ISOLATION OF RAT LIVER LYSOSOMES BY ISOPYCNIC CENTRIFUGATION IN A METRIZAMIDE GRADIENT

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ABSTRACT

A preparation, similar to the light mitochondrial fraction of rat liver (L fraction of de Duve et al. [1955, *Biochem. J.* **60:** 604–617]), was subfractionated by isopycnic centrifugation in a metrizamide gradient and the distribution of several marker enzymes was established. The granules were layered at the top or bottom of the gradient. In both cases, as ascertained by the enzyme distributions, the lysosomes are well separated from the peroxisomes. A good separation from mitochondria is obtained only when the L fraction is set down underneath the gradient. Taking into account the analytical centrifugation results, a procedure was devised to purify lysosomes from several grams of liver by centrifugation of an L fraction in a discontinuous metrizamide gradient. By this method, a fraction containing 10–12% of the whole liver lysosomes can be prepared. As inferred from the relative specific activity of marker enzymes, it can be estimated that lysosomes are purified between 66 and 80 times in this fraction. As ascertained by plasma membrane marker enzyme activity, the main contaminant could be the plasma membrane components. However, cytochemical tests for 5′AMPase and for acid phosphatase suggest that a large part of the plasma membrane marker enzyme activity present in the purified lysosome preparation could be associated with the lysosomal membrane.

The procedure for the isolation of rat liver lysosomes described in this paper is compared with the already existing methods.

KEY WORDS metrizamide · gradient centrifugation · rat liver · lysosomes · plasma membrane

Isopycnic centrifugation in a metrizamide gradient of a total mitochondrial fraction of rat liver (M + L according to de Duve et al. [15]), allows a good separation of peroxisomes from the other major constituents of the fraction, mitochondria, and lysosomes (13). On the other hand, lysosomes are poorly separated from mitochondria as ascertained by the distribution curves of their reference enzymes (13). In the present paper, we show that by using a light mitochondrial fraction (L fraction of de Duve et al. [15]), in certain conditions it is possible to purify rat liver lysosomes extensively by centrifugation in a metrizamide gradient, with satisfactory yield.

MATERIALS AND METHODS

Tissue Fractionation

Experiments were performed on male Wistar rats weighing 200–250 g. The animals were decapitated after

being starved for 20 h. The livers were removed, chilled in ice-cold 0.25 M sucrose, and homogenized in the same medium by means of a smooth glass tube fitted with a Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.) rotating at 3,000 rpm. The homogenate was made up to a volume of 7 ml/g of liver. After filtration on two layers of cheesecloth, a small sample was kept for enzyme and protein determination; the remainder was centrifuged at an integrated force of 30,000 $g \cdot$min in the N° 40 rotor (r_{avg} 5.9 cm) of the Spinco preparative ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). After centrifugation, the supernate was removed, without disturbing the top of the pellet. This was then rehomogenized as described above, made up to a volume of 7 ml/g of liver, and centrifuged once more at 30,000 g·min. The sediment was resuspended in 0.25 M sucrose. It is called the NM fraction because it corresponds to a combination of the N and M fractions of de Duve et al. (15). The pooled supernate were subjected to a centrifugation at 250,000 g·min in the N° 40 Spinco rotor. The supernate was thoroughly removed together with the fluffy layer located at the surface of the sediment. After resuspension, the pellet was washed by centrifugation under the same conditions. Removal of the supernate was carried out with the same precautions. The sediment obtained after the second centrifugation was resuspended in 0.25 M sucrose. We call this preparation the L fraction because it essentially corresponds to the light mitochondrial fraction L of de Duve et al. (15). The pooled supernate constitute the PS fraction; it is made up of microsomes and of soluble fraction (P and S fraction of de Duve et al. [15]).

In density gradient centrifugation, the L fraction was used. Experiments with linear density gradients were performed according to Beaufay et al. (9) with the aid of a Spinco model L2-75B ultracentrifuge (rotor SW 65). Metrizamide solutions were adjusted to pH 7.4 with 0.01 N NaOH. The centrifugation temperature was 4°C. Details concerning the centrifugation made with a discontinuous density gradient are given in the text.

Enzyme Assays

Marker enzymes of the main subcellular membranes were assayed according to the following references: monoamine oxidase (32), cytochrome oxidase (1), acid phosphatase (1), β-galactosidase (27), *N*-acetylglucosaminidase (27), catalase (5), glucose-6-phosphatase (15), NADPH cytochrome *c* reductase (8), galactosyltransferase (8), alkaline phosphodiesterase (8) alkaline phosphatase (8), and 5′AMPase (31). To test a possible effect of metrizamide on enzyme activity, preliminary experiments were performed. The granules were maintained in the presence either of 0.25 M sucrose or of metrizamide and at concentrations covering the range the enzymes can be exposed to, in the gradient. Enzymatic tests were performed as soon as possible and repeated 24 h later. Only galactosyltransferase was found to be deeply inhibited by metrizamide. No significant differences were found for the other enzymes whether sucrose or metrizamide were present in the suspending medium.

In the Lowry procedure (19), metrizamide interferes with the determination of proteins. Therefore proteins in the fraction were first precipitated by 8% TCA (metrizamide does not precipitate under these conditions); the precipitate was recovered by centrifugation, dissolved in 0.5 N NaOH for 20 min at 37°C, and the Lowry procedure was then applied to these solutions. Serum albumin was used as standard.

Units of enzymic activity are defined as the amount of enzyme causing the transformation of 1 μmol of substrate per min under the conditions of the assay, except for cytochrome oxidase and for catalase. One cytochrome oxidase unit is defined as the amount of enzyme causing the decadic logarithm of the concentration of reduced cytochrome *c* to decrease by 1 U/min/100 ml incubation mixture (15). According to Baudhuin et al. (5), one catalase unit corresponds to the amount of enzyme causing the destruction of 90% of the substrate in 1 min in a volume of 50 ml under the assay conditions.

Morphological Examinations

Small aliquots of selected fractions of the gradient were poured into an ice-cold solution containing 1.5% (wt/vol) glutaraldehyde in 0.05 M phosphate buffer, pH 7.4. After 20 min, they were diluted to obtain 120 μg of protein per amount of material to be filtered. After this, according to the procedure described by Baudhuin et al. (6), 0.75 ml of each fixed suspension was filtered through Millipore membranes (Millipore Corp., Bedford, Mass.) of 0.025 μm pore size. After filtration, the Millipore filters were washed in 0.05 M phosphate buffer and postfixed with 1% (wt/vol) osmium tetroxide in 0.05 M phosphate buffer, pH 7.4, for 2 h at 0°C. Then, they were stained with 0.5% (wt/vol) uranyl acetate in 0.05 M maleate buffer, pH 5.2. After dehydration through cold graded ethanol, the filters were transferred to propylene oxide for about 15 min and the films of filtered materials were embedded in Epon 812 (10). Sections were obtained with an LKB Ultratome III microtome (LKB Produkter, Bromma, Sweden) and mounted on unsupported grids. They were stained with 2% (wt/vol) uranyl acetate in alcohol solution for 1.5 min, followed by lead citrate for 2 min (21), and examined with a Philips EM 301 electron microscope.

