ELECTRON MICROSCOPY OF LYSOSOME-RICH FRACTIONS FROM RAT LIVER

By ALEX B. NOVIKOFF, Ph.D., H. BEAUFAY, M.D.,* and C. de DUVE, M.D.

(From the Department of Pathology, Albert Einstein College of Medicine, Yeshiva University, New York, and the Department of Physiological Chemistry, University of Louvain, Belgium)

Plates 60 to 62

Several investigators have demonstrated that the particulate form of acid phosphatase of rat and mouse liver shows a somewhat atypical intracellular distribution, indicating that the enzyme is associated mostly with the smaller mitochondria and the larger microsomes, or, alternatively, with a special class of particles intermediate between these two cytoplasmic entities (1–5). The latter interpretation has been upheld by de Duve and coworkers (4, 6) on the basis of differential centrifuging and washing experiments, and extended by these authors to a number of other hydrolases, showing cytological properties very similar to those of acid phosphatase. They have proposed the name "lysosomes" for the particles containing these enzymes, which include acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin, and β-glucuronidase. Uricase is another enzyme which appears to be attached to lysosomes or to particles of a similar nature (3, 6).

Present knowledge concerning the properties of lysosomes, or even their existence as a separate class, rests only on indirect evidence, since these particles have never been isolated in pure form. It has been deduced from experimental observations that lysosomes belong to the parenchymatous cells of the liver, are associated with no more than 4 per cent of the cell's nitrogen, have diameters ranging between 0.25 and 0.8 μ if their density is low (1.10) or between 0.13 and 0.4 μ if their density is high (1.30), and possess a saclike structure with a semipermeable membrane, within which the hydrolases are present in freely diffusible form (4, 6, 7).

It follows from the second of these properties that pure lysosomes should have specific enzymic activities at least 25 times those of a homogenate. Such figures have never been reached, but fractions showing a 7- to 10-fold increase in specific activity over the homogenate can be prepared fairly easily, leaving the possibility that even greater purification could be achieved by a few additional steps. It seemed to us that such fractions, though still heavily contaminated with mitochondrial or microsomal material, might be sufficiently enriched in the alleged particles to render them directly accessible to morphological exam-

* Aspirant du F.N.R.S.

ination. The present note describes the preliminary observations which have been made in the electron microscope on thin sections of particulate fractions which, from biochemical analyses, should contain a fairly large proportion of lysosomes.

Materials and Methods

Fractionation Procedures.—Several methods were tried to isolate these fractions from rat liver homogenates. In two experiments, L fractions were prepared as described by de Duve and coworkers (6), care being taken to remove as much of the microsomal and fluffy material as possible. In the third experiment the L pellet was resuspended in 10 ml. of 0.25 M sucrose and further fractionated by spinning repeatedly at 30,000 *g*-min. in the No. 40 rotor of the Spinco Model L preparative ultracentrifuge, and removing after each run the heavier particles which were sedimented under these conditions. As shown in Table I, each centrifuging furnished a sediment of higher specific cytochrome oxidase activity and lower specific acid

TABLE I

Subfractionation of L Fraction

Fractions separated by centrifuging each supernatant at 30,000 *g*-minutes. Analyses performed as described by de Duve and coworkers (6), except that the release of bound phosphatase was insured by running the assays in the presence of 0.1 per cent triton X-100 (8).

	Relative specific activity*			
	Cytochrome oxidase		Acid phosphatase	
	Sediment	Supernatant	Sediment	Supernatant
Original L fraction		2.7		7.0
First centrifuging	3.2	2.0	5.2	10.4
Second centrifuging	2.6	1.6	8.0	11.8
Third centrifuging	2.0	1.9	11.2	14.0

* Ratio: $\frac{\text{Activity/mg. N of fraction.}}{\text{Activity/mg. N of cytoplasmic extract.}}$

phosphatase activity than those of the starting material, and a supernatant fluid with reverse properties. A 14-fold purification of the acid phosphatase-containing particles was achieved in the final supernatant, from which these particles could be recovered by centrifuging at 250,000 *g*-min.

In the fourth experiment, the L fraction was subfractionated by the packing procedure of Kuff and Schneider (9), with the aid of the horizontal SW-39 rotor. This experiment was based on preliminary trials performed by Dr. B. C. Pressman, which indicated that lysosomes have a somewhat higher density than the average mitochondria, of the order of 1.20. The denser material was indeed found in most cases to have a higher specific acid phosphatase activity than the less dense granules, but the purification never exceeded that obtained by the method of repeated centrifuging.

