THE FINE STRUCTURAL LOCALIZATION OF

ENDOGENOUS AND EXOGENOUS PEROXIDASE

ACTIVITY IN KUPFFER CELLS OF RAT LIVER

H. DARIUSH FAHIMI

From the Harvard Pathology Unit, Mallory Institute of Pathology, Boston City Hospital, Boston, Massachusetts 02118

ABSTRACT

Endogenous peroxidase activity has been demonstrated in sections of rat liver fixed briefly by glutaraldehyde perfusion and incubated in Graham and Karnovsky's medium for cytochemical demonstration of peroxidase activity (29). In 25–40% of sinusoidal cells, an electron-opaque reaction product is localized in segments of the endoplasmic reticulum, including the perinuclear cisternae, a few Golgi vesicles and saccules and in some large membrane-bounded granules. This staining is abolished after prolonged fixation or boiling of tissue sections in glutaraldehyde, and in the absence of H_2O_2 or DAB from the incubation medium. Furthermore, the reaction is inhibited completely by sodium azide and high concentrations of H_2O_2, and partially by KCN and aminotriazole. Among the different cells in hepatic sinusoids, the nonphagocytic "fat-storing" cells (39) are always peroxidase negative, whereas the lining cells in process of erythrophagocytosis are consistently peroxidase positive. The possible biological significance of endogenous peroxidase in Kupffer cells is discussed. In addition, the uptake of exogenous horseradish peroxidase by Kupffer cells has been investigated. The exogenous tracer protein, which in contrast to endogenous peroxidase of Kupffer cells is not inhibited by prolonged aldehyde fixation, is taken up by micropinocytosis and remains confined to the lysosomal system of Kupffer cells. The significance of these observations in respect to some recent studies suggesting localization of exogenous peroxidases in the endoplasmic reticulum of Kupffer cells and peritoneal macrophages (22, 23) is briefly discussed.

INTRODUCTION

The hepatic reticuloendothelial cells (Kupffer cells)[1] constitute the largest group of fixed macrophages in mammalian organism and are responsible for the phagocytosis of most circulating foreign particles including living microorganisms (1–5). In several recent studies the fine structure of Kupffer cells in normal animals and after reticulo-endothelial stimulation, as well as the morphologic changes accompanying phagocytosis of foreign particles by these cells, have been in-

[1] The following abbreviations have been used in this paper: DAB, 3,3′ diamino-benzidine tetrahydrochloride; Tris, Tris-hydroxy-amino-methane; ER, endoplasmic reticulum; HRP, horseradish peroxidase; RES, reticulo-endothelial system. Because of the general agreement that all hepatic sinusoidal lining cells are capable of phagocytosis and are probably the functional variants of the same single cell types (6, 7, 24, 25), the terms sinusoidal lining cells and Kupffer cells are used interchangeably in this report.

vestigated (6–10). Cytochemical studies on Kupffer cells have dealt mostly with the localization of acid phosphatase, in Kupffer cell lysosomes (11–15). Wachstein and Meisel in their light microscopic histochemical survey of peroxidase localization in mammalain tissues noted a positive peroxidase reaction in Kupffer cells of rats, mice, rabbits, and guinea pigs (16), and recently Novikoff et al. (17) mentioned briefly the localization of peroxidase in the endoplasmic reticulum and lysosomes of rat Kupffer cells. In the course of recent studies on localization of exogenous and endogenous peroxidase activity in rat liver (18–21), we noted a consistently positive endogenous peroxidase reaction in some of the sinusoidal lining cells. These observations, which will be presented in detail in this paper, indicate that, in some sinusoidal lining cells of rat liver, endogenous peroxidase activity is localized in the cisternae of the endoplasmic reticulum (ER) including the nuclear envelope, a few Golgi saccules and vesicles, and in some larger granules.

In view of recent reports (22, 23) on the uptake of exogenous peroxidases by Kupffer cells and peritoneal macrophages, and the suggestion that such exogenous tracer proteins may be localized in the endoplasmic reticulum and the nuclear envelope, the results of our experiments on the uptake of horseradish peroxidase by rat Kupffer cells will also be described. These findings indicate that the exogenous peroxidase is taken up by micropinocytosis and remains confined to the lysosomal system of Kupffer cells.

MATERIALS AND METHODS

Endogenous Peroxidase Activity in Kupffer Cells

Normal male and female adult albino rats (Charles River Strain) weighing 200–250 g and fasted for 24 hr were used. The liver was fixed by perfusion through the portal vein (21, 26) with 1.25% distilled glutaraldehyde (27) in 0.15 M cacodylate buffer, pH 7.3, followed by brief immersion for 20 min in the same fixative at room temperature. After several changes in the same buffer, 50-μ thick sections were cut on a Sorvall TC-2 tissue chopper (28) (Ivan Sorvall Inc., Norwalk, Conn.), collected in 0.1 M Tris-HCl buffer, pH 7.6, and incubated in an incubation medium containing 10 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis,

Mo.) in 10 ml of 0.1 M Tris-HCl buffer, pH 7.6, and 0.02% H_2O_2 (29). In other experiments the pH of the incubation medium was varied from 7.1 to 9.0. The incubation was carried out at room temperature for 30–60 min, followed by postfixation for 60 min in 2% osmium tetroxide in distilled water (30), and dehydration in graded ethanol solutions and embedding in Epon 812 (31). 1 μ thick sections were examined by light microscopy without counterstain. Thin sections for electron microscopy were cut on LKB ultratome III (LKB Instruments Inc., Rockville, Md.) and examined either unstained or lightly counterstained with lead citrate (32), in a Phillips EM200 electron microscope.

Controls for Endogenous Peroxidase Activity in Kupffer Cells

(a) Chopped sections were incubated in the absence of DAB or H_2O_2 from the incubation medium. (b) Inhibitors: chopped sections were first treated for 10 min at room temperature in 0.1 M Tris buffer, pH 7.6, containing 10^{-3}–10^{-1} M KCN, 10^{-5}–10^{-3} M sodium azide, 10^{-3}–10^{-1} M 3-amino-1:2:4-triazole (Mann Research Biochemical Co., New York), and 2% H_2O_2 and then were incubated for 60 min in the complete medium containing the same concentrations of these inhibitors. (c) Sections were boiled for 5 min in 1.25% glutaraldehyde fixative and then incubated for 60 min in the full incubation medium. After the incubation, all control sections were postosmicated and treated like regularly incubated sections.

Studies on the Uptake of HRP by Kupffer Cells

Normal male adult rats of Wistar-Furth strain (Microbiological Associates, Bethesda, Md.) (33), weighing 200–250 g, and fasted for 24 hr, were injected with 10 mg of horseradish peroxidase (type II, Sigma Chemical Co.) per 100 g body weight. Animals were sacrificed at different time intervals, ranging from 1 min to 24 hr following the injection of tracer, and 0.5 mm thick slices of liver were fixed for 3–5 hr at room temperature by immersion in a modified Karnovsky's fixative (34) containing 4% formaldehyde prepared from paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. After an overnight wash in 0.15 M cacodylate buffer, pH 7.3, 50-μ thick sections were chopped and incubated for 60 min in the same regular incubation medium for peroxidase (29), followed by postosmication, dehydration, and embedding in Epon 812, as described above.

RESULTS

Endogenous Peroxidase Activity in Kupffer Cells

DISTRIBUTION OF STAINING: Examination by light microscopy of 1 μ Epon sections revealed diffuse gray staining in the cytoplasm of some of the cells lining the hepatic sinusoids. In addition, a few brown granules were observed in the cytoplasm of some Kupffer cells. The nuclei did not stain. Approximately 25–40% of all sinusoidal lining cells reacted positively. The staining was present in both male and female rats. In addition, staining was noted in red blood cells, in white cell and mast cell granules, and in microbodies of some hepatocytes on the surface of tissue blocks.