When cytochemical tests for acid phosphatase were carried out, the samples were fixed with 1.5% (wt/vol) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. After 10-min fixation at 0°C, the filtration was performed for 40 min and the resulting pellicles were rinsed three times at 0°C with 0.2 M sucrose, 0.05 M cacodylate buffer, pH 7.4. Then, the pellicles were immersed in an incubation mixture containing 1 mM β-glycerophosphate, 0.1 M Tris maleate buffer, pH 5.0, and 2.4 mM lead nitrate. The bottles were placed on a mechanical shaker and incubated for 90 min at 37°C. After incubation, the pellicles were rinsed three times at 0°C with 0.2

M sucrose, 0.04 M acetate-veronal buffer, pH 7.4, postfixed at 0°C for 2 h with 1% (wt/vol) osmium tetroxide in 0.05 M acetate-veronal buffer, pH 7.4, stained with 0.5% (wt/vol) uranyl acetate, and processed as discussed above for usual EM examinations. Controls were run in an incubation mixture without β-glycerophosphate.

Cytochemical tests for 5'AMPase were performed according to Farquhar et al. (17) on unfixed granules as recommended by these authors, with slight modifications. 0.25 ml of unfixed fraction was placed in a Spinco cellulose nitrate centrifuge tube (0.8 ml) and 0.25 ml of a solution made up of 2 mM 5'AMP, 0.2 M Tris-acetate buffer, pH 7.5, 2 mM Pb $(NO_3)_2$, 10 mM NaF (to inhibit acid phosphatase), and 2 mM Mg $(NO_3)_2$ was added. The tubes were incubated at 25°C for 30 min with mechanical shaking, and the reaction was stopped by adding 0.25 ml of cold 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The tubes were kept at 0°C for 10 min, then centrifuged at 25,000 rpm for 20 min in the SW 65 Spinco rotor. The pellets were washed three times with ice-cold 0.029 veronal acetate buffer, pH 7.4, containing 0.2 M sucrose, postfixed for 1 h with 1% (wt/vol) osmium tetroxide in 0.05 M acetate veronal buffer, pH 7.4, 0.2 M sucrose, and stained with 0.5% (wt/vol) uranyl acetate. Then the pellets were processed as described by Farquhar et al. (17). Controls were incubated in a medium without 5'AMP.

Materials

Metrizamide (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) (analytical grade) was purchased from Nyegaard and Co. (Oslo, Norway). UDP-(U-^{14}C)Gal and tryptamine bisuccinate (side chain, 2-^{14}C) were purchased from NEN Chemicals (Dreieichenchain, West Germany). *p*-Nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-2-acetamido-2-deoxy-D-glucopyranoside, Na-glucose-6-phosphate, *p*-nitrophenylthymidine-5'-phosphate, 5'AMP, and NADPH were obtained from Sigma Chemical Co. (St. Louis, Mo.). Horse heart cytochrome *c* was a product of Boehringer and Soehne (Mannheim, West Germany). Na-glycerophosphate and Triton X-100 were purchased from Merck A G Darmstadt (West Germany), and *p*-nitrophenyl phosphate from Calbiochem (San Diego, Calif.).

RESULTS

Centrifugation of the Particles in a Linear Density Gradient of Metrizamide

As our main goal was to purifiy lysosomes, we decided to make use of the light mitochondrial fraction L of de Duve et al. (15) instead of the total mitochondrial fraction ML. On average, the L fraction contains half the amount of lysosomes sedimenting in the total mitochondrial fraction and about 10% of the mitochondria recovered in this fraction (5). As mitochondria are the main components, the distribution of which overlaps that of the lysosomes after density equilibration in a metrizamide gradient, it was hoped that the L fraction would be more suitable than the total mitochondrial fraction M + L.

In a first experiment, the L fraction was layered at the top of a linear density gradient of metrizamide. Table I gives the composition in enzymes and proteins of this L fraction together with that of the other fractions isolated by differential centrifugation in 0.25 M sucrose. The enzymes are

TABLE I

Enzyme Distributions after Differential Centrifugation in 0.25 M Sucrose

		NM		L		PS		Recovery	
	Homogenate	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Cytochrome oxidase	100	86.1	86.8	6.9	4.5	7.7	2.7	100.7	94.0
Acid phosphatase	100	39.7	39.0	39.8	35.6	30.1	23.6	109.6	98.2
β-Galactosidase	100	51.2	52.7	31.0	26.0	15.8	15.4	98.0	94.1
Catalase	100	19.9	26.0	22.0	20.2	60.4	40.5	102.3	86.7
NADPH cytochrome *c* reductase	100	16.7	20.1	1.8	2.8	85.9	88.9	104.4	111.8
Alkaline phosphodiesterase	100	51.9	45.0	5.5	2.9	66.5	58.6	123.9	106.5
Proteins	100	41.1	41.4	3.7	2.9	63.1	58.3	107.9	102.6

Experimental details and meanings of the symbols NM, L, and PS are given in the text. L fraction of fractionation (a) was used for gradient centrifugation, the results of which are reported in Fig. 1; L fraction of fractionation (b) was used for gradient centrifugation, the results of which are reported in Fig. 2. Results are given as percentage values.

markers of different organelles:cytochrome oxidase is located in the inner mitochondrial membrane (22), acid phosphatase and β-galactosidase are associated with lysosomes (15, 23), and catalase is situated in peroxisomes (5). NADPH cytochrome *c* reductase is an endoplasmic reticulum enzyme (8), and alkaline phosphodiesterase is located in the plasma membrane (8). As ascertained by the amount of acid phosphatase and β-galactosidase and of proteins recovered in the L fraction, lysosomes are purified between 8 and 10 times in this fraction.

The enzyme distributions after isopycnic centrifugation are depicted in Fig. 1 A. As shown by the acid hydrolase distributions, most of the lysosomes are recovered in the upper layers, as far down as a density of about 1.15 g/ml, around a median equilibrium density of 1.136 g/ml. Most of the cytochrome oxidase, the marker for mitochondria, is found in the same region, with a main peak of activity in the fraction of 1.136 g/ml mean density. As ascertained by the distribution of catalase, peroxisomes equilibrate in the denser fractions of the gradient, exhibiting a median equilibrium den-

