8 min after injection of bovine hemoglobin (75 mg/100 g). Incubated for 2 hr in the DAB medium. Thin section stained with lead. The complex, interconnecting infoldings of the plasma membrane (*PM*) into the cytoplasm are evident. × 32,000.

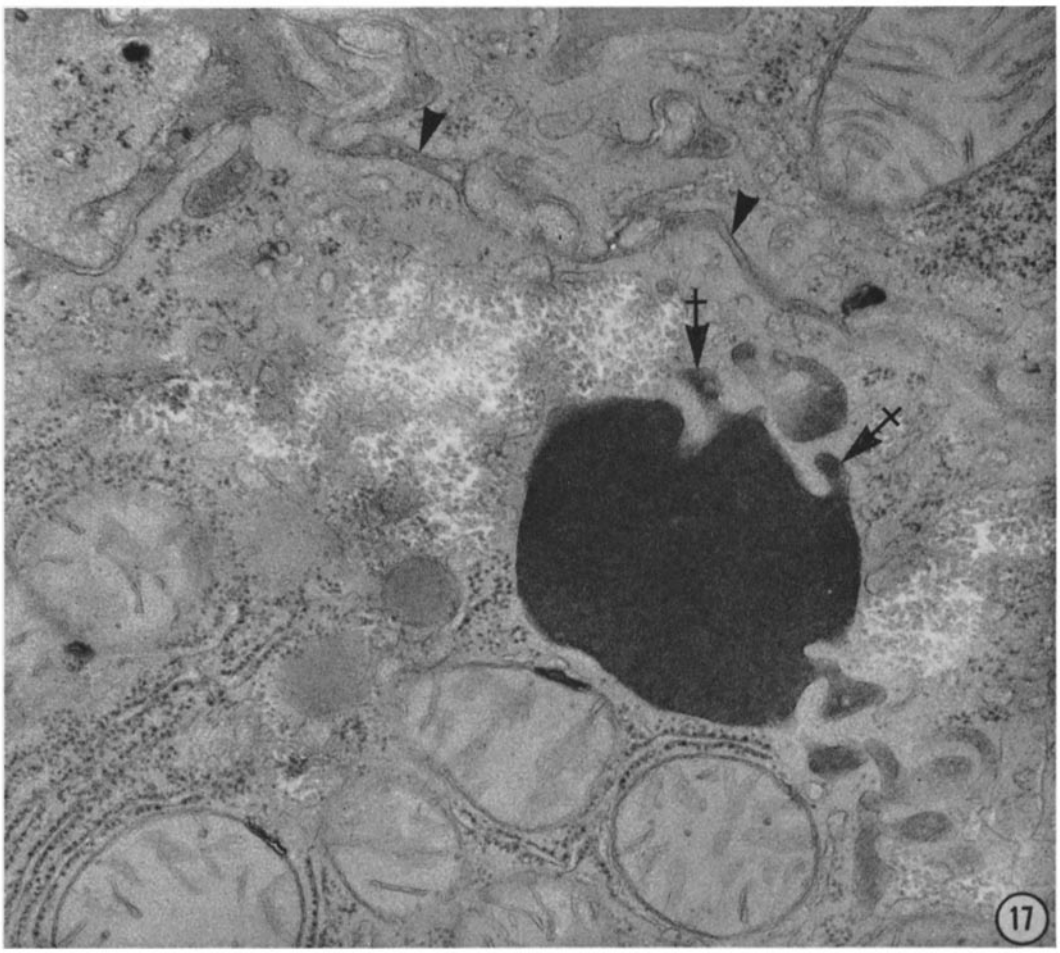


FIGURE 17 Rat liver 3 min after injection of bovine hemoglobin (200 mg/100 g). Incubated for 2 hr in a DAB medium. Thin section stained with uranium and lead. Hemoglobin reaction product is present in the intercellular space (arrows) and a vacuole with tubular extensions (crossed arrows). The similarities in the contents of these extensions, nearby tubules, and the intercellular space suggest that the contents probably come from the cell surface. × 23,000.

g), hemoglobin droplets are found only in Kupffer cells. Hemoglobin droplets are not seen in saline-injected controls.