By electron microscopy the positively reacted Kupffer cells exhibited an electron-opaque reaction product in the cisternae of the endoplasmic reticulum including the nuclear envelope (Figs. 1–10). All segments of the rough ER stained positively, but some smooth-surfaced tubular structures remained unstained (Fig. 2). In addition, reaction product was noted in some outer cisternae, and in a few small vesicles (0.05 μ in diameter) in the periphery of the Golgi complex (Figs. 3 and 4). The intensity and the extent of the staining reaction in the Golgi region varied markedly from cell to cell and even from one Golgi complex to the other in the same cell (Fig. 3). In general, however, the staining along the concave (inner) face of the Golgi complex appeared stronger and was seen more consistently than along the convex (outer) face (Fig. 4). Furthermore, electron-opaque reaction product was noted in a few large (0.1–0.3 μ in diameter), membrane-bounded granules (Figs. 1, 2, 7). The membrane around these granules appeared accentuated because of a region of low electron opacity immediately subadjacent to it (Fig. 2). Electron-opaque deposits were also observed within the lysosomes and phagosomes of many Kupffer cells (Fig. 2). However, because of inherent electron opacity of such lysosomal deposits, the exact relationship of their staining to the peroxidase reaction in the ER could not be clearly elucidated. Similar electron-opaque deposits have also been noted in some peribiliary dense bodies of hepatocytes (19, 21).

The presence of staining in the ER did not seem to correlate with the size or the degree of maturation of individual Kupffer cells. Thus, a positive peroxidase reaction was noted in small Kupffer cells with only a few cytoplasmic organelles (Figs. 5 and 8), as well as in large, hypertrophic Kupffer cells containing many phagolysosomes, mitochondria, and vermiform tubular invaginations (or the so-called "micropinocytosis vermiformis") (35, 36) (Figs. 1 and 6). The latter structures have been described in normal as well as in stimulated Kupffer cells (10, 35–37).

Whereas the peroxidase reaction in the so-called "fat storing cells" (lipocytes) (38, 39) of hepatic sinusoids was always negative (Fig. 5), a positive reaction was noted in those Kupffer cells which appeared in the process of erythrophagocytosis (Figs. 8–10). This latter group, however, consisted of only 5% or less of all sinusoidal lining cells in our animals, whereas the peroxidase reaction was noted in 25–40% of all sinusoidal cells. Figs. 8–10 illustrate three such Kupffer cells at different stages of erythrophagocytosis, all of which show a positive peroxidase reaction in the ER and the nuclear envelope. The prominent staining of red blood cells with DAB is due to the peroxidatic activity of hemoglobin (40).

TECHNICAL CONSIDERATIONS: Concerning fixation, best staining results were obtained with the material fixed by perfusion with distilled glutaraldehyde followed by brief immersion for 20 min in the same fixative. Fixation for longer periods and use of impure commercial preparations of glutaraldehyde resulted in weak and inconsistent staining. Fixation by immersion for 3–5 hr in glutaraldehyde-formaldehyde mixture (34), as used in studies with exogenous horseradish peroxidase, inhibited the staining of endogenous peroxidase in Kupffer cells. The endogenous peroxidases in rat uterus (41) and colon (42) have also been noted to be highly sensitive to prolonged aldehyde fixation.

As far as the variables in the staining reaction were concerned, by raising the pH of the incubation medium from 7.1 to 9 the intensity of the staining in Kupffer cells did not change appreciably. At lower pH values (7.1), however, the background staining of the tissue with DAB appeared quite strong. Best staining results were obtained by incubation for 60 min at 22°C, and at pH 7.6.

CONTROLS FOR CHARACTERIZATION OF THE ENDOGENOUS PEROXIDASE IN KUPFFER CELLS: The results of control experiments are recorded in Table I. The lack of staining in the absence of DAB or H_2O_2 from the incubation

SINUSOID

MV

G
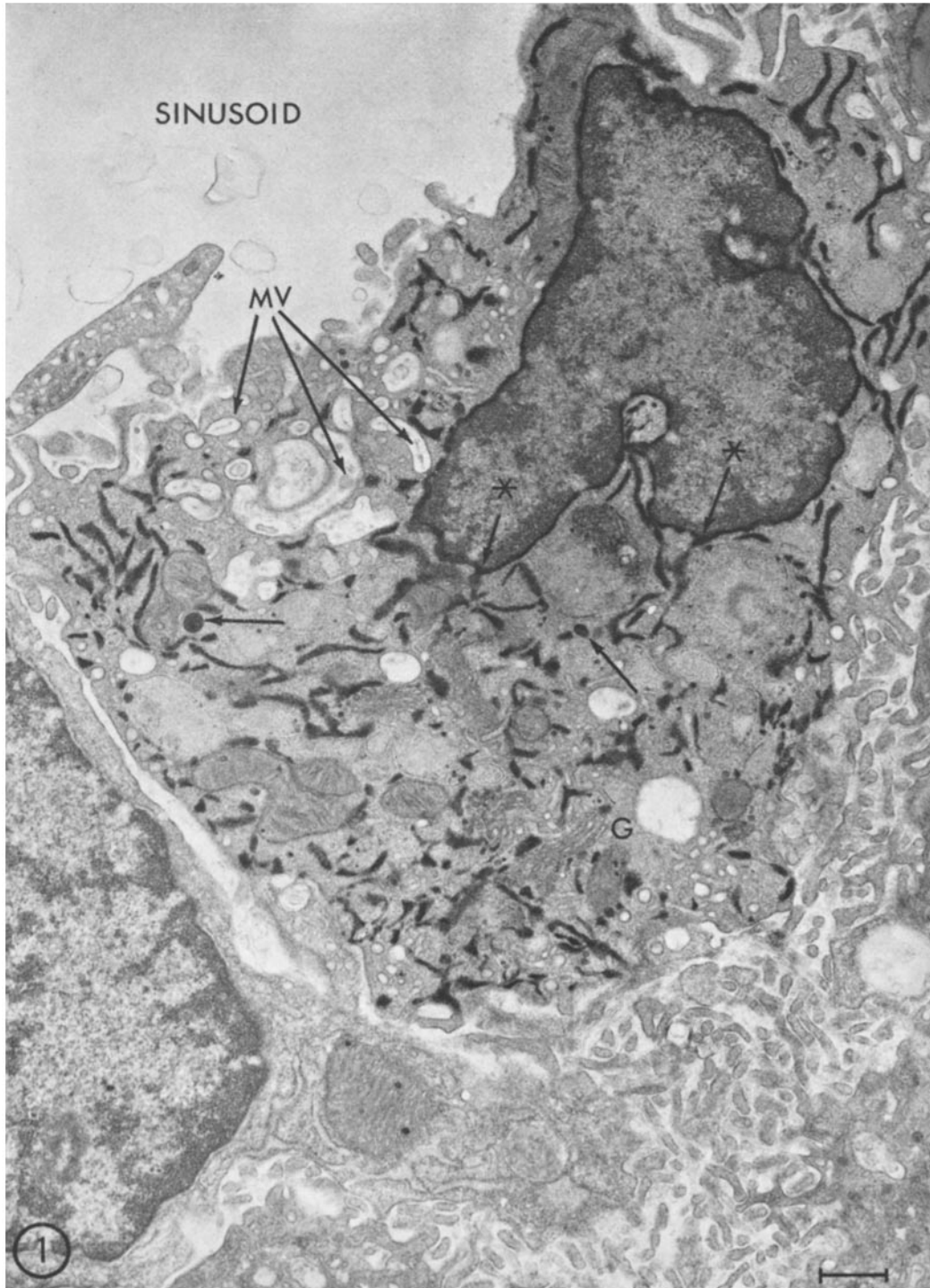
All figures, except Fig. 3, are from sections counterstained with lead citrate (32). Length of scale line for all figures is 1 μ.