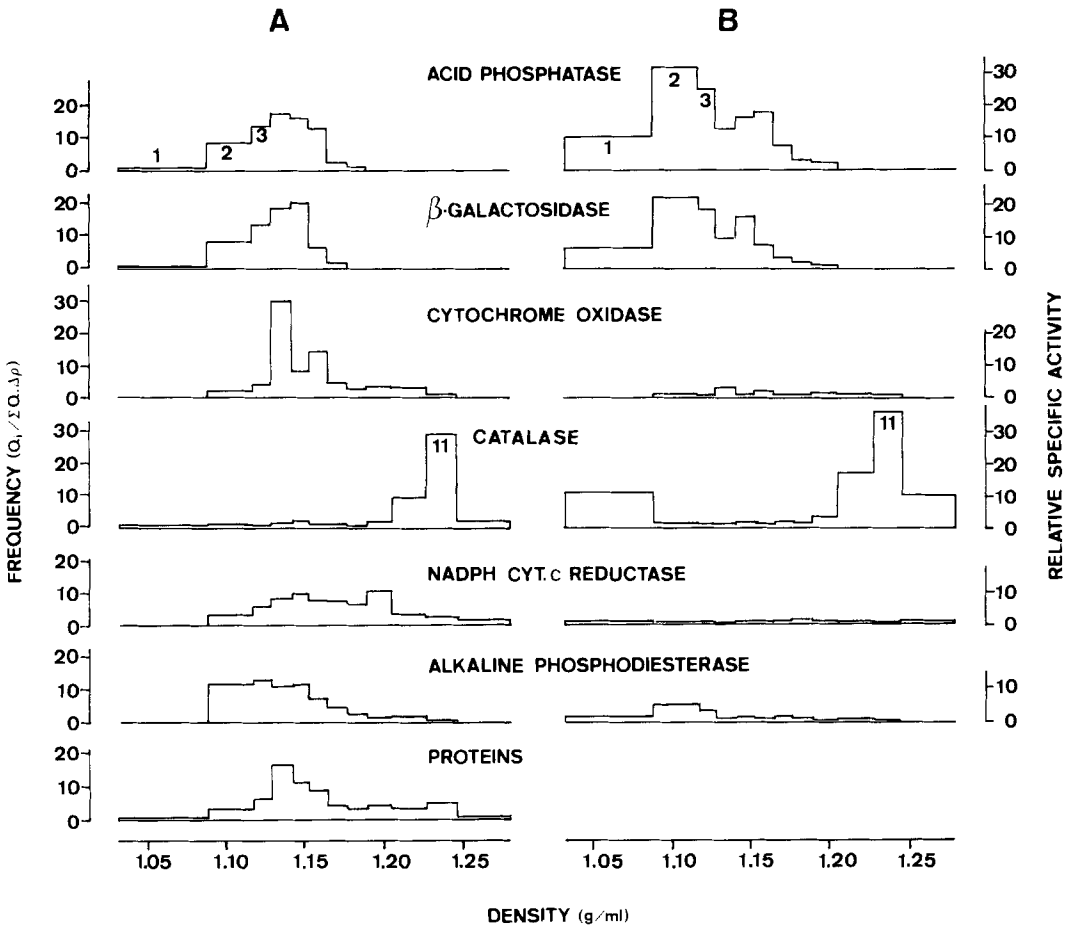

FIGURE 1 Distributions of enzymes after isopycnic centrifugation in a metrizamide gradient, of a rat liver L fraction. The granules, suspended in 0.25 M sucrose, were initially layered at the top of the gradient. The SW 65 Spinco rotor was operated for 150 min at 4°C and 108,000 *g* (r_{avg} 6.4 cm). The gradient extended from 1.105 to 1.280 g/ml density. Ordinate: (A) Average frequency of the components for each fraction: $Q/\sum Q \cdot \Delta\rho$, where Q represents the activity found in the fraction, $\sum Q$, the total recovered activity, and $\Delta\rho$, the increments of density from top to bottom of the fraction, (B) Relative specific activity, i.e., the ratio of the specific activity found in the fraction to that measured in the homogenate. Frequency and relative specific activity were plotted against density in a histogram form. Numbers are given to fractions to which reference is made in the text.

sity of 1.230 g/ml. The distribution of the endoplasmic reticulum marker NADPH cytochrome *c* reductase is flattened. Alkaline phosphodiesterase, the reference enzyme of the plasma membrane, is mainly recovered in the regions where lysosomes are found, but with a distribution shifted towards lower densities: the median equilibrium density is 1.128 g/ml. The extent to which the different components are purified in the fractions of the gradients can be appreciated in Fig. 1 B. It shows the relative specific activity of the marker enzymes, i.e., the ratio of the specific activity found in the fraction to that measured in the homogenate, as a function of the density. The highest acid hydrolase relative specific activity is found in the two upper fractions of the gradient (fractions 2 and 3) and reaches about 30 for acid phosphatase and 20 for β-galactosidase. Catalase exhibits a relative sp act of 36 in fraction 11 where it is mainly recovered. The relative specific activity of the other enzymes is considerably lower, except for phosphodiesterase which is purified 5.3 times in fraction 2.

In a second experiment, instead of layering the L fraction at the top of the gradient, we set down the granules at the bottom of the tube below the density gradient. The main reason was because we expected, as had been found by Beaufay et al. (9) with sucrose gradients, that mitochondria would exhibit under these conditions a higher equilibrium density probably as a result of the high hydrostatic pressure they are subjected to during an important part of the run (29). Thus, we hoped that a more clear-cut separation would be observed between lysosomes and mitochondria.

The composition of the fractions isolated by differential centrifugation in 0.25 M sucrose is given in Table I. It can be seen that the L fraction is comparable to the one used in the preceding experiment. The enzyme distributions observed after centrifugation in the metrizamide gradient are given in Fig. 2 A. The distributions of acid hydrolases are similar to those found in the first experiment, the median equilibrium density being 1.133 g/ml. It is to be noted that the frequency value attributed to enzymes recovered in the first fraction is higher than that found for enzymes recovered in the fraction exhibiting the same upper density limit in the previous gradient (fraction 2). This is due to the differences between the lower density limits of these fractions. Particles in 0.25 M sucrose are layered at the top of the gradient in the first experiment and, because some mixing occurs between sucrose and metrizamide at the interface, a decrease of the lower density limit of the first fraction of the gradient takes place. Cytochrome oxidase exhibits a distribution pattern comparable to the one reported in Fig. 1 but shifted towards regions of high densities; the main peak of activity is now found in a fraction of 1.162 g/ml mean density. The distribution of catalase is bimodal; a high proportion of the enzyme stays at the bottom of the tube and does not migrate. This probably originates from a disruption of peroxisomes by hydrostatic pressure, with a release of their content in the medium. It is to be noted that peroxisomes are very susceptible to hydrostatic pressure (12). The remainder of the activity is recovered around a density of 1.225 g/ml. Alkaline phosphodiesterase is again recovered in the fractions of low density. NADPH cytochrome *c* reductase shows a narrower distribution pattern than in the previous experiments, perhaps owing to the fact that the lighter endoplasmic reticulum fragments have not reached their equilibrium position. Only a few percent of the enzyme is found above a density of 1.15 g/ml. In Fig. 2 B, the purification of the components in the different fractions is illustrated. Most remarkable is the purification of lysosomes. About 25% of acid phosphatase and β-galactosidase of the homogenate is recovered in the first three fractions above a density of 1.142 g/ml, with a mean relative specific activity for the sum of the three fractions of 42.5 (acid phosphatase) and 38.0 (β-galactosidase). The most purified fraction is fraction 3; it contains 7.4% of the homogenate β-galactosidase with a relative specific activity of 53.0 and 8.9% of the homogenate acid phosphatase, exhibiting a relative sp act of 60.0. The high purification of lysosomes originates mainly from the shift of the mitochondrial distribution and to a lesser extent to the lower amount of endoplasmic reticulum fragments that equilibrate in the lower density regions.