Kupffer cells, in addition to showing the hemoglobin which is taken up from the circulation and seen as droplets (Figs. 2 and 18), occasionally show an endogenous peroxidatic activity that has been described previously (Wachstein and Meisel, 1964; Novikoff et al. 1968). Curiously, with high doses larger numbers of hemoglobin droplets in Kupffer cells are more numerous 16 hr after injection than at earlier intervals.

Electron Microscopy

The smaller and more numerous vacuoles appear to form from trenchlike infoldings of the lateral and sinusoidal surfaces of the hepatocyte (Fig. 15). These infoldings project deeply into the cytoplasm and appear to swell and fuse at the sites of deepest penetration (Figs. 16 and 17). The hemoglobin content of these vacuoles is high, as judged from their electron opacity in unincubated preparations and their intense staining in the DAB medium.

The huge vacuoles seem to form by a massive infolding of the plasma membrane at the surface

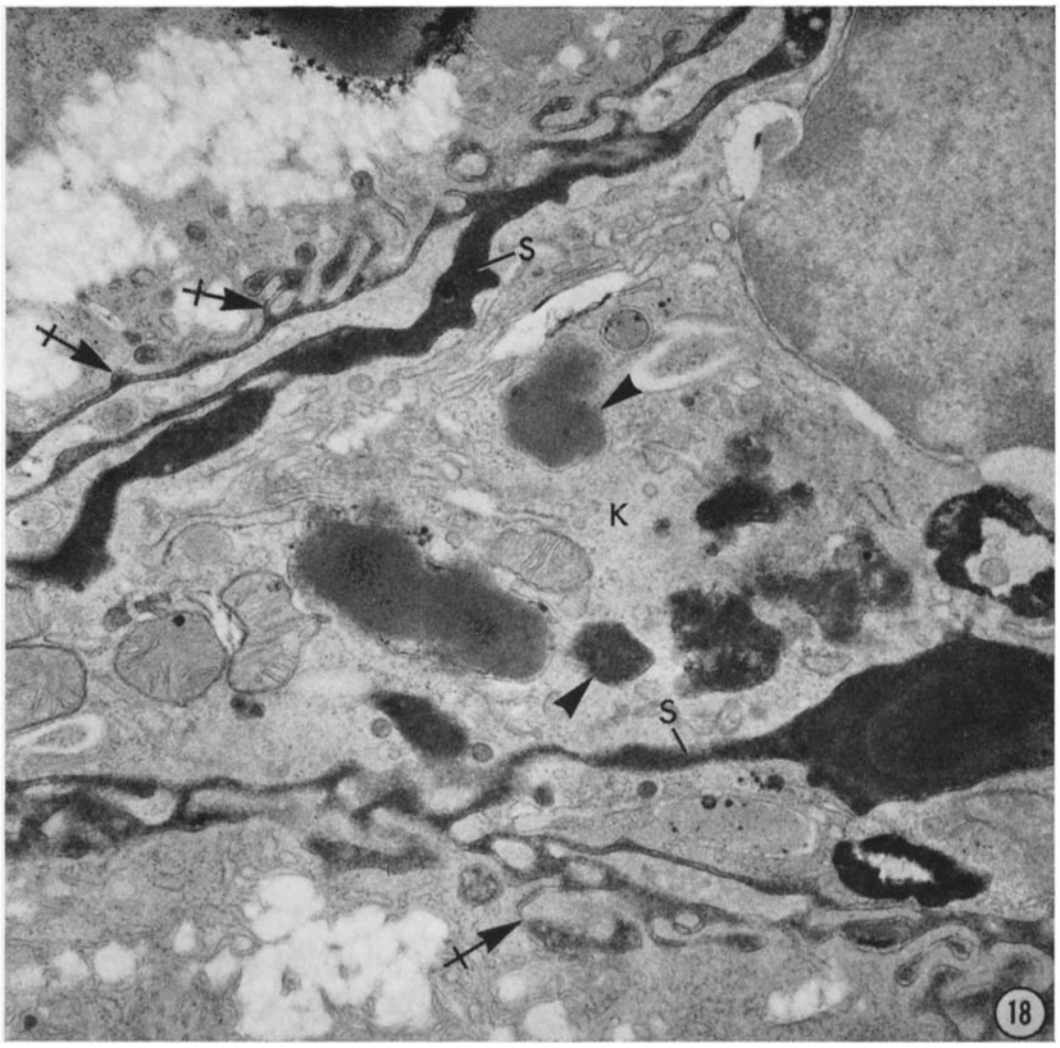


FIGURE 18 Rat liver 8 min after injection of bovine hemoglobin (400 mg/100 g). Incubated for 2 hr in DAB medium. Thin section stained with lead. Hemoglobin reaction product is seen in the sinusoid (*S*), in vacuoles (arrows) within a Kupffer cell (*K*), and in tubular extensions (crossed arrows) into the hepatocytes. × 23,000.

