BRIEF NOTES

ISOLATION OF LIVER LYSOSOMES BY IRON LOADING

Ultrastructural Characterization

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Since the biochemical (8), morphological (16), and cytochemical definition of lysosomes (cf. reference 7), much effort has been focused on establishing procedures for isolating these organelles in a pure fraction. This has met with several difficulties. First, the lysosomal population is not homogeneous but consists of a number of different morphological entities (cf. references 3 and 7) with variable sizes and densities. Second, this heterogeneity implies that lysosomes overlap with other organelles with respect to sedimentation rates and equilibrium densities. The use of differential or isopycnic centrifugation will therefore result in a relatively pure lysosomal fraction only at the expense of the recovery. For this reason, other experimental approaches have been found necessary in order to obtain a pure lysosomal fraction (1, 3, 15, 17, 18). The most widely used procedure is based on injections of Triton WR-1339 into experimental animals. This compound accumulates in secondary lysosomes with resultant decrease of their density, thus making their isolation possible (15). Much important information has been gained with this method. Some drawbacks (lysosomal swelling, interference with lipid metabolism, etc.) have been summarized previously (3).

Recently, we described an alternative procedure for isolating lysosomes from liver (1, 2). In short, this method is based on repetitive injections of an iron sorbitol citric acid complex which causes increased ferritin synthesis in the liver (6). Some of the ferritin apparently accumulates in the lysosomes and, since ferritin is slowly degraded by the hydrolytic enzymes, it remains in the lysosomes for days (4). This accumulation leads to an increase in the density of the liver lysosomes, thereby making it possible to isolate them by a combination of differential pelleting and density gradient centrifugation procedures. In the lysosomal fraction isolated with the iron-loading method, hydrolytic enzymes were found to be highly enriched (1). The purpose of the present study was to characterize the lysosomal fraction morphologically. This was facilitated by the introduction of fixative to the sucrose gradient and the use of en bloc staining which improved the preservation of the lysosomal membrane. A slight modification of the sucrose gradient is also introduced in this paper. With this procedure, no gradient mixer is necessary.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180–220 g were used. Before sacrifice, the animals were starved for 15 h. They were always decapitated between 8:00 and 10:00 A.M.

Biochemical Procedures

A liver *lysosomal fraction* was isolated as described earlier (1), with some modifications. The rats were injected intramuscularly with Jectofer (Astra, Södertälje, Sweden) diluted 1:1 with Ringer solution once daily for 10 days (5 mg Fe^{++} daily). The rats were sacrificed 1 or 2 days after the last Jectofer injection. The livers were perfused with 0.3 M sucrose at 37°C via the inferior vena cava to remove the blood (retrograde perfusion) and the portal vein and hepatic artery were cut open (12). After the liver was minced it was homogenized in 0.30 M sucrose in a Teflon-glass homogenizer (200 rpm with four complete strokes). A 10% homogenate was sedimented at 700 g for 10 min, and the supernate further centrifuged at 6,000 g for 8 min. The brown portion of the resulting two-layered pellet was resuspended in 0.30 M sucrose (about 1 g/ml) and 2 ml

were layered onto a discontinuous sucrose gradient (25 ml) containing 5 ml of 1.4, 1.6, 1.8, 2.0, and 2.2 M sucrose. After sedimentation at 100,000 g for 2 h in an SW-27 rotor (L-65B ultracentrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), a pellet was obtained which was designated *lysosomal fraction*. The fractionation procedure is schematically presented in Fig. 1. Plasma membranes and microsomes were isolated as described previously (5, 10, 11).