FIGURE 1   This figure illustrates the general distribution of the peroxidase staining in a large Kupffer cell. The reaction product is localized in the perinuclear cisternae and other segments of the endoplasmic reticulum, and in a few membrane-bounded granules (arrows). There are several vermiform tubular invaginations (micropinocytosis vermiformis, [35, 36]) (MV) facing the sinusoidal lumen. Points of continuity between the ER and the nuclear envelope are demonstrated at the asterisks. Another sinusoidal cell at the lower left has remained negative and does not contain reaction product in the perinuclear cisternae and ER. G, Golgi complex. × 9600.

FIGURE 2   This high-power electron micrograph of a portion of a Kupffer cell illustrates the localization of peroxidase in the cisternae of the granular endoplasmic reticulum. In addition, there are two granules (arrows) with a distinct limiting membrane which also show a positive reaction. A larger phagolysosome (L) contains electron-opaque deposits, but due to inherent electron opacity of such deposits the exact relationship of this reaction to the peroxidase staining in the ER could not be elucidated. The segments of the ER in the hepatocyte (H) do not show any evidence of staining. × 30,000.
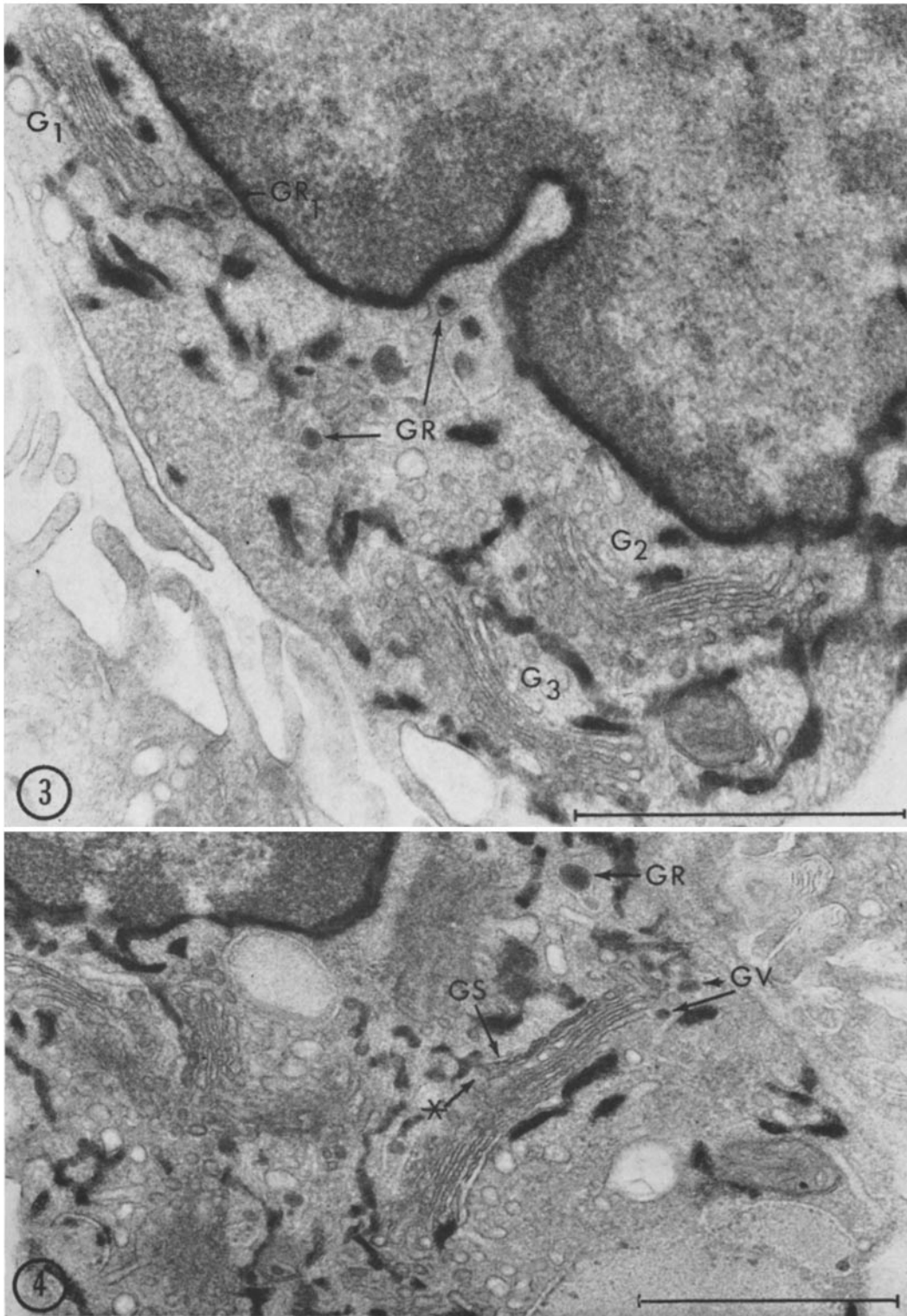
TABLE I

Controls for Kupffer Cell Peroxidase

	Staining in Kupffer cells*
1. Regular incubation	4+
2. −DAB	0
3. −H_2O_2	0
4. KCN (10^{-3} M)	4+
5. KCN (10^{-1} M)	1+
6. NaN_3 (10^{-5} M)	4+
7. NaN_3 (10^{-3} M)	0
8. Aminotriazole (10^{-3} M)	4+
9. Aminotriazole (10^{-1} M)	1+
10. 2% H_2O_2	0
11. Boiling	0

* Intensity of reaction in Kupffer cells arbitrarily graded 0 to 4+.

medium and the inhibition of the reaction by boiling of sections for 5 min strongly suggest that the reaction is enzymatic in nature and that the enzyme is most probably a peroxidase. The sensitivity of the reaction to azide and excess concentrations of H_2O_2 is also in agreement with this notion (43). Furthermore, the persistence of staining in the presence of high concentrations of KCN, even though somewhat unusual for peroxidases (43), resembles the results obtained for peroxidase in guinea pig eosinophil granules (44), and in rat colon (42). The lack of complete inhibition of staining by aminotriazole (AT), which is an inhibitor of hepatic catalase (45, 46), would suggest that the staining in Kupffer cells is probably due to a peroxidase rather than catalase. This agent (AT), however, has also been reported to inhibit peroxidase activity in thyroid (47), but not in eosinophils (44) and colon (42).

Uptake of Horseradish Peroxidase by Kupffer Cells

Horseradish peroxidase was localized in hepatic sinusoids 60 sec after the intravenous injection,

and it appeared to be taken up by Kupffer cells in micropinocytotic vesicles. At 10 min numerous phagosomes and multivesicular bodies containing reaction product were present in Kupffer cells (Fig. 11). The intensity of the peroxidase reaction in hepatic sinusoids decreased gradually after 6–24 hr following the injection, but staining in numerous phagolysosomes and multivesicular bodies of Kupffer cells persisted. It must be emphasized that in all these experiments a positive peroxidase reaction was never observed in the endoplasmic reticulum or the perinuclear cisternae of the Kupffer cells.

DISCUSSION

The findings presented in this report indicate that 25–40% of all sinusoidal lining cells in rat liver have cytochemically demonstrable peroxidase activity. Fine structurally, the reaction product is localized in the endoplasmic reticulum, including the perinuclear cisternae, a few Golgi cisternae and vesicles, and in some larger, membrane-bounded granules. These findings confirm the light microscopic histochemical observations of Wachstein and Meisel (16) and are also in agreement with the findings mentioned by Novikoff et al. on localization of peroxidase in a variety of cell types including Kupffer cells (17). Furthermore, a somewhat similar fine structural pattern of localization of peroxidase has been observed in maturing leukocytes (51–53), epithelial cells of rat uterus (41), colon (42), follicular cells of rat thyroid (47), and recently in peritoneal macrophages of guinea pigs[2] (48). This latter finding is of great interest since both peritoneal macrophages and Kupffer cells represent different forms of tissue macrophages and are functionally closely related (49).

---

[2] R. S. Coltran and M. Litt. 1970. Ultrastructural localization of endogenous and exogenous peroxidase activity in guinea pig peritoneal macrophages. *J. Immunol.* 1970. In press.