Centrifugation of the Particles in a Discontinuous Density Gradient of Metrizamide

Taking into account the analytical centrifugation results reported in the previous secton, we devised a procedure to purify lysosomes from several grams of liver by centrifugation in a discontinuous density gradient. An L fraction was prepared and made up to a volume of 1 ml/3 g of liver with 0.25 M sucrose. 1 vol of this fraction

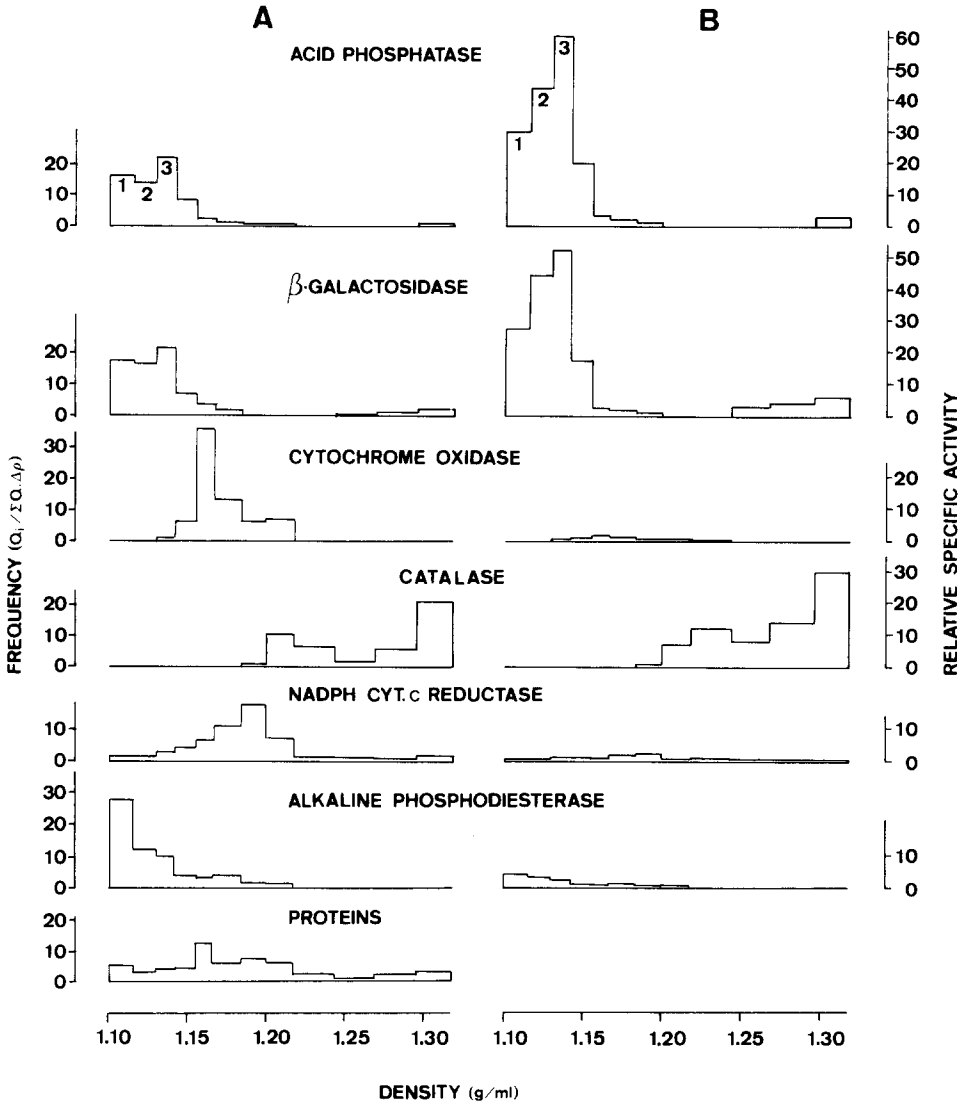

FIGURE 2 Distributions of enzymes after isopycnic centrifugation in a metrizamide gradient of a rat liver L fraction. The granules suspended in 56.7% (wt/vol) metrizamide were initially layered below the gradient. The conditions of centrifugation and the mode of representation are the same as in Fig. 1.

was added to 2 vol of 85.6% (wt/vol) metrizamide solution. 10 ml of this preparation was layered at the bottom of an SW 27 Spinco rotor tube. Then, 6 ml of 32.82% (wt/vol) metrizamide (density 1.181 g/ml), 6 ml of 26.34% (wt/vol) metrizamide (density 1.145 g/ml), 6 ml of 24.53% (wt/vol) metrizamide (density 1.135 g/ml), and 9 ml of 19.78% (wt/vol) metrizamide (density 1.109 g/ml) were successively added. Centrifugation was performed for 2 h at 95,000 *g* (r_{avg} 11.8 cm) in the Spinco SW 27 rotor at 4°C. After centrifugation, four fractions, numbered 1, 2, 3, and 4, were collected by slicing the tube with a tube slicer similar to the one described by de Duve et al. (14) as illustrated in Fig. 3.

The percentages of enzymes found in the different fractions together with their recovery are found in Table II, and in Table III, the relative specific activities with respect to the whole liver. Additional enzymes have been measured to assess more thoroughly the composition of the fractions: monoamine oxidase, marker of the outer mito-

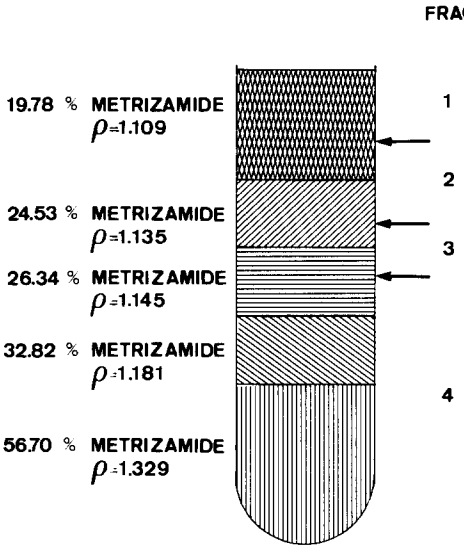

FIGURE 3 Schematic representation of the discontinuous metrizamide gradient used to purify lysosomes. Arrows indicate where the sections are made after centrifugation to give fractions 1, 2, 3, and 4.

chondrial membrane (22), and glucose-6-phosphatase, which is associated with endoplasmic reticulum. Glucose-6-phosphatase was measured in the presence of 10 mM tartrate to prevent the action of acid phosphatase on glucose-6-phosphate (11). This precaution is necessary when the preparation contains high amounts of acid phosphatase and is endowed with low glucose-6-phosphatase activity. Three plasma membrane marker enzymes were determined: alkaline phosphodiesterase, alkaline phosphatase, and 5′ AMPase; the latter enzyme was tested in the presence of 10 mM tartrate to inhibit acid phosphatase. *N*-acetylglucosaminidase, a lysosomal enzyme (23), and galactosyltransferase, an enzyme present in the Golgi apparatus membrane (8), were also measured. However, as reported in Material and Methods, the transferase is inhibited by metrizamide and cannot be measured directly as can the other enzymes in the gradient fractions. In the experiments reported in Table II, the enzyme was measured only on the fractions obtained by differential centrifugation, showing that only 1.5% of the homogenate activity is found in the L fraction.