of the cell facing the space of Disse and at the lateral surface (Figs. 13 and 14). Neither actual continuities between vacuoles and Disse's space have been seen, nor have serial sections been done. However, the membranes delimiting these vacuoles sometimes show projections resembling the microvilli that are characteristic of the plasma membrane at the sinusoidal surface (Fig. 13). The vacuoles are surrounded by a cytoplasm that is homogeneous and free of glycogen and organelles and thus resembles in both these respects the cytoplasm adjacent to the sinusoidal plasma membrane (Figs. 13 and 14). The contents of these vacuoles are flocculent and of moderate electron capacity. Although the vacuoles show DAB reaction product in the light microscope (Figs. 3 and 8), staining was not apparent by electron microscopy, probably because some of the contents of these huge vacuoles are lost when the tissue is processed.

Light microscopy demonstrates (*a*) that lyso-

somes surround the hemoglobin vacuoles and (*b*) that these vacuoles acquire acid hydrolase activities (Figs. 6, 7, 9–11). Electron microscopy suggests that the hydrolases are brought to the vacuoles by lysosomes of the dense body type that appear to fuse with the vacuoles. Evidence for fusion of the large vacuoles and lysosomes comes from (*a*) the large numbers of dense bodies that are very often seen closely apposed to the edges of hemoglobin vacuoles (Figs. 12, 21, and 22) and that are identified as lysosomes by their acid phosphatase activity (Figs. 19, 23, and 24), (*b*) images that suggest fusion of the delimiting membranes of dense bodies and hemoglobin vacuoles (Figs. 20 and 22), and (*c*) the presence within hemoglobin vacuoles of acid phosphatase reaction product (Figs. 23–25) and electron-opaque material, similar to the contents of dense bodies (Figs. 22, 25, and 26). Images showing fusion of large and small hemoglobin vacuoles are common (Fig. 19).

Acid phosphatase activity is seen in areas of Golgi-associated smooth-surfaced endoplasmic reticulum (a region called GERL by Novikoff, 1967) of hemoglobin-injected animals (Fig. 25). Evidence for a relationship between Golgi vesicles and hemoglobin vacuoles was not encountered.

In Kupffer cells, the infoldings of the plasma membrane and the vacuoles (Fig. 18) are seen to contain hemoglobin at all doses tested.

Neither light microscopy nor electron microscopy reveals the presence of any DAB reaction product in bile canaliculi.

DISCUSSION

Our results demonstrate that rat hepatocytes have the capacity of absorbing large amounts of circulating hemoglobin. This capacity is apparent, under the conditions of our experiments, only when moderate to large doses (40–400 mg/100 g) are administered. With doses of 15 mg/100 g, hemoglobin uptake is detectable, by our methods, only in the Kupffer cells. At this level, circulating hemoglobin is probably bound to haptoglobin. It is possible that hemoglobin complexed with haptoglobin is taken up exclusively by reticuloendothelial cells.

The uptake of hemoglobin by hepatocytes occurs by pinocytosis, mainly through the trenchlike infoldings of the plasma membrane where small vesicles and large vacuoles separate from the membrane at these sites. Although the uptake of peroxidase by pinocytosis has been described in hepatocytes (Daems et al., 1964; Graham and Kellermeyer, 1968; Fahimi and Karnovsky, 1968), the relationship between pinocytotic vacuoles and lysosomes was not discussed. Protein uptake in liver cells has been described in a child with Pompe's disease, a disorder in which the lysosomal enzyme, acid maltase, is deficient and large quantities of glycogen accumulate in hepatic lysosomes (Hers, 1963). Hug and Schubert (1967) have shown that following the intravenous administration of an enzyme extract containing acid maltase, lysosomal glycogen is broken down, indicating that the enzyme is taken up by liver cells and reaches the lysosomes. The incorporation of asialoceruloplasmin, a copper protein, into hepatocytes has been shown by radioautography (Morell et al., 1968). Jacques (1968) has recently determined the rate at which injected peroxidase and invertase are cleared from the plasma and the uptake and fate of these proteins in the liver. However, the participation of hepatocyte lysosomes and the participation of Kupffer cell lysosomes could not be separated by cell fractionation techniques.