FIGURES 3–4   Figs. 3 and 4 illustrate the localization of peroxidase in the Golgi region. Staining in the Golgi cisternae on the convex (outer) surface was less frequent and less intense (G_1, Fig. 3; *GS*, Fig. 4), than along the concave (inner) surface (G_1, G_2, G_3 in Fig. 3; Fig. 4). The asterisk in Fig. 4 shows a point of continuity between a segment of ER and a Golgi cisterna. A few small vesicles in the periphery of the Golgi apparatus also show evidence of staining (*GV*, Fig. 4). Reaction product is also noted in larger, membrane-bounded granules (*GR* in both figures). In Fig. 3, one such granule (*GR1*) seems to arise from the periphery of the Golgi complex (G_1). Fig. 3, × 50,000; Fig. 4, × 39,000.
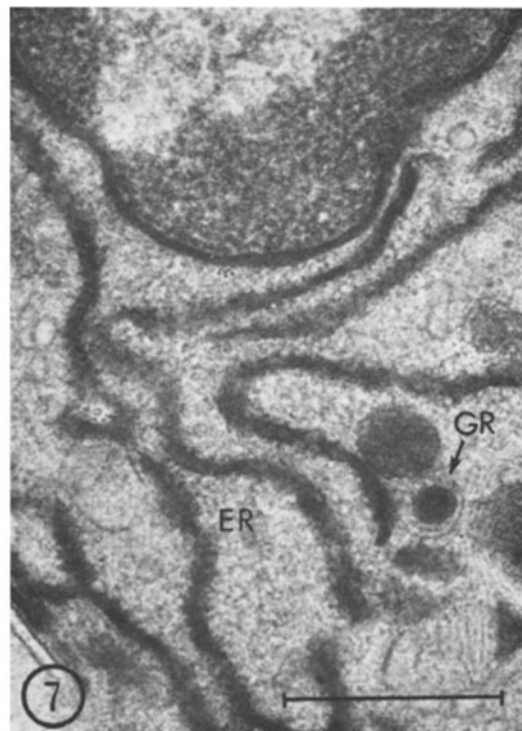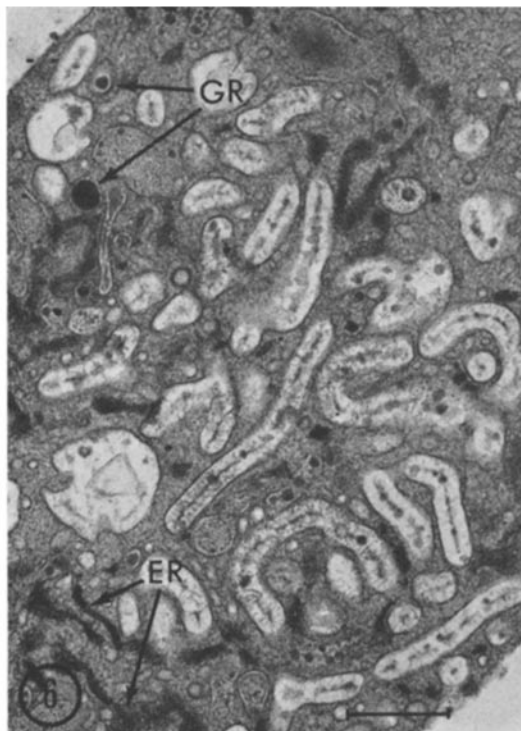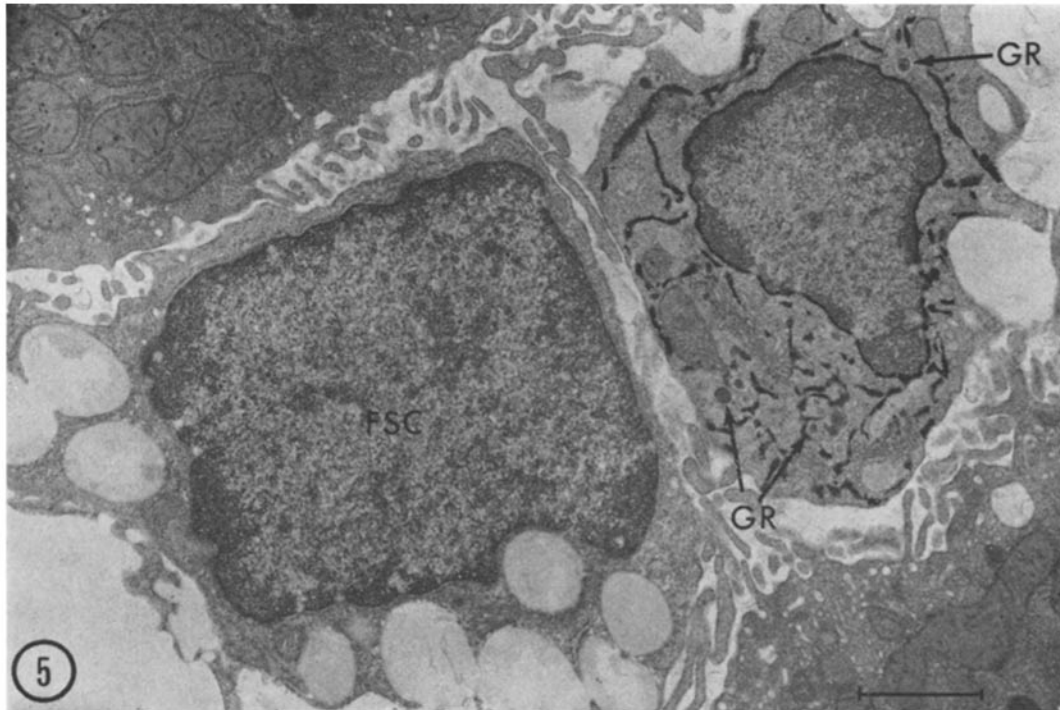
Possible Functional Significance of Peroxidase in Kupffer Cells

The exact function of peroxidase activity in rat Kupffer cells remains obscure but the pattern of localization in the cisternae of the endoplasmic reticulum, parts of the Golgi apparatus, and in a few membrane-bounded granules is reminiscent of similar patterns seen in hematopoetic cells involved in synthesis of peroxidase. Thus, promyelocytes and myelocytes of eosinophils and neutrophils from the bone marrow of several mammalian species have been shown to contain peroxidase in the cisternae of ER, including the nuclear envelope and the Golgi complex (50–52). These cytochemical observations corroborate essentially the original studies of Palade and co-workers, which established that secretory proteins are synthesized on ribosomes, transferred to the cisternae of ER and are concentrated and packaged within the elements of the Golgi apparatus (53, 54). The discharge of granule-associated peroxidase (44, 55) and other lysosomal enzymes into the phagocytic vacuoles has been likened to the process of secretion of zymogen granules in pancreas (56). Whether peroxidase in Kupffer cells is destined for secretion can not be answered at this time, since we have not seen any clear evidence of discharge of peroxidase into phagocytic vacuoles or into the extracellular space. However, this may be due to paucity of such granules in normal, unstimulated animals. Recent studies of Rouiller and co-workers (6, 7) indicate that after reticuloendothelial system stimulation the number of granules and vacuoles in the cytoplasm of Kupffer cells increases markedly. Thus, cytochemical studies following the reticulo-endothelial system (RES) stimulation may be helpful in demonstrating the fate of granule-associated peroxidase in Kupffer

cells. Such an observation would support the hypothesis that Kupffer cell peroxidase has probably a somewhat similar function as the neutrophil peroxidase. Recent studies of Klebanoff and others (57, 59) indicate that myeloperoxidase (and also salivary gland and lacto-peroxidase), in association with H_2O_2 and a halide (or thiocyanate), exerts a strong bactericidal, virucidal, and fungicidal activity. The role of Kupffer cells in clearing living microorganisms from the blood is well known, and thus one may speculate that Kupffer cell peroxidase participates in a bactericidal mechanism like other peroxidases in neutrophils, milk, and saliva.