In the fifth experiment a somewhat modified centrifugation schedule was used, designed to prepare an L fraction from 0.25 M sucrose-7.3 per cent polyvinylpyrollidone (PVP) homogenate. This medium has been found very effective in preserving the structure of mitochondria and other intracellular components (10, 11). The biochemical data showed that a good separation of lysosomes from mitochondria and microsomes had not been achieved.

The fraction contained 27.5 per cent of the total acid phosphatase (lysosomal), 14.5 per cent of the total cytochrome oxidase (mitochondrial), and 14 per cent of the total glucose-6-phosphatase (microsomal) of the homogenate.

Electron Microscopy Procedures.—As the work progressed, the pellets used for fixation became progressively smaller; at the end, 0.1 to 0.2 ml. of a dilute suspension of the fraction was centrifuged at high speed, yielding a thin pellet. The pellets were cut out with the bottoms of the plastic centrifuge tubes, quickly dropped in cold fixative, and lifted away from the plastic with a fine glass needle.

The fixative consisted of 2 per cent osmium tetroxide added to the medium from which the fraction was isolated, adjusted with alkali to pH 7.0. After overnight fixation at refrigerator temperature, the material was rapidly dehydrated and embedded in *n*-butyl methacrylate by the usual procedures. Thin sections, usually about 30 mμ, were cut with the Porter-Blum ultramicrotome, mounted on formvar-coated copper grids and photographed with the RCA EMU-2E electron microscope. In most cases, only one or two pieces of the fraction have been sectioned, and the frequently different appearance of different areas underlines the need for more adequate sampling before comparison of particle distributions among the fractions can be made quantitative.

OBSERVATIONS

Morphology of Isolated Fractions.—In every fraction showing high acid phosphatase activity (L fraction and subfractions from it) we have found distinctive particles ranging in length from 0.25 μ to 0.50 μ (mean 0.37 μ) which we shall refer to as "dense bodies." On the other hand, the fractions with low acid phosphatase activity only rarely showed a dense body. Thus the M (large mitochondria) fractions from 0.25 M sucrose homogenates were composed almost exclusively of mitochondria—spheres of three types in which the cristae (12) were either only slightly enlarged, or entirely indistinguishable in the material flattened against the outer mitochondrial membrane, or localized at one pole of a much enlarged mitochondrial sphere. Similarly, the P (microsome) fraction showed, in agreement with the findings of Palade and Siekevitz (13), mostly small sacs composed of the membranes of ergastoplasm or endoplasmic reticulum, on which were present the granules described by Palade (14).

As could be expected from the cytochrome oxidase measurements, even the purest L fraction still contained many mitochondria. Thin membranes were also often present, the origin of which is not clear; they may have originated in such diverse structures as separated portions of the external mitochondrial membranes, converted internal mitochondrial membranes (cristae), or the Golgi membranes. Some microsomal membranes, with their attached granules, were also encountered.

Fig. 1 shows four dense bodies in the fraction obtained from the PVP sucrose homogenate. They are considerably smaller than most mitochondria, and are dotted with granules of high electron density. As can be seen in the figure, these granules are more dense and smaller than those of the microsomal membranes. On photographs from two experiments, the average diameters of these new

granules were 56 and 76 A while those of the microsomal membranes were 162 and 181 A. The extremes in the diameters of the former were 40 and 100 A. Higher resolution photographs would unquestionably reduce these figures somewhat.

The highest concentration we have found of dense bodies is shown in Fig. 2. This was a section of a brown material at the bottom of the original L fraction shown in Table I; its specific activities were 9.6 for acid phosphatase and 1.7 for cytochrome oxidase. As the photograph shows, there was considerable polymorphism among the dense bodies. About half the particles were found to possess large internal cavities (see also Fig. 3). (It is possible that all particles were hollow but only those properly sectioned reveal cavities.) In some cases, these cavities were lined with a broad layer of dense material.

In Fig. 4 is seen the external membrane found around some of the dense bodies. Since these were found more frequently in the better sections, the possibility exists that they were destroyed in the others.

"Dense Bodies" in Liver Sections.—As first described by Rouiller (15), electron micrographs of liver sections show that the parenchymatous cells contain dense polymorphic particles, particularly along the bile canaliculi.

As Figs. 6 and 7 show, these particles are like the isolated dense bodies in size, in their possession of small electron-dense granules, in the presence of internal cavities lined with a broad dense zone, and in polymorphism.