Our results indicate further that in hepatocytes lysosomes of the dense body type probably fuse with the pinocytotic vacuoles that contain hemoglobin, to form digestive vacuoles. All three lysosomal enzymes tested (acid phosphatase, β-glucuronidase, and glucosaminidase) are present in these secondary lysosomes, and the number of lysosomes is greatly reduced. Transformation of pinocytotic vacuoles into digestive vacuoles ("phagolysosomes") by fusion with lysosomes is a common phenomenon in other cell types (see de Duve and Wattiaux, 1966 for review). Fusion with lysosomes other than dense bodies, e.g. Golgi vesicles or multivesicular bodies, was not seen but the possibility that this occurs cannot be excluded.

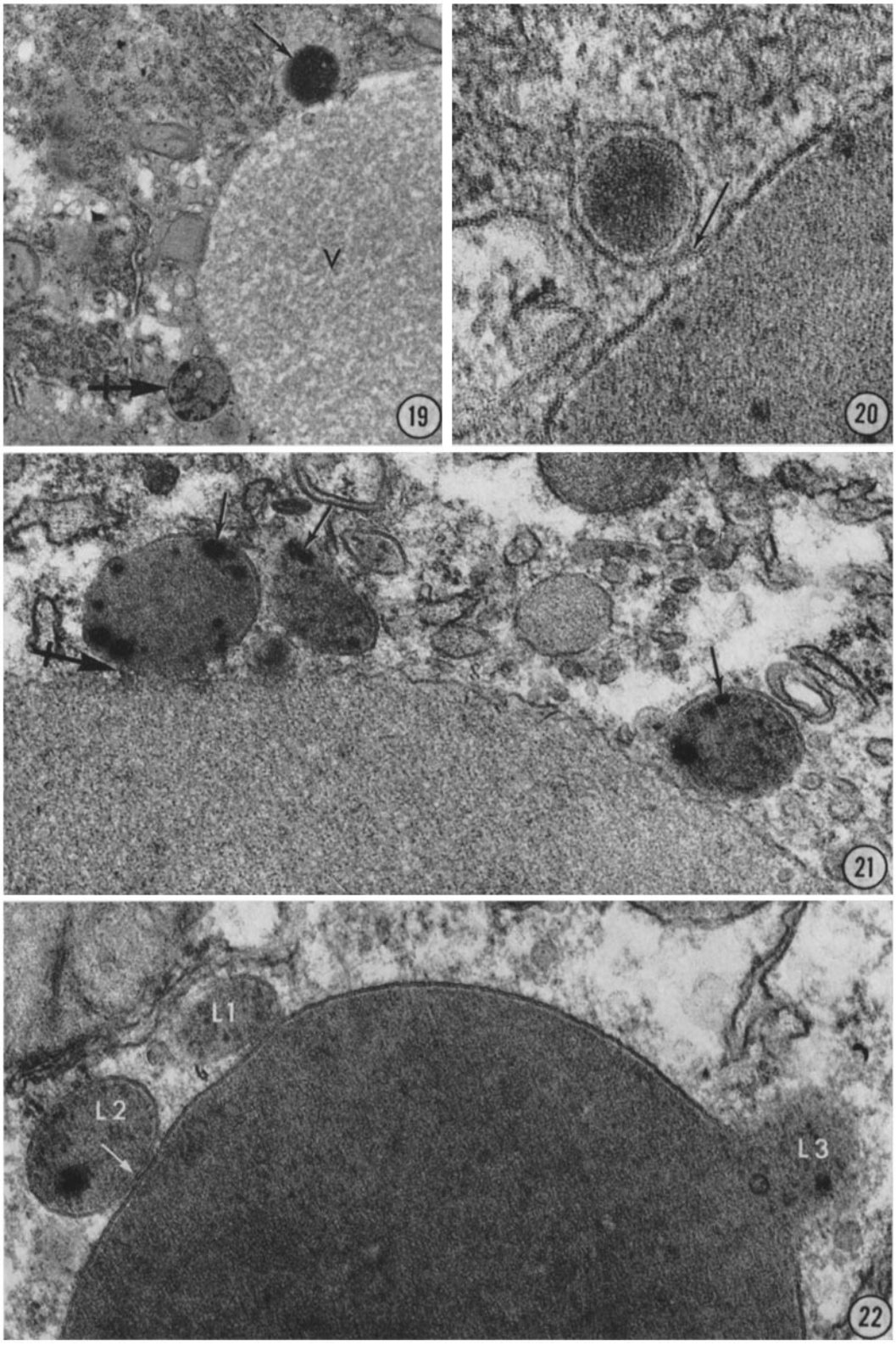
Our animals were in a shocklike state following anesthesia and hemoglobin injections, and this may have been responsible for formation of the huge vacuoles that were seen within minutes after injection. Hepatocellular vacuoles, with a similar ultrastructure, have been described in endotoxin shock (Boler and Bibighaus, 1967; Levy, Slusser, and Ruebner, 1968), but they were not reported in hypovolemic shock (Blair et al., 1968). The huge vacuoles were not present in our control animals that were anesthetized and injected with equal volumes of saline. Finally, such pinocytotic vacu-