Later, we found that, by comparing aliquots of the L fraction suspended in 0.25 M sucrose or in various metrizamide concentrations, >90% of the galactosyltransferase could be recovered by centrifuging the preparations and discarding the supernate which contains the metrizamide, on condition that metrizamide concentrations to not exceed 25% (wt/vol). Table IV gives the results of two experiments in which the Golgi enzyme was determined by this procedure in the subfractions isolated by gradient centrifugation. The metrizamide concentration in fractions 1 and 2 is lower than 25% (see Fig. 3): therefore galactosyltransferase activity measured in these fractions is a reliable estimation of the amount of the Golgi enzyme present in these fractions. On the other hand, the activity found in fractions 3 and 4 is surely underestimated. It can be seen that the purified lysosome preparation (fraction 2) only contains 0.2–0.3% of the homogenate galactosyltransferase activity.

As estimated by the acid hydrolase activity, lysosomes are markedly purified in fractions 1, 2, and 3 of the gradient. The most interesting fraction is fraction 2 which contains material collected at the interface between the two upper layers. 10–12% of the acid hydrolases, exhibiting a mean relative sp act of 66–80, are recovered in this fraction.

Using the relative specific activity of the marker enzymes present in the gradient fractions and the percentage of total liver proteins constituted by the different membranes, we estimated the proportion of protein of each fraction contributed by the different membranes. We made use of the relationship given by Leighton et al. (18):

$$100 = aA + bB + cC + dD + eE + fF + X,$$

in which a, b, c, d, e, and f are the percentages of the total liver proteins constituted by the mitochondria, outer mitochondrial membrane fragments, endoplasmic reticulum, plasma membrane, Golgi, and peroxisomes, respectively. A, B, C, D, E, and F are the relative specific activities of cytochrome oxidase, monoamine oxidase, glucose-6-phosphatase, plasma membrane enzymes (mean of the three enzymes), galactosyltransferase, and catalase. We supposed that cytochrome oxidase is a marker for intact mitochondria and that the percentage of monoamine oxidase, minus that of cytochrome oxidase, gives the proportion of the free outer mitochondrial membrane.

The products aA, bB, cC, dD, eE, and fF give the percentage of the proteins present in the fraction that may be attributed to each membrane component. X corresponds to the percentage of lysosomal proteins together with that of the unas-

TABLE II

Enzyme Distributions after Fractionation of the L Fraction in a Discontinuous Metrizamide Gradient

Enzyme	No. of exp	Content of liver	NM	1	2	3	4	PS	Recovery
Proteins	10	225.4 ± 39.3	43.5 ± 4.2	0.13 ± 0.02	0.15 ± 0.05	0.12 ± 0.03	1.98 ± 0.35	50.6 ± 5.6	96.5 ± 3.6
Acid phosphatase	10	11.19 ± 2.52	40.4 ± 3.6	4.6 ± 1.3	12.0 ± 2.1	4.3 ± 1.0	7.4 ± 1.2	25.5 ± 4.2	94.2 ± 7.3
β-Galactosidase	9	0.646 ± 0.186	54.2 ± 3.4	3.8 ± 1.4	9.5 ± 3.0	4.2 ± 0.8	7.6 ± 0.7	18.4 ± 3.2	97.7 ± 2.7
N-Acetylglucosaminidase	5	2.95 ± 0.48	53.3 ± 8.5	3.3 ± 0.9	11.1 ± 4.2	5.1 ± 0.8	9.7 ± 0.9	16.2 ± 3.2	98.7 ± 7.3
Monoamine oxidase	6	0.206 ± 0.030	61.2 ± 11.3	0.10 ± 0.03	0.10 ± 0.08	0.32 ± 0.19	3.75 ± 0.97	25.5 ± 4.3	91.0 ± 9.5
Cytochrome oxidase	7	39.2 ± 17.4	81.1 ± 12.4	0.02 ± 0.01	0.03 ± 0.03	0.04 ± 0.01	1.96 ± 0.56	6.4 ± 2.8	89.7 ± 11.9
NADPH Cyt *c* reductase	7	6.93 ± 2.01	23.2 ± 10.2	0.06 ± 0.03	0.04 ± 0.02	0.08 ± 0.05	1.48 ± 0.90	77.2 ± 11.8	102.1 ± 6.7
Glucose-6-phosphatase in presence of 10 mM tartrate	5	18.5 ± 2.3	17.7 ± 5.3	0.03 ± 0.02	0.04 ± 0.02	0.07 ± 0.08	2.80 ± 1.36	74.9 ± 8.7	95.5 ± 3.0
Alkaline phosphodiesterase	9	17.3 ± 5.7	46.1 ± 5.7	1.26 ± 0.17	1.27 ± 0.30	0.62 ± 0.28	1.85 ± 0.42	58.0 ± 6.1	109.1 ± 5.9
Alkaline phosphatase	5	1.66 ± 0.54	32.3 ± 2.2	0.83 ± 0.23	1.42 ± 0.60	0.70 ± 0.18	2.20 ± 1.16	53.6 ± 4.8	91.0 ± 3.3
5'Nucleotidase in presence of 10 mM tartrate	6	4.17 ± 1.18	59.8 ± 10.7	1.25 ± 0.65	0.88 ± 0.33	0.44 ± 0.18	0.93 ± 0.25	44.6 ± 6.0	107.9 ± 9.2
Catalase	3	62.0 ± 15.6	30.7 ± 7.3	0.04 ± 0.03	0.02 ± 0.01	0.02 ± 0.00	31.8 ± 8.4	20.5 ± 2.7	83.0 ± 15.4
Galactosyltransferase	7	0.016 ± 0.003	12.4 ± 3.1		1.4 ± 0.7 (unfractionated L)			77.9 ± 4.7	91.7 ± 2.3

Absolute values are given in mg/g for proteins and in U/g fresh weight of liver for enzymes. Content of fractions are given as percentage values. The results are given as means ±SD. Fractions 1, 2, 3, and 4 originate from the fractionation of L by centrifugation in metrizamide gradient.