DISCUSSION

Since pure preparations have not yet been examined, it is not possible to decide whether the "dense bodies" identified in the isolated fractions and in the liver sections are actually identical with the lysosomes, whose existence has been postulated on the basis of biochemical data. At the present time one can only point to a set of correlations, whose true significance will have to be established by further work. The correlations are the following:

(*a*) Thus far, all methods leading to the isolation of lysosome-rich fractions have been equally useful in concentrating the dense bodies.

(*b*) The observed dimensions of the dense bodies are in good agreement with those predicted for lysosomes.

(*c*) At least some of the dense bodies show internal cavities and well defined outer membranes. This, too, agrees well with the postulated nature of lysosomes (sac-like structure with a semipermeable membrane).

(*d*) The observations of Wattiaux *et al.* (7) indicate that lysosomes belong to the parenchymatous cells of the liver. The same appears true of the dense bodies (Figs. 6 and 7). Their localization along the bile canaliculi fits in with the cytochemical observations of Holt (16) who found acid phosphatase (and esterase) to be localized there.

To the above correlations should finally be added the statement that no other

characteristic particle showing the required distributions among the fractions could be identified in any of the examined sections besides mitochondria and dense bodies. One could therefore suggest that if the dense bodies are not identical with lysosomes, the latter must either be extremely rare, or have essentially the same structural properties as mitochondria.

Though fairly impressive, the above arguments should not be overestimated or oversimplified. As pointed out by de Duve and coworkers (6), the biochemical data do not entirely support the existence of a single homogeneous class of lysosomes. In the present work, acid phosphatase has been the only guiding enzyme and it is not known whether the methods used to fractionate the L fractions have also led to a further purification of the other enzymes of the same group. On the other hand, the morphological data available so far are scanty and it is possible that a more extensive survey may lead to finer distinctions. The meaning of the polymorphism of the dense bodies, observed in tissue sections as well as isolated fractions, remains to be established. Particularly interesting is its significance for a relationship between dense bodies and mitochondria. Thus far, the main basis for grouping all the dense bodies in one class has been the presence of the small granules with which their matter is studded.

The nature of these granules raises an interesting problem. They are distinctly smaller and of greater electron density than the ergastoplasmic granules. It is not known if they are highly osmiophilic or possess considerable intrinsic electron-absorbing power. A possibility which fits in with the latter interpretation and with the observed dimensions of these granules is that they represent ferritin-iron micelles. To illustrate this possibility, Fig. 5 reproduces Farrant's (17) electron micrograph of purified ferritin micelles at the same magnification as Figs. 3 and 4. Preliminary experiments have indeed shown that the L fraction contains significantly more iron per milligram of nitrogen than the neighboring M and P fractions (heavy mitochondrial fraction and microsomes) and that the specific iron content of this fraction is roughly proportional to its specific acid phosphatase activity. The data are compatible with the hypothesis that approximately 10 per cent of the total ferritin of the cell is associated with lysosomes, the remainder being in the soluble fraction.

SUMMARY

A preliminary electron microscope study has revealed the presence in lysosome-rich fractions, isolated from rat liver, of hitherto undescribed cytoplasmic particles, called "dense bodies."

Approximately 0.37 μ in length, the dense bodies often possess an internal cavity and external membrane. They contain many electron-dense granules 55 to 77 A, or less, in diameter.

Such dense bodies are also visible in electron micrographs of parenchymatous cells in liver sections.

The correlations between dense bodies and lysosomes are listed, but until pure preparations are available it is not possible to assert that dense bodies and lysosomes are identical.