Electron Microscopy

For electron microscope analysis of the lysosomal pellet, 0.5% glutaraldehyde was included in a "cushion" of 2.3 M sucrose and placed at the bottom of the gradient (Fig. 1). After centrifugation, the lysosomal fraction was carefully resuspended by hand in 2.2 M sucrose-4% glutaraldehyde and slowly diluted with 4% glutaraldehyde-0.1 M cacodylate buffer (pH 7.4) to a final concentration of 0.5 M sucrose. This suspension was sedimented at 20,000 g for 30 min in a swinging bucket rotor. Small cubes were cut from the pellets and washed in 0.1 M cacodylate buffer containing 0.1 M sucrose (pH 7.4) for 1 h and postfixed for 1 h in 1% OsO₄ buffered with s-collidine. To increase the contrast of lysosomal membranes, it was found to be of great advantage to perform en bloc staining with uranyl acetate, as described by Farquhar and Palade (9). If this techique was not performed, the membranes surrounding the isolated lysosomes were indistinct and almost invisible. For electron microscope examination of liver from Jectofer-treated rats, primary fixation was performed by vascular perfusion via the vena cava with 2% glutaraldehyde containing 0.1 M cacodylate buffer, and 0.1 M sucrose (pH 7.4). Small pieces of glutaraldehyde-fixed liver were postfixed in s-collidine buffered 1% OsO₄ for 1 h. In some experiments, primary fixation was performed with OsO₄.

RESULTS AND DISCUSSION

The gross chemical compositions (protein, phospholipids, and cholesterol) of liver homogenate, plasma membranes, microsomes, 100,000 g supernate, and lysosomal fraction are illustrated in Table I. Our findings are in agreement with the available data in the literature on the protein and lipid composition of subcellular fractions of liver (cf. reference 3 and 10). Of special interest in this context is the relative enrichment of cholesterol in the iron-loaded lysosomes when related to phospholipids. Such an enrichment of cholesterol has also been shown with other methods for isolation of lysosomes.

Treatment with Jectofer results in the occurrence of electron-dense "ferritin granules" in the lysosomes of both parenchymal and sinusoidal lining cells (endothelial cells and Kupffer cells). The granule-filled lysosomes in these cells differ somewhat in their appearance. Thus, in Jectofer-treated rats the parenchymal cell lysosomes (Fig. 2) containing ferritin granules are relatively uniform in appearance (sphere-shaped), and show moderate variation in size. Also, the ferritin gran-
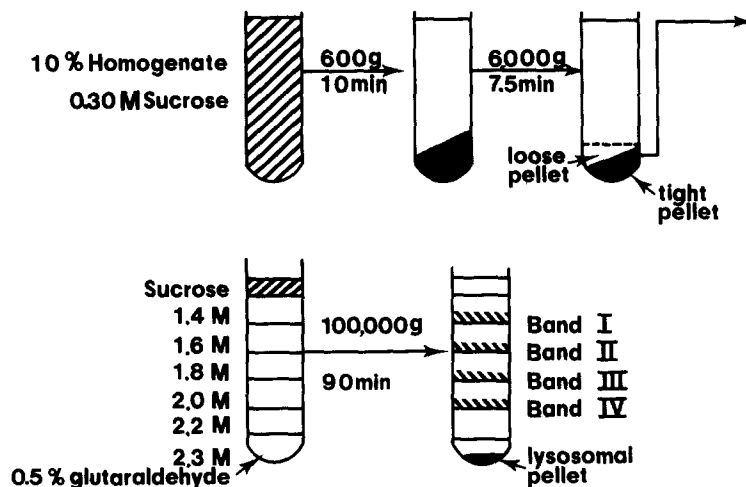

FIGURE 1 Schematic representation of the procedure for isolating lysosomes for electron microscope analysis. Bands I-IV have been analyzed biochemically and ultrastructurally and are mainly composed of mitochondria and microsomes. For the biochemical analyses the same gradient was used but the bottom layer containing glutaraldehyde was omitted.

TABLE I

Protein, Phospholipid, and Cholesterol Content in Liver Homogenate, Supernate, Microsomal Fraction, Plasma Membrane Fraction, and Lysosomal Fraction

Fraction	Protein*	Phospholipid*	Cholesterol*	Cholesterol Phospholipid
Homogenate	200 ± 44	20.7 ± 2.7	2.5 ± 0.21	0.12
Supernate	60 ± 1.3	0.10 ± 0.06	0.15 ± 0.05	0.15
Plasma membranes	1.3 ± 0.2	0.36 ± 0.06	0.14 ± 0.02	0.39
Microsomes	13.5 ± 2.7	4.1 ± 0.7	0.40 ± 0.05	0.10
Lysosomes	0.75 ± 0.1	0.060 ± 0.005	0.015 ± 0.003	0.25