TABLE III

Purification of Lysosomes by Centrifugation of the L Fraction in a Discontinuous Metrizamide Gradient

Enzymes	Gradient fractions from L			
	1	2	3	4
Acid phosphatase	37.1 ± 10.7	80.0 ± 11.8	39.4 ± 11.9	3.9 ± 0.9
β-Galactosidase	31.2 ± 11.7	64.1 ± 15.5	38.7 ± 6.9	4.0 ± 1.0
N-Acetylglucosaminidase	26.2 ± 6.5	66.5 ± 8.4	45.3 ± 7.5	4.8 ± 1.1
Monoamine oxidase	0.78 ± 0.18	0.56 ± 0.42	2.82 ± 1.12	1.92 ± 0.36
Cytochrome oxidase	0.17 ± 0.06	0.17 ± 0.17	0.33 ± 0.05	0.72 ± 0.40
NADPH cyt. *c* reductase	0.51 ± 0.31	0.27 ± 0.20	0.77 ± 0.40	0.76 ± 0.47
Glucose-6-phosphatase	0.25 ± 0.11	0.23 ± 0.08	0.56 ± 0.41	1.32 ± 0.58
Alkaline phosphodiesterase	10.5 ± 1.9	8.8 ± 2.2	5.4 ± 1.5	1.0 ± 0.2
Alkaline phosphatase	6.0 ± 1.5	8.7 ± 1.5	5.5 ± 1.4	1.0 ± 0.5
5'Nucleotidase	9.3 ± 4.1	5.4 ± 1.1	3.3 ± 0.9	0.4 ± 0.1
Catalase	0.28 ± 0.20	0.13 ± 0.07	0.13 ± 0.04	15.1 ± 3.0

Results are given in relative specific activity, i.e. specific activity found in the fraction/specific activity measured in the homogenate. Values given are means ± SD. Calculations were made with the data of experiments reported in Table II.

TABLE IV

Galactosyltransferase and Acid Hydrolase Activity of the Gradient Fractions

Enzyme	no. exp	NM	1	2	3	4	PS	Recovery
Galactosyltransferase	1	16.1	0.48	0.28	0.04	0.02	78.2	96.1
		(0.4)	(5.8)	(1.9)	(0.3)	(0.01)	(1.6)	
	2	11.6	0.65	0.38	0.1	0.13	68.5	80.4
		(0.3)	(4.3)	(3.8)	(0.7)	(0.06)	(1.4)	
Acid Phosphatase	1	38.8	4.2	12.8	5.7	7.6	28.0	97.1
		(0.97)	(38.0)	(85.5)	(41.0)	(4.1)	(0.58)	
	2	43.5	3.8	10.6	6.1	7.9	21.0	92.9
		(0.96)	(25.4)	(106.0)	(44.5)	(3.9)	(0.42)	
β-Galactosidase	1	51.5	3.6	10.4	5.3	7.5	17.2	95.5
		(1.3)	(30.0)	(69.5)	(38.1)	(4.0)	(0.35)	
	2	57.2	3.2	7.6	3.8	7.2	16.3	95.3
		(1.3)	(21.3)	(76.0)	(27.2)	(3.5)	(0.32)	
Proteins	1	40.0	0.12	0.15	0.14	1.85	49.5	91.8
	2	45.1	0.15	0.10	0.14	2.04	50.5	98.0

Activities of fractions 1, 2, 3, and 4 have been measured on pellets obtained by centrifuging the fractions for 30 min at 78,100 *g* (r_{avg} 7.8 cm) in the Spinco 30 rotor. Contents of fractions are given as percentage values. Relative specific activity in parentheses.

signed proteins. Results of the calculation are given in Table V. X reaches a value of 68.1 in fraction 2 where lysosomes are mainly recovered and are the most extensively purified. Taking into account the different marker enzymes we have measured, it seems justified to suppose that the unidentified components are negligible in the fraction. Thus, lysosomal proteins would represent about 70% of the fraction 2 proteins.

According to these calculations, the main contaminant of the lysosomes appears to be the plasma membrane. So the question arises: Does alkaline phosphodiesterase, alkaline phosphatase, and 5'AMPase activity only originate from plasma membrane fragments? Generally, reference enzymes of plasma membranes are hydrolases: in this case they are phosphodiesterase, 5'-nucleotidase, and alkaline phosphatase. In our work, a particular problem arises because these enzymes have to be measured in a purified lysosome preparation. Indeed, acid hydrolases located in lysosomes may still be endowed with a slight but significant activity at alkaline pH on substrates used to determine plasma membrane enzymes. In some cases, one can remedy this situation, by making use of a selective inhibitor for the acid

TABLE V

Percentage of Total Proteins of Gradient Fractions Contributed by Different Components

Components	Whole liver	Gradient fractions from L			
		1	2	3	4
Mitochondria	20.2	3.4	3.4	6.7	14.5
Outer mitochondrial membrane	0.8	0.6	0.4	2.3	1.5
Endoplasmic reticulum	21.5	5.4	4.9	12.0	26.4
Plasma membrane*	2.6	22.4	19.7	12.2	2.1
Peroxisomes	2.53	0.7	0.3	0.3	38.2
Golgi	1.15	5.8	3.2	0.6	0.03
Lysosomes and unassigned components (X)		61.7	68.1	65.3	17.3

Calculations were performed as described in the text; percentages of total liver protein constituted by different membrane components were taken from published results; mitochondria, endoplasmic reticulum, and peroxisomes from Leighton et al. (18), outer mitochondrial membrane from Schnaitman and Greenawalt (22), plasma membrane and Golgi membranes from Beaufay et al. (8).

* See text for the meaning of that contamination.

hydrolase; for example, 10 mM of tartrate inhibits lysosomal acid phosphatase but does not inhibit 5′-nucleotidase of the plasma membrane. Another point must also be considered, i.e., neutral or alkaline hydrolases may be present in lysosomes after being taken from the blood by endocytosis. This has been illustrated by Bartholeyns et al. (3). These authors show that rat liver lysosomes contain a neutral RNase, identical to the pancreatic enzyme and originating from the blood plasma. Thus, contamination of the lysosomes by plasma membrane components may be overestimated if only the total activity of the supposed plasma membrane enzyme is measured. If part of the alkaline phosphodiesterase, 5′-AMPase and alkaline phosphatase activity present in fraction 2 is due to the hydrolases present inside the lysosomes, it would probably be solubilized after disrupting the lysosomal membrane.

We therefore subjected a purified lysosome preparation to treatments which disrupted the lysosomal membrane: freezing and thawing, hypotonicity, treatment in an ultraturrax. After high-speed centrifugation, alkaline phosphodiesterase, alkaline phosphatase, and 5′-AMPase were determined in the pellet and the supernate. The disruption of the lysosomal membrane was checked by measuring the release of β-galactosidase. We found that the treatment solubilized 25–40% of the three alkaline enzymes. This result indicates that part of the alkaline phosphodiesterase, the alkaline phosphatase, and the 5′-AMPase measured in the lysosome preparation originates from enzymes, that, like lysosomal hydrolases, are present in the granule matrix and are not associated with a membrane component. It strongly suggests that the total activity of the enzymes taken as markers for the plasma membranes gives an overestimation of contamination by this component.