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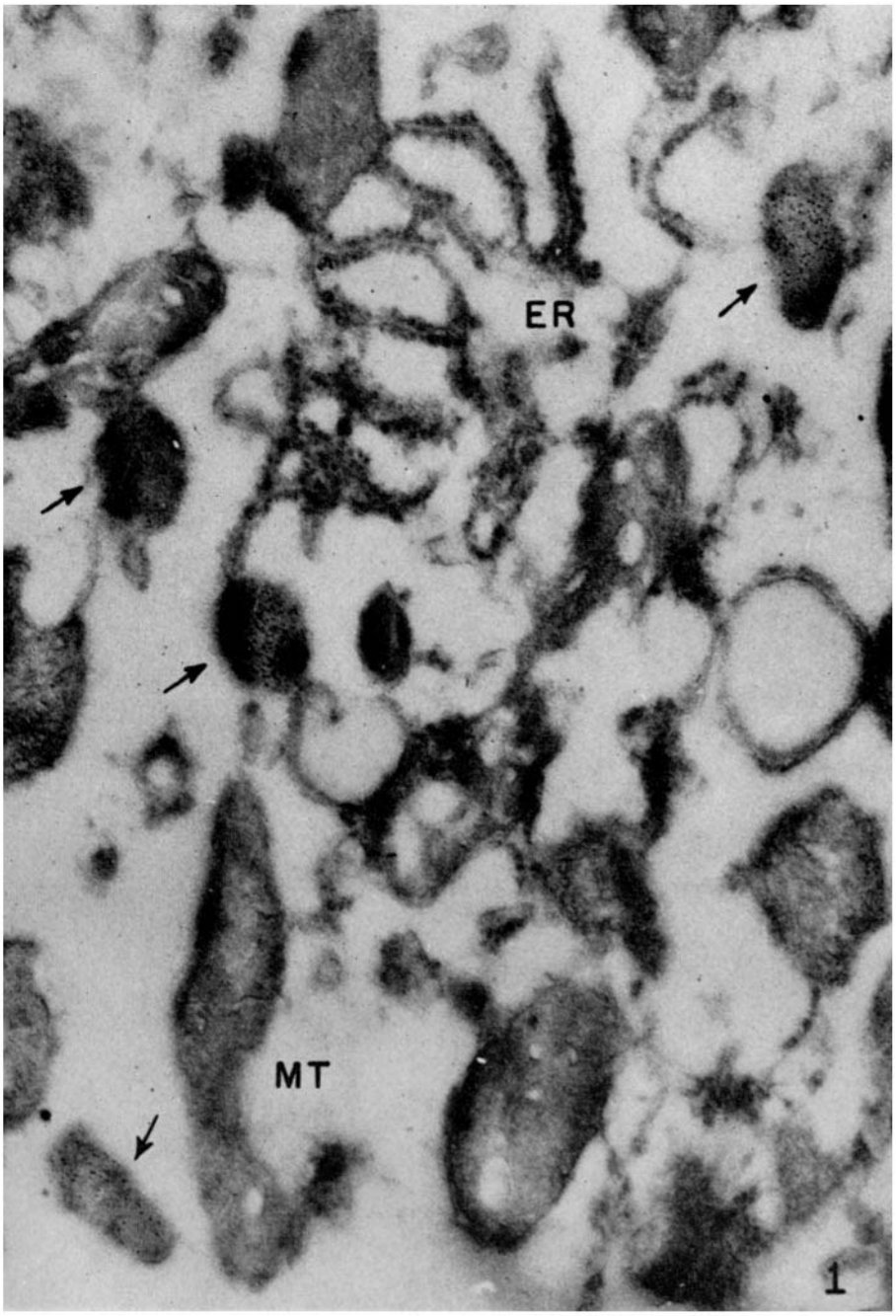
EXPLANATION OF PLATES

PLATE 60

FIG. 1. Fraction isolated from 7.3 per cent PVP-0.25 M sucrose homogenate of rat liver. A pellet of the fraction was fixed in 7.3 per cent PVP- 0.25 M sucrose - 2 per cent OsO_4, pH 7.0, at 0°C. for 13 hours. Arrows indicate the dense bodies; note the dense granules within them. Microsomal membranes are indicated by *ER*; note that their granules are considerably larger than those of the dense bodies. The mitochondria (MT) were not well preserved in this portion of the pellet. × 59,500.



(Novikoff *et al.*: Lysosome-rich fractions from rat liver)