The livers were perfused with 0.30 M sucrose via the inferior caval vein to wash out the blood (12). All subcellular fractions were washed with 0.15 M Tris-buffer at pH 8.0 to release adsorbed proteins and lipids (10, 11). "Supernate" denotes 100,000 g × 60 min. supernate. "Cholesterol" denotes free and esterified cholesterol.
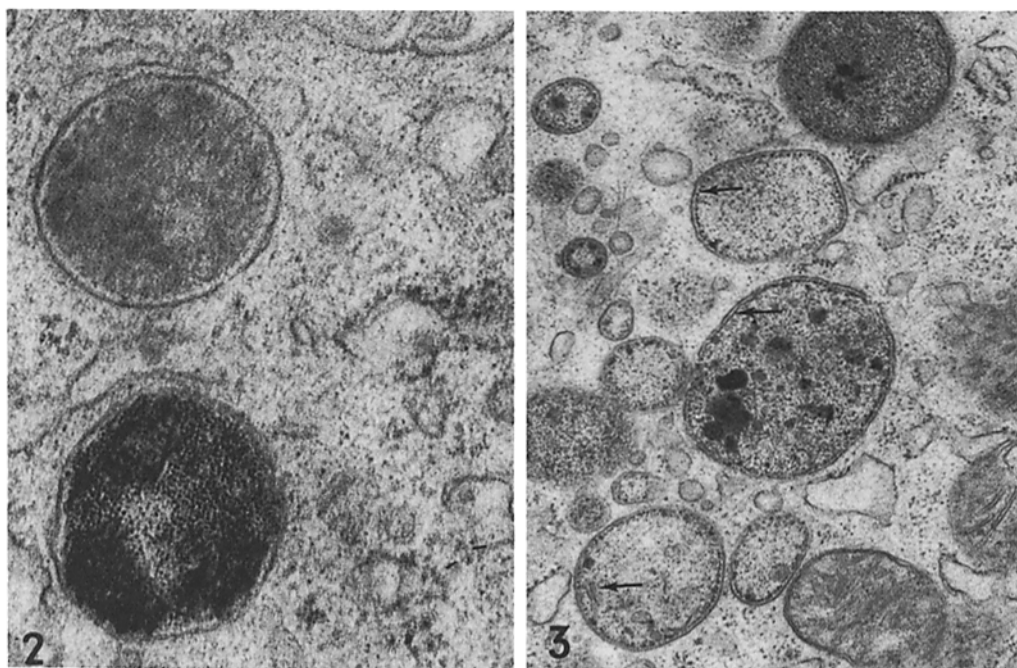* mg per gram of liver (wet weight after perfusion).

FIGURE 2   Peribiliary portion of liver parenchymal cell with two lysosomes, the upper of which has a dense matrix containing few and randomly distributed ferritin granules. The lower lysosome is filled with similar granules. Note clear peripheral zone in both lysosomes, and the absence of piling up of granules adjacent to the clear zone (cf. Figs. 3 and 5). Thin section stained with lead citrate. × 68,000.

FIGURE 3   Portion of Kupffer cell containing at least 10 lysosomes of highly variable size, all showing presence of ferritin granules in their matrix. Note peripheral aggregation of the ferritin granules, especially in lysosomes marked by arrows. Thin section stained with lead citrate. × 25,000.