Structure-Linked Latency of Acid Hydrolases in Purified Lysosomes

To appreciate the structural integrity of the membranes of lysosomes purified in the metrizamide gradient, the free activity of acid phosphatase was measured in fraction 2 of two preparations, and also in the L fraction. In the latter, the free activity represented 5.3 and 9.4% of the total activity, but in fraction 2, only 6.9 and 6.1%. This indicates that the lysosomal membrane did not deteriorate during centrifugation in the metrizamide gradient.

Morphological Examinations

In three experiments, fractions 1, 2, and 3 were examined in the electron microscope. Results of one of these experiments are illustrated in Figs. 4–8. The enzyme composition of these fractions is given in Table VI.

Fig. 4 shows the general aspect of the most purified fraction, fraction 2, and Fig. 5 illustrates, at a higher magnification, the main constituents of this fraction. These constituents are typical dense bodies, endowed with a electron-lucent rim beneath their membrane. They are rounded (Fig. 5*A*) or elongated (Fig. 5*B*). Frequently they contain a vacuole that may give to the organelle a ring shape (Fig. 5*C*). It should be noted that such a picture could also originate from the area of a horseshoe structure open to the cytosol and which appears as a vacuole because of the plane of the

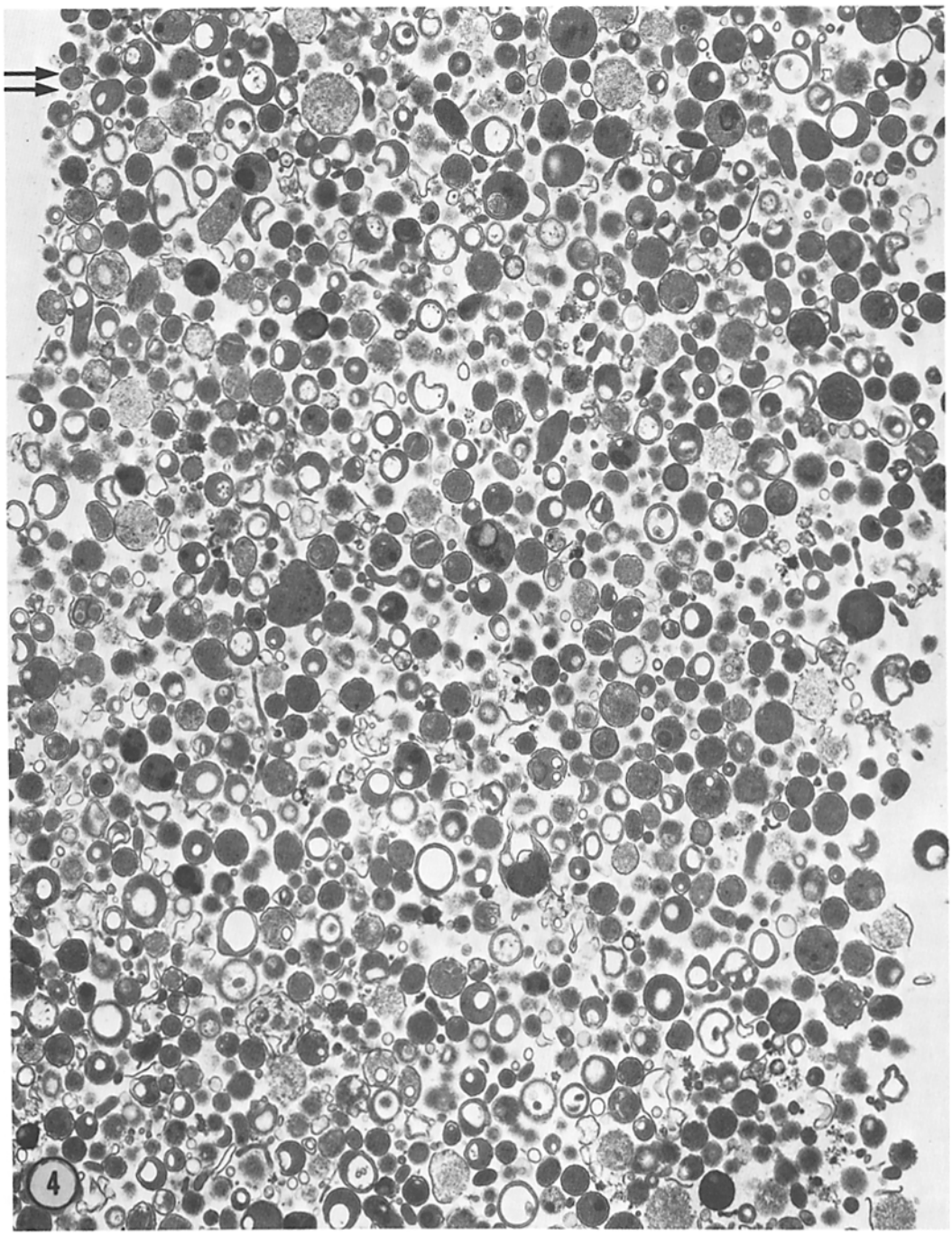

FIGURE 4 Random sample through the depth of the pellicle of fraction 2, the most purified lysosomal fraction. Double arrow: surface contact with the Millipore filter. × 14,200.

section. Other frequent constituents are residual bodies (Fig. 5 *D*). In addition to these typical lysosomes, several kinds of organelles are present in smaller amount, in particular annular structures (Fig. 5 *E*), and coated vesicles (Fig. 5 *F*).

Figs. 6 and 7 show the morphological aspects of fractions 1 and 3. In both fractions, the main elements observed in fraction 2 are present. One