Another possible explanation for the localization of peroxidase activity in the ER of Kupffer cells could be that peroxidase is not synthesized in ER for secretion but is rather associated with the ER, like other microsomal enzymes. Indeed, there is a heme protein, the cytochrome P-450, which has been most extensively studied in microsomal fractions derived from liver and which plays an important role in microsomal electron-transport functions (60, 61). It is conceivable that this enzyme, like several other heme proteins, could oxidize DAB (62) and thus be responsible for the staining of ER in Kupffer cells. Such an explanation, however, is unlikely, since combined biochemical and fine structural studies of Ernster and Orrenius (63) and others (64) strongly suggest that a close association exists between the amount of cytochrome P-450 and the degree of differentiation and complexity of ER in hepatocytes rather than in Kupffer cells. In our material, however, no concomitant staining of the endoplasmic reticulum in liver cells has been observed (Fig. 2), thus indicating that the peroxidase staining of ER in Kupffer cells is probably not related to the microsomal heme proteins of hepatocytes. Similar attempts by Brökelmann and Fawcett (41) to local-

---

FIGURE 5 This figure illustrates positive peroxidase reaction in a small Kupffer cell and negative reaction in an adjacent "fat-storing cell" (*FSC*) (38, 39). All such fat-storing cells in hepatic sinusoids were consistently peroxidase negative. In the positively reacted cell, there is staining in the perinuclear cisternae ER, and in a few, large, membrane-bounded granules (*GR*). × 16,000.

FIGURE 6 Portion of the cytoplasm of a large, hypertrophic Kupffer cell with many vermiform tubular invaginations (so-called "micropinocytosis vermiformis" [35, 36]). There is positive peroxidase reaction in a few cisternae of the ER (*ER*), and in some large, membrane-bounded granules (*GR*). × 13,000.

FIGURE 7 This figure illustrates at higher magnification peroxidase localization in perinuclear cisternae, granular *ER*, and in a membrane-bounded granule (*GR*). × 28,000.
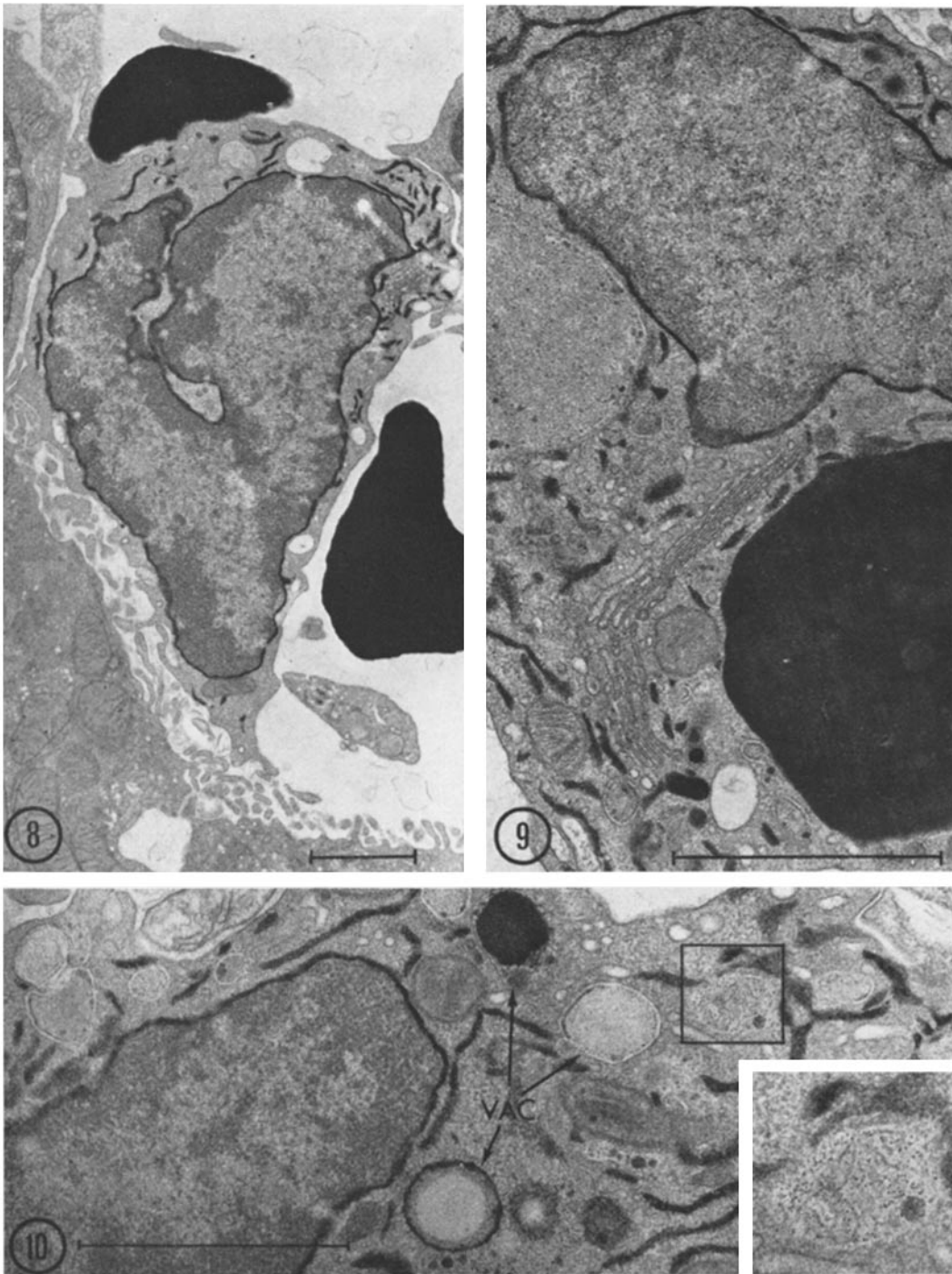
FIGURE 8–10 Figs. 8–10 illustrate positive peroxidase reaction in Kupffer cells in process of erythrophago-
cytosis. In Fig. 8, the red cell has just become attached to the cell surface of the Kupffer cell and is be-
coming engulfed by the pseudopods of the latter, whereas in Fig. 9, the red cell is lodged in a large vacuole
in the cytoplasm of the Kupffer cell. In Fig. 10, only several large vacuoles (*VAC*) with remnants of red
cells are left. One such vacuole (enlarged in the *inset*) contains very fine, electron-opague granules of ferri-
tin. Note the positive peroxidase reaction in the ER of all three cells in Figs. 8–10. All Kupffer cells in the
process of erythrophagocytosis (about 5% of all sinusoidal cells in our animals) gave a positive peroxidase
reaction in the ER. The strong staining of red cells with DAB is due to peroxidatic activity of hemoglobin
(40). Fig. 8, × 14,000; Fig. 9, × 39,000; Fig. 10, × 39,000; *inset*, × 78,000.

ize cytochrome P-450 with the DAB reaction have not been successful. Since Kupffer cells can be isolated from liver (65, 66), further biochemical studies on microsomal fractions derived from such isolated Kupffer cells may clarify the relationship of Kupffer cell peroxidase to cytochrome P-450.

Recently Brökelmann and Fawcett described the fine structural localization of a peroxidase in the epithelium of rat uterus (41) and suggested that this peroxidase probably participates in binding of estrogen-hormones (67). Even though the distribution of peroxidase in Kupffer cells resembles that of peroxidase in rat uterus, some basic differences exist between the two enzymes: Kupffer cell peroxidase is observed in both male and female rats and is not inhibited by high concentrations (10^{-1} M) of KCN. The rat uterine peroxidase, in contrast, is dependent on estrogens and disappears from the epithelial cells of uterus after ovariectomy and is sensitive to KCN (41).