FIGURE 19 Rat liver 13 min after injection of bovine hemoglobin (200 mg/100 g). Double incubation to visualize both acid phosphatase activity and hemoglobin. Incubated for 25 min in the CMP medium and for 2 hr in the DAB medium. Thin section stained with lead. Acid phosphatase reaction product is coarse and granular; DAB reaction product is more homogeneous. Two small bodies are seen very close to a large hemoglobin vacuole (*V*). The body at the top of the field (arrow) contains a high concentration of hemoglobin. Acid phosphatase reaction product is seen in the body at the bottom of the field (crossed arrow), identifying it as a lysosome. × 15,000.

FIGURE 20 Rat liver 30 min after injection of bovine hemoglobin (300 mg/100 g). Unincubated preparation. Tissue stained with uranyl acetate, in block. Thin section stained with lead. A high power view of the periphery of a huge hemoglobin vacuole and an adjacent body. The body could be either a lysosome, in which electron-opaque areas (see Fig. 21) have not been included, or a small hemoglobin vacuole. The unit membranes surrounding the body and the large vacuole appear to have fused, and a continuity of the outer leaflet is seen (arrow). × 140,000.

FIGURE 21 Rat liver 30 min after injection of bovine hemoglobin (400 mg/100 g). Unincubated preparations. Tissue stained with uranyl acetate in block. Thin section stained with lead. Three lysosomes of the dense body type, with characteristic foci of high electron opacity (arrows) and delimited by a unit membrane, are seen at the periphery of a hemoglobin vacuole. At some sites (crossed arrow) the lysosomes and the vacuole are so close that no cytoplasmic matrix can be seen between the two bodies. However, fusion of membranes is not evident in this field. × 50,000.