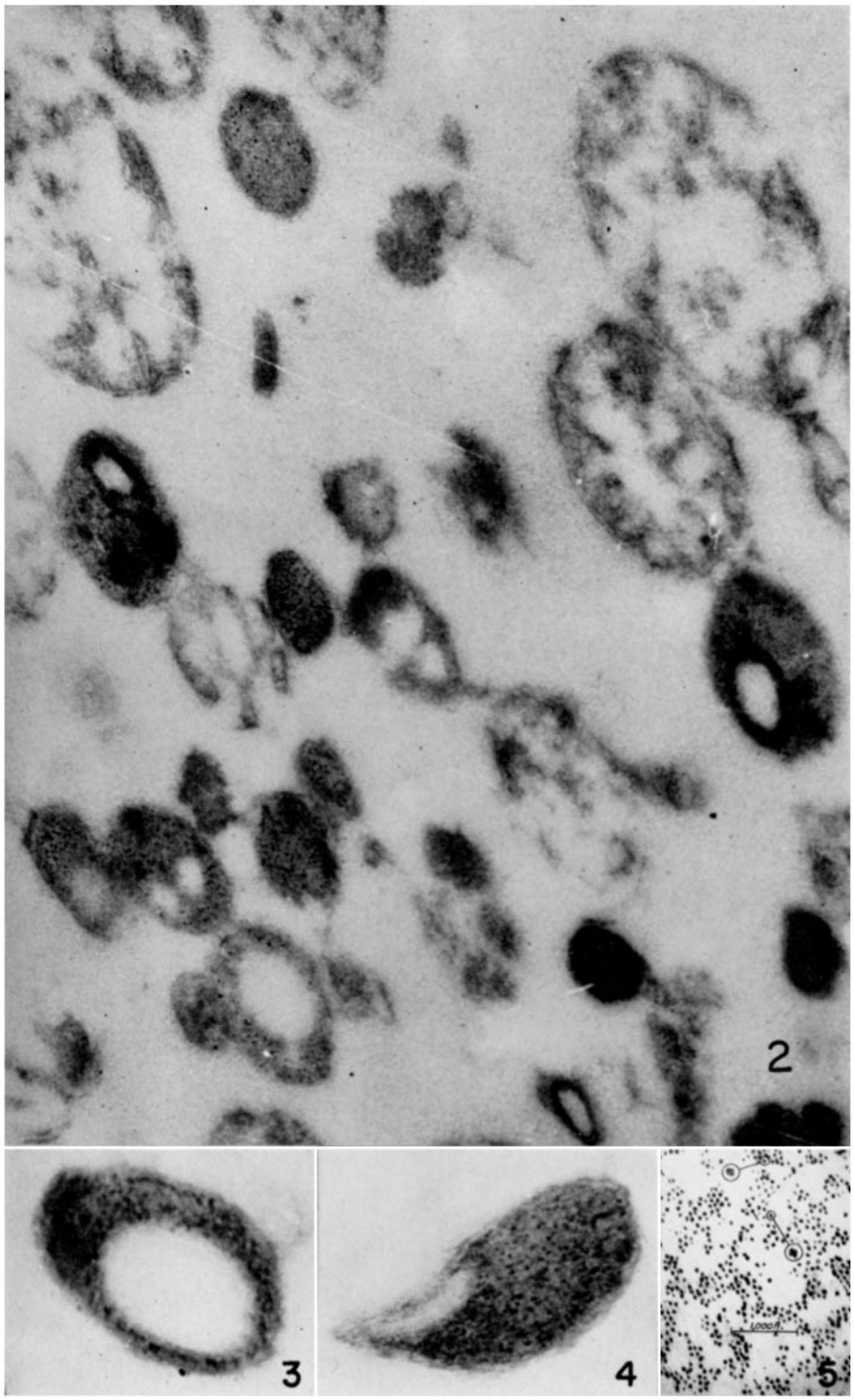
PLATE 61

FIG. 2. Fraction isolated from 0.25 M sucrose - 0.001 M versene homogenate of rat liver. A small brown crescent-shaped area at the bottom of the usual lysosome-containing sediment was fixed in 0.25 M sucrose - 0.001 M versene - 2 per cent OsO_4, pH 7.0, at 0°C. for 18 hours. Note the abundance of dense bodies; they exhibit considerable polymorphism. × 50,000.

FIG. 3. Dense body from 7.3 per cent PVP- 0.25 M sucrose homogenate of rat liver. From the same grid as used for Fig. 1, this dense body shows a large internal cavity. × 90,000.

FIG. 4. Dense body from L fraction prepared from 0.25 M sucrose - 0.001 M versene homogenate of rat liver. The pellet was fixed in 0.25 M sucrose - 0.001 M versene - 2 per cent OsO_4, pH 7.0, at 0°C. for 16 hours. Note the external membrane. × 90,000.

FIG. 5. Electron micrograph of Farrant (17) showing micelles of isolated ferritin. Note that their size is similar to that of the granules within the dense body in Fig. 4. × 90,000.