The Relationship of Kupffer Cells to Other Sinusoidal Cells

The question, "whether all sinusoidal cells of the liver are of a single type, which is found in various phases of physiological activity," is an old one, and most recent investigators tend to answer this question affirmatively (2, 6, 11, 24, 25). Marshall (68), on the other hand, on the basis of silver impregnation studies, divided the sinusoidal cells into two separate types: the metallophilic cells which comprise most of the phagocytic Kupffer cells, and nonmetallophilic endothelial lining cells. The results presented here indicate that only 25–40% of all sinusoidal lining cells in the liver of normal rats exhibit a positive peroxidase reaction. Furthermore, it is noted that all so-called "fat storing cells" (lipocytes) (38, 39) of hepatic sinusoids give a negative peroxidase reaction (Fig. 5), whereas all Kupffer cells in process of erythrophagocytosis are consistently peroxidase positive (Figs. 8–10). Even though the function of "fat storing cells" is not yet known, it is generally agreed that they do not participate in phagocytosis of foreign particles (39). On the other hand, the positive peroxidase reaction in Kupffer cells active in erythrophagocytosis would suggest a possible participation of peroxidase in the process of breakdown of red blood cells. Indeed, it has been shown that lipid peroxidation of red cell membrane causes hemolysis (69). Furthermore,

the initial step of hemoglobin breakdown in the synthesis of bile pigments involves a poorly understood "oxidative step" at the α-bridge of the hemoglobin molecule (70). Since the Kupffer cells are the most important site of catabolism of hemoglobin (71) the role of Kupffer cell peroxidase in the catabolism of hemoglobin deserves further investigation. It should be emphasized, however, that the staining in the ER of Kupffer cells can not be due to peroxidatic activity of red cell hemoglobin following erythrophagocytosis because (a) ER staining for peroxidase can be inhibited by prolonged fixation (3–5 hr) in glutaraldehyde/formaldehyde fixative which does not affect the red cell staining, and (b) conversely, KCN (at 10^{-2} M) inhibits the red cell peroxidase but does not affect the ER staining. Furthermore, the majority of cells exhibiting a positive peroxidase reaction in ER do not show any evidence of erythrophagocytosis.

Uptake of Exogenous Peroxidase by Kupffer Cells

The most important function of Kupffer cells seems to be related to the marked phagocytic capacity of these cells. Fine structural studies have demonstrated the uptake of colloidal mercuric sulfide, and Thorotrast (72), colloidal iron (73), polystyrene latex particles (74), hemoglobin (40), and bacteria (9, 10). Straus, who introduced horseradish peroxidase as a tracer in microscopy (75), indicated the uptake of this tracer by sinusoidal lining cells of the liver (14, 15). In a recent electron microscope study on the uptake of peroxidase tracers by mouse liver, Graham et al. (22) confirmed the uptake of peroxidases by Kupffer cells, but in addition reported that "in occasional Kupffer cells, reaction product was seen in the cisternae of the rough ER and within the perinuclear space." Even though originally the biological significance of this observation remained unexplained (22), in a subsequent publication Catanzaro, Graham, and Schwartz (23) reported the localization of exogenous horseradish peroxidase in the rough ER and the perinuclear space of guinea pig peritoneal macrophages, and interpreted the previous observation in liver as indicating that "in mouse Kupffer cells the exogenous protein, lactoperoxidase, enters the perinuclear space and the rough surfaced ER as well as lysosomes."
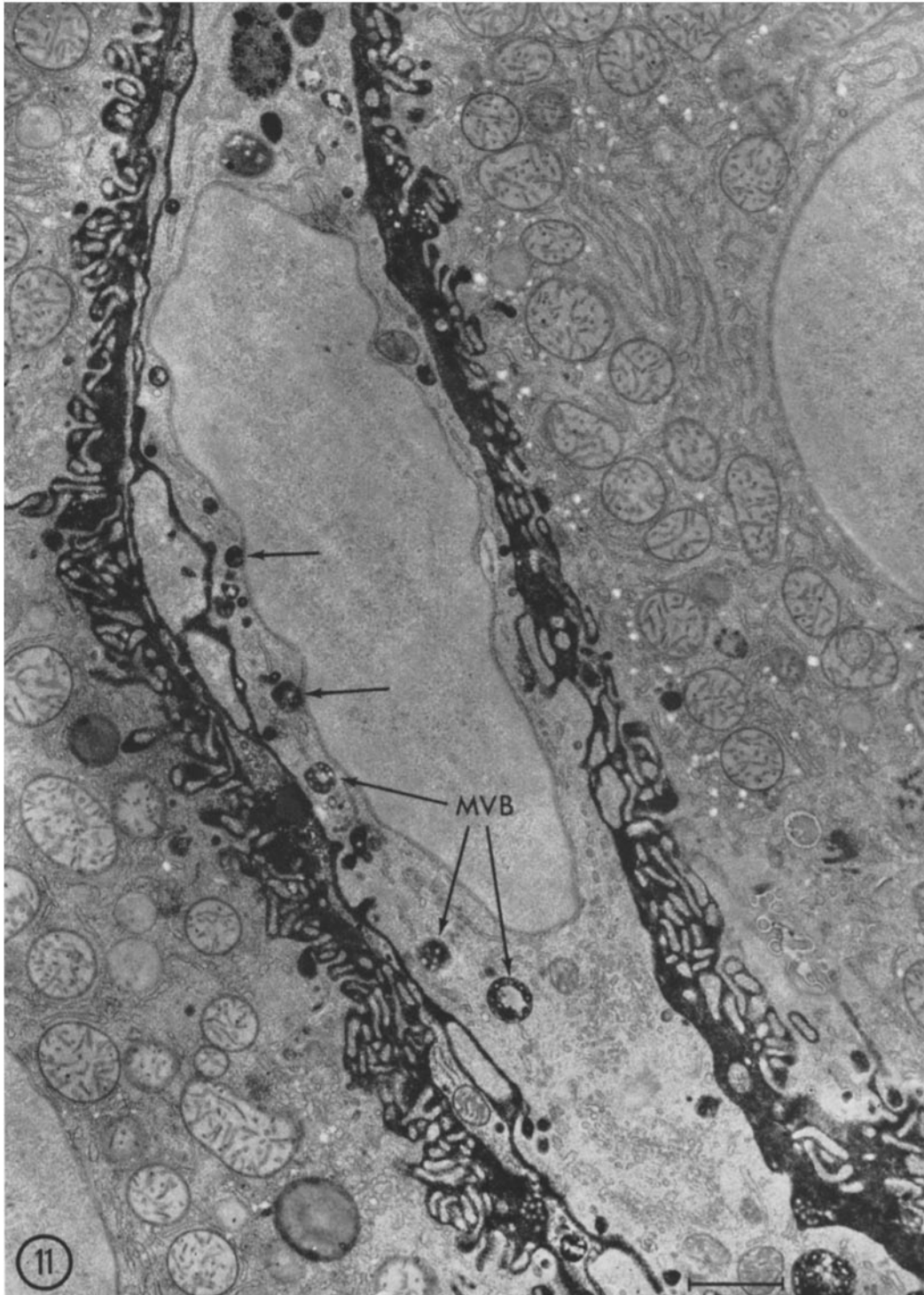
The observations reported in this paper indicate

FIGURE 11   This figure illustrates the localization of exogenous horseradish peroxidase in a Kupffer cell of rat liver, 10 min after intravenous injection of the tracer. This material was fixed for 5 hr, at room temperature, in a mixture of glutaraldehyde and formaldehyde, and thus endogenous peroxidase in the ER and the perinuclear cisternae of Kupffer cells is inhibited, but the exogenous horseradish peroxidase survives and is localized in sinusoids and in numerous micropinocytotic vesicles and multivesicular bodies (MVB) in Kupffer cells. Two vesicles containing peroxidase appear in the vicinity of the nuclear envelope of the Kupffer cell (arrows), but there is no evidence of discharge of their contents into this space and no evidence of staining in the perinuclear cisternae. × 14,000.