FIGURE 22 Rat liver 30 min after injection of bovine hemoglobin (400 mg/100 g). Unincubated preparation. Tissue stained with uranyl acetate in block. Thin section stained with lead. One lysosome (*L1*) is directly apposed to the hemoglobin vacuole. The membrane delimiting a second (*L2*) has probably fused (arrow) with the membrane of the hemoglobin droplet. The contents of a third lysosome (*L3*) and that of hemoglobin droplet have merged; the delimiting membrane of this lysosome is not evident, probably because of the tangential plane in which this body is sectioned. × 65,000.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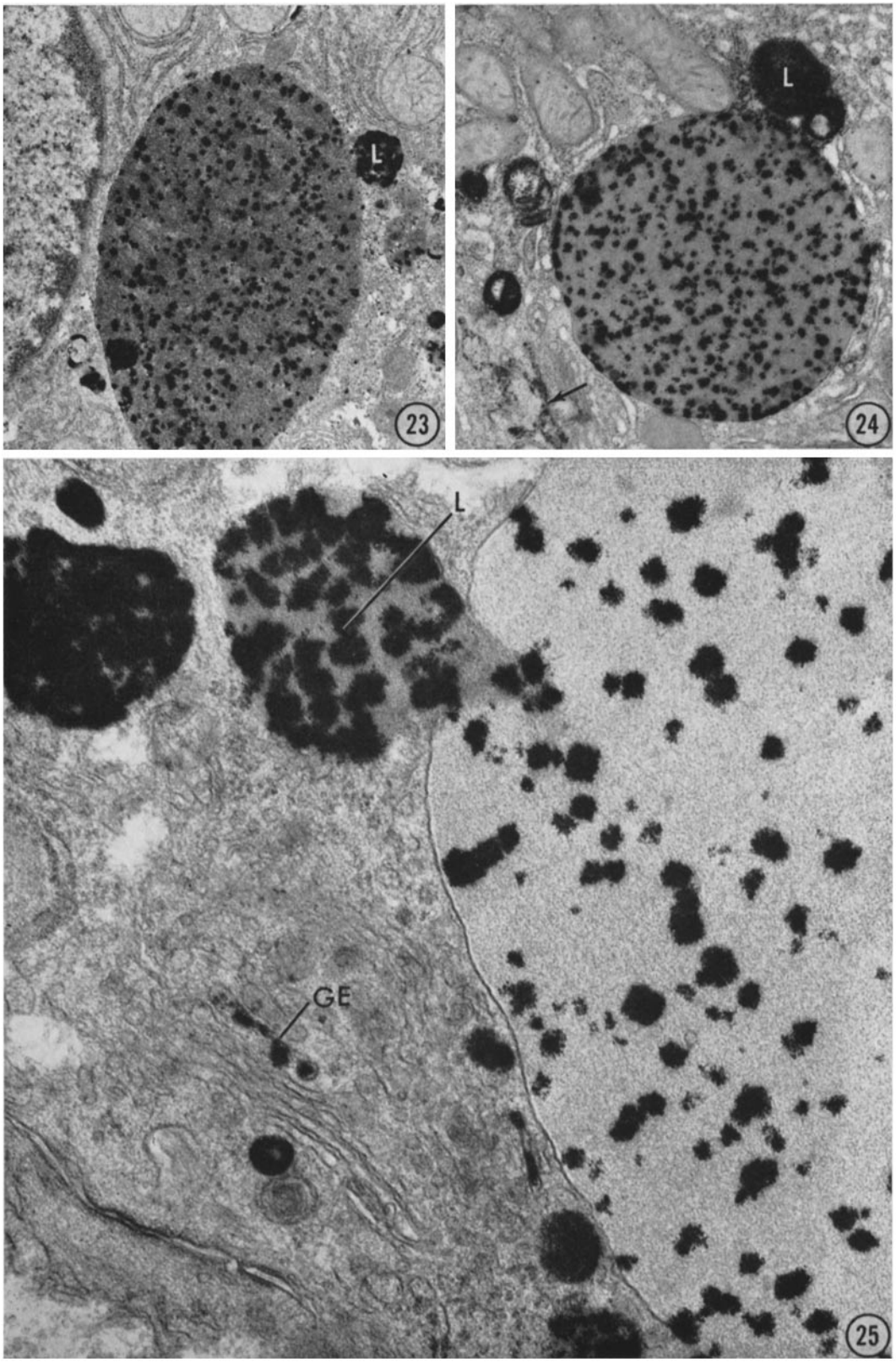
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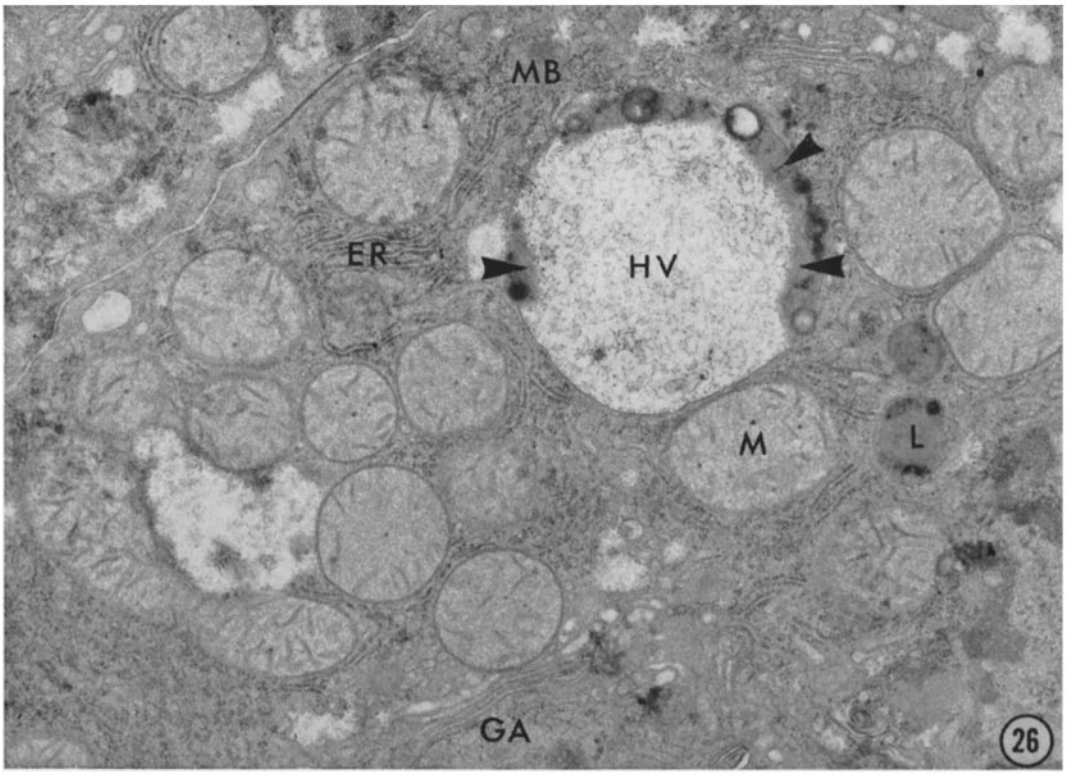
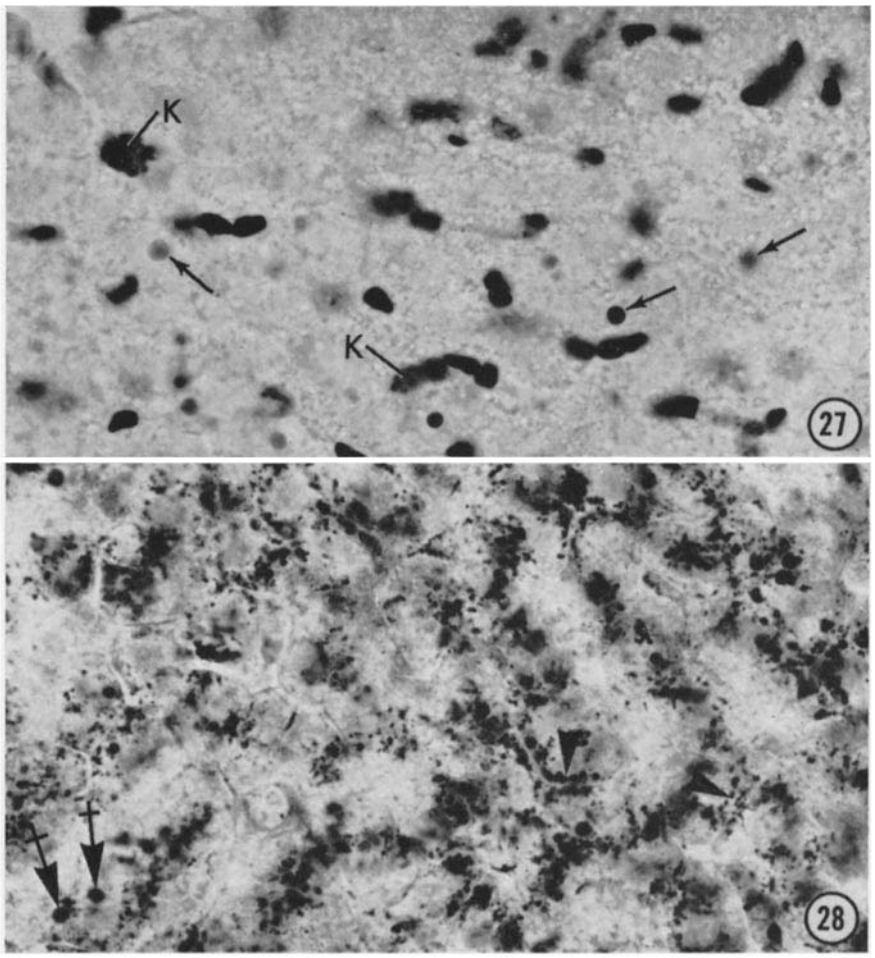