ules are randomly distributed within these lyso-somes except in a 100-200 Å wide clear peripheral zone. The lysosomes of sinusoidal lining cells (Fig. 3) vary to a great extent in both size and shape, and are generally small and few in number in endo-thelial cells. Furthermore, the electron-dense ma-terial within these lysosomes often tended to form aggregates and filaments, and a piling up of
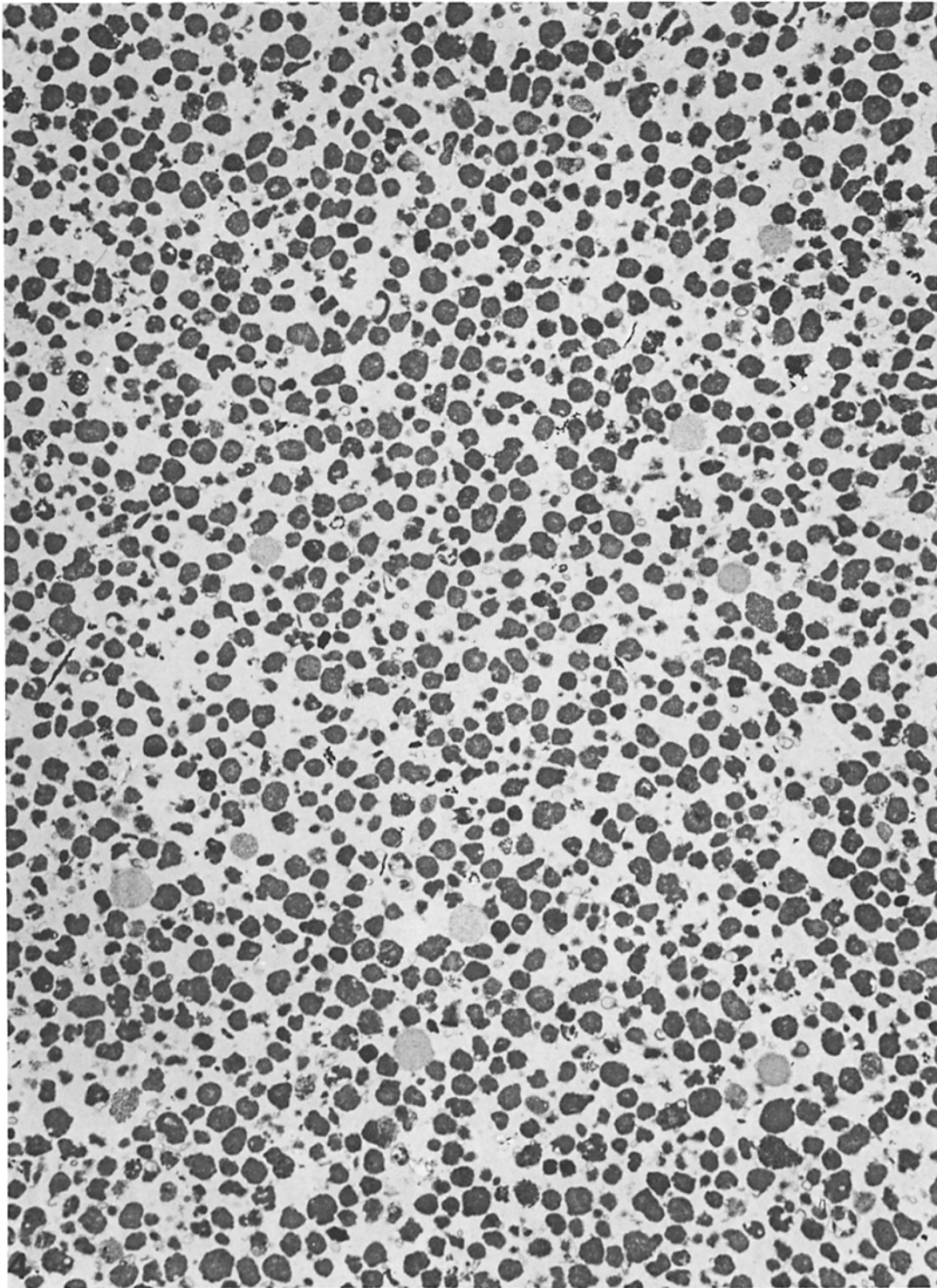
FIGURE 4.   Electron micrograph of the isolated lysosomal fraction. The fraction consists almost entirely of lysosomes with dense matrix. En bloc staining according to Farquhar and Palade (9). Thin section stained with uranyl acetate and lead citrate. × 5,000.

electron-dense material was typically seen at the periphery of the Kupffer cell lysosomes immediately inside a granule-free zone of 100–200 Å width. This peripheral concentration of granules was also observed in lysosomes containing very few granules.