that endogenous peroxidase activity is demonstrable in the cisternae of ER and the nuclear envelope of 25–40% of rat Kupffer cells. This endogenous peroxidase, which was originally noted by Wachstein and Meisel (16) and which is similar to endogenous peroxidases of peritoneal macrophages (49), uterus (41), thyroid (47), and colon (42), is quite sensitive to fixation with aldehydes, and is inhibited by fixation for 3–5 hr at room temperature in a glutaraldehyde/formaldehyde mixture. In contrast, exogenous horseradish peroxidase is not inhibited by this same fixation procedure (29). Therefore, by fixing the livers of rats, injected with horseradish peroxidase, for 3–5 hr at room temperature in the above-mentioned fixative, we can assume that all the staining which survives is attributable to exogenous horseradish peroxidase. In such preparations peroxidase is localized in micropinocytic vesicles, phagosomes, phagolysosomes, and multivesicular bodies (Fig. 11), but is not seen in the endoplasmic reticulum or the perinuclear space. This finding is in complete agreement with all previous reports on the uptake of foreign substances by Kupffer cells (6–10, 72, 74) and on the uptake of peroxidase by a variety of other cell types (75). The study of Graham et al. (22) was performed in mice rather than in rats, as in this report, and it is possible that there may be species differences in localization of peroxidase and in the sensitivity of this enzyme to fixation procedures. It is conceivable, however, that the staining of both lysosomes and ER in Kupffer cells of peroxidase-injected mice, as reported by Graham et al. (22), could be due to simultaneous visualization of endogenous and exogenous peroxidase activity in these cells, the staining in ER reflecting the endogenous enzyme. and the localization in lysosomes reflecting the exogenous tracer. Further studies on localization of endogenous peroxidase in mouse Kupffer cells will clarify this controversy.

REFERENCES

1. BENACERRAF, B., G. BIOZZI, B. N. HALPERN, and C. STIFFEL. 1957. Physiology of phagocytosis of particles by the R.E.S. *In* The Physiopathology of the Reticulo-Endothelial System. B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, editors. Charles C Thomas Publisher, Springfield, Ill. 52.

2. BENACERRAF, B. 1964. Functions of the Kupffer Cells. *In* The Liver, Morphology, Biochemistry, Physiology. CH. Rouiller, editor. Academic Press Inc., New York. 2:37.

3. BIOZZI, G., B. N. HALPERN, B. BENACERRAF, and C. STIFFEL. 1957. Phagocytic activity of the reticuloendothelial system in experimental infections. *In* The Physiopathology of the Reticulo-Endothelial System. B. N. Halpern, B. Benacerraf, and J. F. Delafresnaye, editors. Charles C Thomas Publisher, Springfield, Ill. 204.

4. WARDLAW, A. C., and J. G. HOWARD. 1959. A comparative survey of the phagocytosis of different species of bacteria by Kupffer cells. *Brit. J. Exp. Pathol.* 40:113.

5. ROGERS, D. E. 1960. Host mechanisms which act to remove bacteria from the blood stream. *Bacteriol. Rev.* 24:50.

6. ROUILLER, CH., R. PICTET, P. NICOLESCU, and L. ORCI. 1967. Les cellules reticulo-endotheliales du foie et l'espace de Disse. *Rev. Int. Hepatol.* 17:827.

7. NICOLESCU, P., and CH. ROUILLER. 1967. Beziehungen zwichen den Endothelzellen der Lebersinusoide und den von Kupfferschen Sternzellen. Elektronenmikroskopische Untersuchung. *Z. Zellforsch. Mikroskop. Anat.* 76:313.

8. CARR, I. 1968. Some aspects of the fine structure of the reticuloendothelial system; the cells which clear colloids from the blood stream. *Z. Zellforsch. Mikroskop. Anat.* 89:355.

9. CASLEY-SMITH, J. R., and P. C. READE. 1965. An electron microscopical study of the uptake of foreign particles by the livers of fetal and adult rats. *Brit. J. Exp. Pathol.* 46:473.

10. HORN, R. G., M. G. KOENIG, J. S. GOODMAN, and R. D. COLLINS. 1969. Phagocytosis of *Staphylococcus aureus* by hepatic reticulo-endothelial cells. An ultrastructural study. *Lab. Invest.* 21:406.

11. NOVIKOFF, A. B., and E. ESSNER. 1960. The liver cell. Some new approaches to its study. *Amer. J. Med.* 29:102.

12. THORBECKE, G. I., L. J. OLD, B. BENACERRAF, and D. A. CLARKE. 1961. A histochemical study of acid and alkaline phosphatase in mouse livers during various conditions modifying activity of the reticulo-endothelial system. *J. Histochem. Cytochem.* 9:392.

13. BÖHME, D., M. WACHSTEIN, and H. SCHNEIDER. 1961. Behavior of acid phosphatase in the re-

ticulo-endothelial system of genetically susceptible and resistant mice infected with *S. typhimurium*. *Amer. J. Pathol.* **39**:103.

14. STRAUS, W. 1964. Cytochemical observations on the relationships between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase. *J. Cell Biol.* **20**:497.

15. STRAUS, W. 1967. Changes in intracellular location of small phagosomes (micropinocytotic vesicles) in kidney and liver cells in relation to time after injection and dose of horseradish peroxidase. *J. Histochem. Cytochem.* **15**:381.

16. WACHSTEIN, M., and E. MEISEL. 1964. Demonstration of peroxidase activity in tissue sections. *J. Histochem. Cytochem.* **12**:538.

17. NOVIKOFF, A. B., L. BIEMPICA, N. QUINTANA, A. ALBALA, and R. DOMINITZ. 1968. Peroxidatic activity in cell organelles. *J. Cell Biol.* **39**:100a. (Abstr.)

18. FAHIMI, H. D., and M. J. KARNOVSKY. 1968. Fine structural localization of exogenous peroxidase in rat liver. *In* Third International Congress of Histochemistry and Cytochemistry, Summary Reports. R. M. Rosenbaum, editor. Springer-Verlag New York Inc., New York. 55. (Abstr.)

19. FAHIMI, H. D. 1968. Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). *J. Histochem. Cytochem.* **16**:547.

20. VENKATACHALAM, M. A., and H. D. FAHIMI. 1969. The use of beef liver catalase as a protein tracer for electron microscopy. *J. Cell Biol.* **42**:480.

21. FAHIMI, H. D. 1969. Cytochemical localization of peroxidatic activity of catalase in rat hepatic microbodies (peroxisomes). *J. Cell Biol.* **43**:275.

22. GRAHAM, R. C., S. LIMPERT, and R. W. KELLER-MEYER. 1969. The uptake and transport of exogenous proteins in mouse liver. Ultrastructural cytochemical studies with peroxidase tracers. *Lab. Invest.* **20**:298.

23. CATANZARO, P. J., R. C. GRAHAM, JR., and H. J. SCHWARTZ. 1969. Ultrastructural identification of possible sites of antigen processing in macrophages. *J. Immunol.* **103**:618.

24. ATERMAN, K. 1958. Some observations on the sinusoidal cells of the liver. *Acta Anat.* **32**:193.

25. ATERMAN, K. 1963. The structure of the liver sinusoids and the sinusoidal cells. *In* The Liver, Morphology, Biochemistry, Physiology. CH. Rouiller, editor. Academic Press Inc., New York. 1:61.

26. FAHIMI, H. D. 1967. Perfusion and immersion fixation of rat liver with glutaraldehyde. *Lab. Invest.* **16**:736.

27. FAHIMI, H. D., and P. DROCHMANS. 1965. Essais

de standardisation de la fixation au glutaraldehyde. I. Purification et determination de la concentration du glutaraldehyde. *J. Microsc.* **4**:725.

28. SMITH, R. E., and M. G. FARQUHAR. 1965. Preparation of nonfrozen sections for electron microscope cytochemistry. *Sci. Instrum. (News).* **10**:13.

29. GRAHAM, R. C., JR., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.

30. CLAUDE, A. 1960. Problems of fixation for electron microscopy. Results of fixation with osmium tetroxide in acid and alkaline media. *Pathol. Biol.* **9**:933.

31. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

32. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

33. COTRAN, R. S., and M. J. KARNOVSKY. 1967. Vascular leakage induced by horseradish peroxidase in the rat. *Proc. Soc. Exp. Biol. Med.* **126**:557.

34. KARNOVKSY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* **27**:137A. (abstr.)

35. ORCI, L., R. PICTET, and CH. ROUILLER. 1967. Image ultra-structurale de pinocytose dans la cellule de Kupffer du foie de rat. *J. Microsc.* **6**:413.

36. MATTER, A., L. ORCI, W. G. FORSSMANN, and CH. ROUILLER. 1968. The stereological analysis of the fine structure of the "Micropinocytosis Vermiformis" in Kupffer cells of the rat. *J. Ultrastruct. Res.* **23**:272.

37. TÖRÖ, I., P. RUSZA, and P. RÖHLICH. 1962. Ultrastructure of early phagocytic stages in sinus endothelial and Kupffer cells of the liver. *Exp. Cell Res.* **26**:601.

38. YAMAGISHI, M. 1959. Electron microscopic studies on the fine structure of sinusoidal wall and fat-storing cells of rabbit livers. *Arch. Histol. Jap.* **18**:223.

39. BRONFENMAJER, S., F. SCHAFFNER, and H. POPPER. 1966. Fat-storing cells (lipocytes) in human liver. *Arch. Pathol.* **82**:447.

40. GOLDFISCHER, S., A. B. NOVIKOFF, A. ALBALA, and L. BIEMPICA. 1970. Hemoglobin uptake by rat hepatocytes and its breakdown within lysosomes. *J. Cell Biol.* **44**:513.

41. BRÖKELMANN, J., and D. W. FAWCETT. 1969.

The localization of endogenous peroxidase in the rat uterus and its induction by estradiol. *Biol. Repr.* 1:59.

42. VENKATACHALAM, M. A., M. H. SOLTANI, and H. D. FAHIMI. 1970. The fine structural localization of peroxidase activity in the epithelium of large intestine of rat. *J. Cell. Biol.* 46:168.

43. SAUNDERS, B. C., A. G. HOLMES-SIEDLE, and B. P. STARK. 1964. Peroxidase. The properties and uses of a versatile enzyme and of some related catalysts. Butterworths, Inc., Washington, D.C. 134.

44. COTRAN, R. C., and M. LITT. 1969. The entery of granule-associated peroxidase into the phagocytic vacuoles of eosinophils. *J. Exp. Med.* 129:1291.

45. MARGOLIASH, E., and A. NOVOGRODSKY. 1958. A study of the inhibition of catalase by 3-amino-1:2:4-triazole. *Biochem. J.* 68:468.

46. MARGOLIASH, E., A. NOVOGRODSKY, and A. SCHEJTER. 1960. Irreversible reaction of 3-amino-1:2:4 triazole and related inhibitors with the protein of catalase. *Biochem. J.* 74:339.

47. STRUM, J. M., and M. J. KARNOVSKY. 1970. Cytochemical localization of endogenous peroxidase in thyroid follicular cells. *J. Cell Biol.* 44:655.

48. FAHIMI, H. D., R. S. COTRAN, and M. LITT. 1970. Localization of endogenous and exogenous peroxidase activity in Kupffer cells and peritoneal macrophages. *Fed. Proc.* 29:493. (Abstr.)

49. COHN, Z. A. 1968. The structure and function of monocytes and macrophages. *Advan. Immunol.* 9:163.

50. BAINTON, D. F., and M. G. FARQUHAR. 1970. Segragation and packaging of granule enzymes in eosinophilic leukocytes. *J. Cell Biol.* 45:54.

51. ACKERMAN, G. A. 1968. Ultrastructure and cytochemistry of the developing neutrophil. *Lab. Invest.* 19:290.

52. MILLER, F., and V. HERZOG. 1969. Die Lokalisation von Peroxydase und saurer Phosphatase in eosinophilen Leukocyten während der Reifung. Elektronenmikroskopisch-cytochemische Untersuchungen am Knochenmark von Ratte und Kaninchen. *Z. Zellforsch. Mikroskop. Anat.* 97:84.

53. PALADE, G. E. 1966. Structure and function at the cellular level. *J. Amer. Med. Ass.* 198:815.

54. JAMIESON, J. D., and G. E. PALADE. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* 34:577.

55. BAEHNER, R. L., M. J. KARNOVSKY, and M. L. KARNOVSKY. 1969. Degranulation of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* 48:187.

56. DEDUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Annu. Rev. Physiol.* 28:435.

57. KLEBANOFF, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. *J. Exp. Med.* 126:1063.

58. BELDING, M. E., S. J. KLEBANOFF, and C. G. RAY. 1970. Peroxidase mediated virucidal systems. *Science (Washington).* 167:195.

59. LEHRER, R. I. 1969. Antifungal effects of peroxidase systems. *J. Bacteriol.* 99:361.

60. OMURA, T., and R. SATO. 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J. Biol. Chem.* 239:2379.

61. OMURA, T., R. SATO, D. Y. COOPER, O. ROSENTHAL, and R. W. ESTABROOK. 1965. Function of cytochrome P-450 of microsomes. *Fed. Proc.* 24:1181.

62. HIRAI, K. 1968. Specific affinity of oxydized amine dye (radical intermediater) for heme enzymes: study in microscopy and spectrophotometry. *Acta Histochem. Cytochem. (Tokyo)* 1:43.

63. ERNSTER, L., and S. ORRENIUS. 1965. Substrate-induced synthesis of the hydroxylating enzyme system of liver microsomes. *Fed. Proc.* 24:1190.

64. HUTTERER, F., F. M. KLION, A. WENGRAF, F. SCHAFFNER, and H. POPPER. 1969. Hepatocellular adaptation and injury. Structural and biochemical changes following Dieldrin and methyl butter yellow. *Lab. Invest.* 20:455.

65. ROUS, P., and J. W. BEARD. 1934. Selection with the magnet and cultivation of reticuloendothelial cells (Kupffer cells). *J. Exp. Med.* 59:577.

66. MILLS, D. M., and D. ZUCKER-FRANKLIN. 1969. Electron microscopic study of isolated Kupffer cells. *Amer. J. Pathol.* 54:147.

67. BRÖKELMANN, J. 1969. Peroxidase-associated binding of estradiol by the rat uterus. *J. Histochem. Cytochem.* 17:394.

68. MARSHALL, A. H. E. 1956. An Outline of the Cytology and Pathology of the Reticular Tissue. Charles C Thomas Publisher, Springfield, Ill.

69. MENGEL, C. E., and H. E. KANN, JR. 1966. Effects of in vivo hyperoxia on erythrocytes. III. In vivo peroxidation of erythrocyte lipid. *J. Clin. Invest.* 45:1150.

70. LESTER, R., and R. F. TROXLER. 1969. Recent advances in bile pigment metabolism. *Gastroenterology.* 56:143.

71. KEENE, W. R., and J. H. JANDL. 1965. The sites of hemoglobin catabolism. *Blood.* 26:705.

72. HAMPTON, J. C. 1958. An electron microscope study of the hepatic uptake and excretion of submicroscopic particles injected into the blood stream and into the bile duct. *Acta Anat.* 32:262.

73. RICHTER, G. W. 1959. The cellular transformation of injected colloidal iron complexes into ferritin and hemosiderin in experimental animals. A study with the aid of electron microscopy. *J. Exp. Med.* **109**:197.

74. SANDERS, E., and C. T. ASHWORTH. 1961. A study of particulate intestinal absorption and hepatocellular uptake. Use of polystyrene latex particles. *Exp. Cell Res.* **22**:137.

75. STRAUS, W. 1969. Use of horseradish peroxidase as a marker protein for studies of phagolysosomes, permeability and immunology. *In* Meth. Achievem. Exp. Path. E. Bajusz and G. Jasmin, editors. S. Karger AG., Basel. **4**:54.