FIGURE 26 Rat liver 8 min after injection of bovine hemoglobin (400 mg/100 g). Section not incubated. Thin section stained with uranium and lead. At the periphery (arrows) of the hemoglobin vacuole (*HV*) densities and "vacuoles" are present that strongly resemble the contents of dense bodies (*L*); also see Fig. 21. Mitochondria (*M*), microbody (*MB*), endoplasmic reticulum (*ER*), and Golgi apparatus (*GA*) are unaltered. × 17,000.

FIGURES 23 and 24 Rat liver after injection of bovine hemoglobin. Fig. 23, 30 min after injection of bovine hemoglobin (400 mg/100 g); Fig. 24, 16 hr after injection of bovine hemoglobin (400 mg/100 g). Both specimens doubly incubated: 15 min in CMP medium followed by 90 min in DAB. Thin sections stained with lead. The large droplets have a uniform, light reaction product for hemoglobin activity and dark, irregular deposits of acid phosphatase reaction product. The hemoglobin droplets have been transformed into digestive vacuoles, probably by fusion with dense bodies (*L*). In Fig. 24, acid phosphatase reaction product is also seen in saccular structures (arrow), which are probably part of Golgi-associated smooth-surfaced endoplasmic reticulum (GERL) (Novikoff, 1967). (Also seen in Fig. 25). Fig. 23, × 17,000; Fig. 24, × 18,000.

FIGURE 25 Rat liver 16 min after injection of bovine hemoglobin, 400 mg/100 g. Incubated for 25 min in CMP medium and for 40 min in DAB. Tissue stained with uranyl acetate in block. Thin section stained with lead. This image suggests that the contents of the small lysosome (*L*) is being released into the hemoglobin vacuole. Acid phosphatase reaction product is seen in a saccular structure, which is probably part of Golgi-associated smooth-surfaced endoplasmic reticulum (*GE*). × 41,000.



FIGURES 27 and 28 Rat liver 16 hr after injection of bovine hemoglobin (400 mg/100 g). Stained to visualize hemoglobin (Fig. 27) and lysosomes (Fig. 28). Fig. 27 shows a frozen section incubated for 30 min in the DAB medium. Hemoglobin has almost disappeared from parenchymal cells, and only a few hemoglobin vacuoles may be seen in hepatocytes at this time (arrows). Darkly staining hemoglobin droplets are present in the Kupffer cells (*K*). × 500. Fig. 28 illustrates frozen section incubated for 30 min for acid phosphatase activity. Some large lysosomes are still seen (crossed arrows). However, a return to pericanalicular arrays is evident (arrows). × 500.

oles were also formed when the hemolysis was induced with the injection of distilled water. This last group of animals recovered promptly from anesthesia and showed no overt signs of shock.

Our experimental conditions are comparable to pathologic situations in which the levels of circulating hemoglobin are in excess of available haptoglobin. Preliminary studies of human liver specimens from patients who had received multiple transfusions indicate that hemoglobin is also taken up by human hepatocytes, and suggest that the incorporation of hemoglobin into lysosomes and the accumulation or iron residues in these organelles may result in hepatic hemosiderosis. The common occurrence of hemosiderosis in individuals with chronic hemolytic anemias is consistent with this hypothesis.

The fate of the porphyrin moiety of the hemoglobin molecule cannot be traced by staining. The work of Ostrow, Hammaker, and Schmid

(1962) shows that the major portion of labeled, injected hemoglobin is secreted as bile pigment. Presumably porphyrin leaves the lysosomes of hepatocytes and Kupffer cells. How this occurs and in what form is not known; nor do we know the role, if any, of lysosomal enzymes in the conversion of hemoglobin to bilirubin.

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