The appearance of the lysosomal pellet is presented in Fig. 4. The fraction consists almost exclusively of rounded, markedly electron-dense vacuoles or bodies believed to represent lysosomes. Thus, out of 3,784 bodies counted on randomly selected micrographs, only 14 (i.e. less than 1%) were nonlysosomal (Table II). At high magnification, it can be seen that the vacuoles have a dense matrix and are filled with highly electron-opaque granules resembling ferritin and/or hemosiderin ("ferritin granules") (Fig. 5). The size and appearance of the bulk of the isolated lysosomes are consistent with an origin in parenchymal liver cells (compare Figs. 2 and 4). Lysosomes with an appearance suggesting origin from sinusoidal lining cells were only rarely observed (quantitation, however, could not be performed with satisfactory accuracy). Although some of the parenchymal cell lysosomes in situ contain membrane derivatives (and appear to correspond to "autolysosomes"), this type of lysosome was not recovered in the fraction.

Presumably because of the high electron density of the lysosomal content, as well as the relatively slow rate of penetration of glutaraldehyde (14) with resultant inadequate preservation, the limiting membrane was not clearly distinguishable when routine staining procedures for electron microscopy were performed. However, with the advantage of the en bloc staining technique (9) and the introducion of glutaraldehyde to the bottom of the sucrose gradient, adequate preservation and distinct bordering membranes were obtained (see Fig. 5).

The findings concerning the morphology of the pellet are in agreement with earlier data on the chemical and enzymatic composition of different types of lysosomal fractions (1–3, 13, 15, 17, 18), and also with the enrichment of the three acid hydrolases presented in Table III. In this table the calculation of the purification is made on a phospholipid basis, since with our method proteins (presumably ferritin and hemosiderin) accumulate in the lysosomes (calculating the enrichment of acid hydrolases on a protein basis would give too low values). With the method used, lysosomes become highly (about 60-fold) purified over the

total liver homogenate, and the recovery ranges between 15 and 20%. Both the recovery and the purification are similar to, or in fact higher than, those obtained with the Triton WR-1339 method (3, 18). Evidently, lysosomes isolated by the present method are more "normal" in size than those isolated by the use of Triton WR-1339 (3, 18, 13). Our method, therefore, offers a complementary procedure for studies on isolated liver lysosomes.

Besides hepatocytes, the liver contains non-parenchymal cells, viz. endothelial cells, Kupffer cells and fat-storing cells. These nonparenchymal cells make up about 30% of the total number of liver cells. However, based on volume the percentage is much less, or about 10%, due to the small size of the nonparenchymal cells (19). Theoretically, all liver cells may contribute to the lysosomal fraction.

The endothelial cells are the most numerous nonparenchymal cell type (20). Since these cells are probably not ruptured by the present mild homogenization (10), lysosomes from endothelial cells are unlikely to be recovered in the pellet enriched in lysosomes. Small lysosomes of the type seen in endothelial cells were also encountered very rarely in the pellets. Since the volume of the Kupffer cells is only a few percent of the total liver volume, Kupffer cell lysosomes can only be expected to make a marginal contribution, if any contribution at all, to the lysosomal pellet if these cells become ruptured during homogenization. In agreement with this, only occasional Kupffer cell lysosomes were present in the lysosomal fraction. The contribution from the fat-storing cells can evidently be neglected.