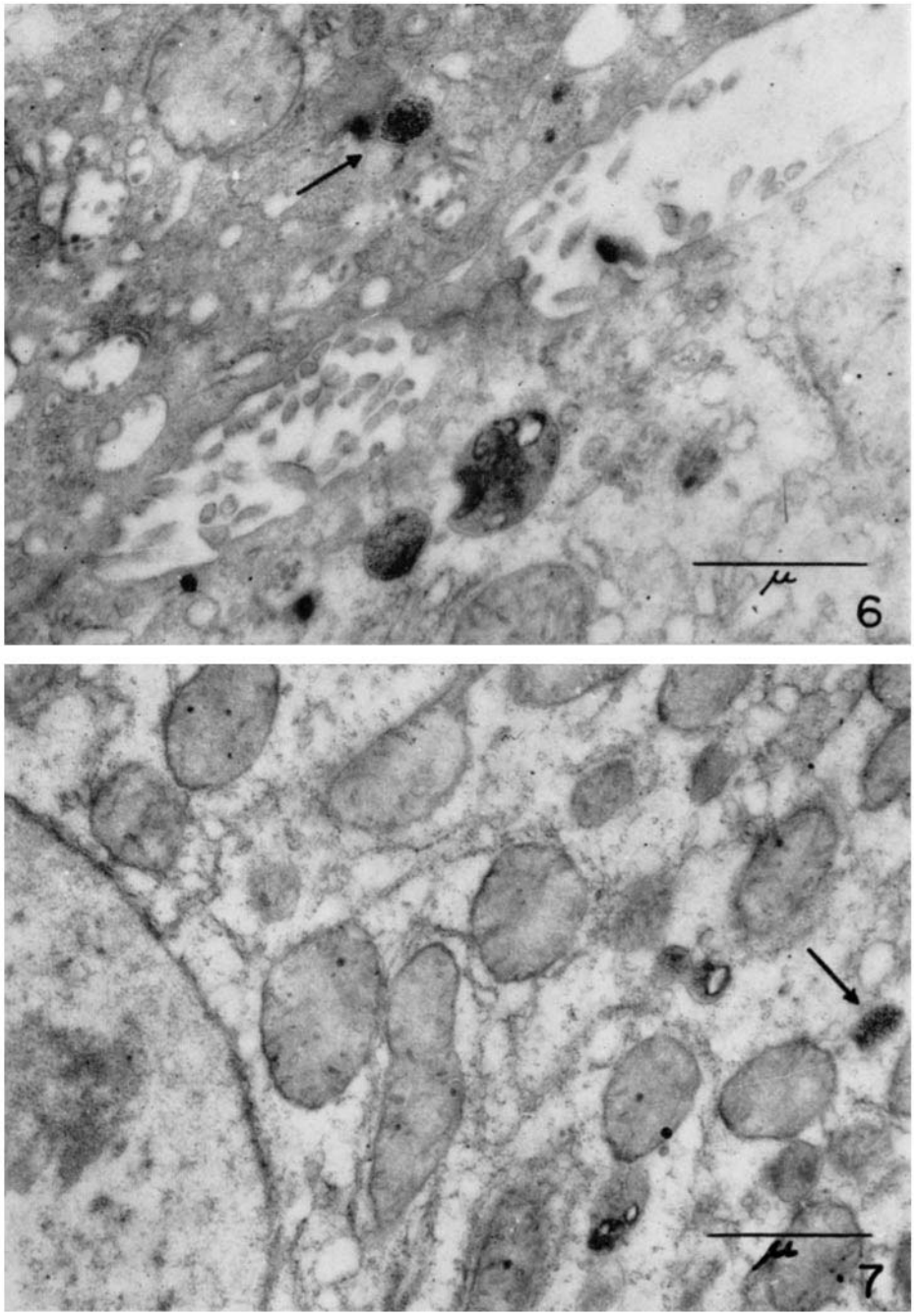
(Novikoff *et al.*: Lysosome-rich fractions from rat liver)

PLATE 62

Sections of liver of rat deprived of food for 4 days, then refed and sacrificed 6 hours later. Tissue fixed in buffered osmium tetroxide. Photomicrographs kindly furnished us by Dr. W. Bernhard and Dr. C. Rouiller.

FIG. 6. Section passing longitudinally through a bile canaliculus. Note alongside the canaliculus a typical dense body (arrow) and others apparently related to it. × 24,000.

FIG. 7. Section passing through nucleus with nucleolus. Note a typical dense body (arrow) and two others nearby apparently related to it. × 22,000.



(Novikoff *et al.:* Lysosome-rich fractions from rat liver)