SUMMARY

In summary, the data demonstrate that, by the use of repeated injections of an iron sorbitol complex,
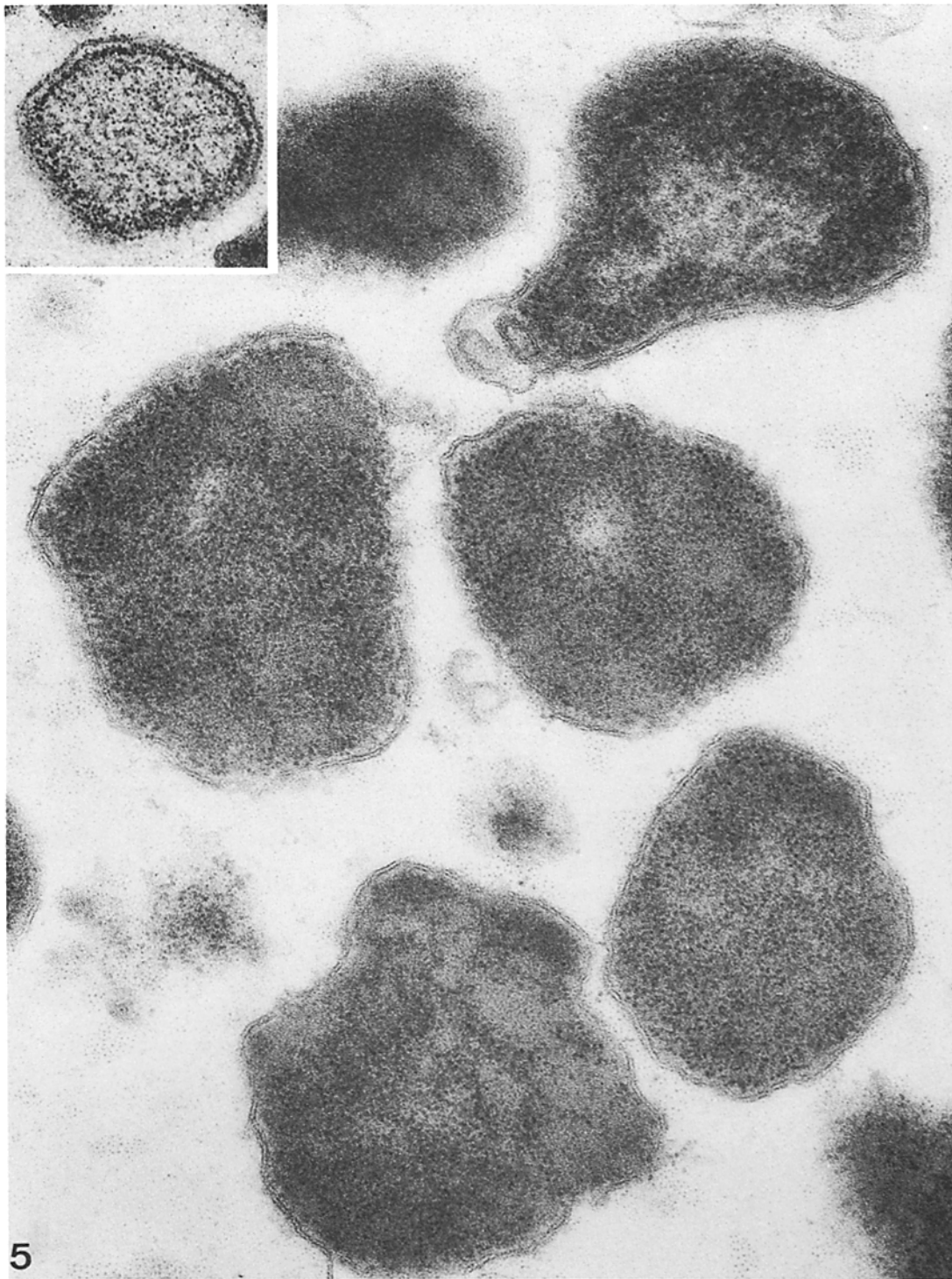
FIGURE 5   High magnification electron micrograph of the lysosomal fraction. The lysosomes are bordered by a triple-layered membrane and contain abundant electron-dense ferritin granules. Electron microscope processing as in Fig. 4. × 125,000; Inset shows a lysosome with peripheral aggregation or piling up of granules (presumably originating from Kupffer cell, cf. Fig. 3). × 81,000.

TABLE III

Distribution of Three Acid Hydrolases in Liver Homogenate and in the Fraction Enriched in Lysosomes

Fraction	Acid phosphatase*	Cathepsin D‡	Aryl sulfatase §
Homogenate	231 ± 7	31 ± 2	58 ± 2
Lysosomal fraction	13,341 ± 509 (58)‖	1,905 ± 127 (61)‖	3,430 ± 190 (59)‖
Recovery	16.7%	17.8%	17.1%

* Moles P_i released/min/gm of phospholipid
‡ Moles tyrosine released/min/mg of phospholipid
§ Moles 4-nitrocatechol released/min/gm of phospholipid
‖ The numbers in parentheses denote the enrichment of apparent specific activity in the lysosomal fraction as compared to the homogenate.

it is possible to isolate a fraction highly enriched in hydrolytic enzymes (60 times over the homogenate) and in well preserved lysosomes emanating almost entirely from liver parenchymal cells. The advantage of adding fixative to the bottom of the gradient and of using en bloc staining with uranyl acetate is also demonstrated.

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