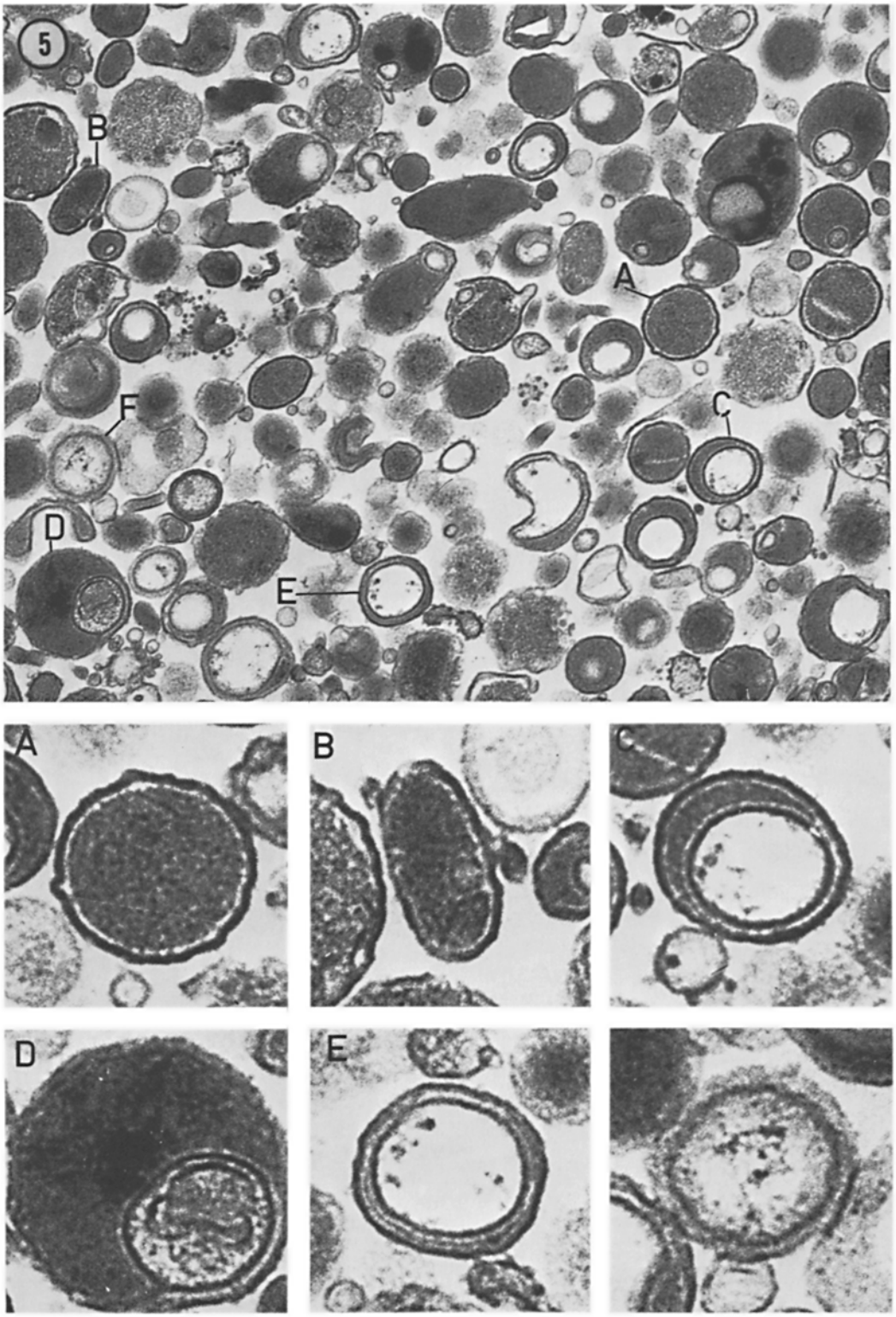

FIGURE 5 Upper part: enlargement of an area of Fig. 4. × 35,500. Lower part: main constituents of fraction 2 shown at high magnification: rounded (*A*), elongated (*B*), ring-shaped (*C*) dense bodies, residual bodies (*D*), annular profile (*E*), and coated vesicle (*F*). × 90,200.

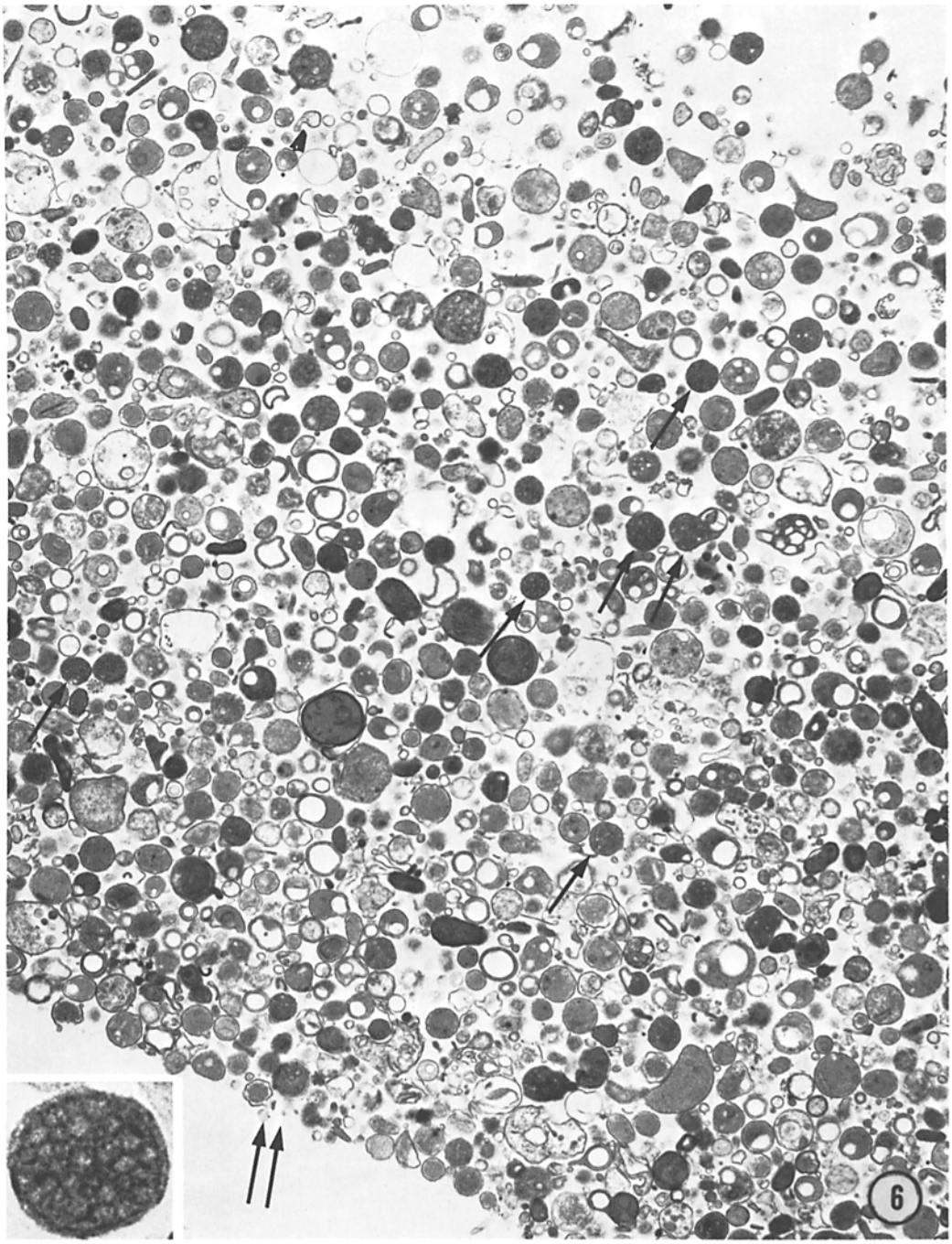

FIGURE 6 Random sample through the depth of the pellicle of fraction 1. The main constituents seen in fraction 2 are present. In addition, several bodies containing vesicles (single arrows and *inset*) are apparent. Double arrow: surface contact with the Millipore filter. × 14, 200; *inset*, × 72,700.

characteristic of fraction 1 seems to be the occurrence of organelles containing many vesicles. They could be multivesicular bodies or Golgi secretory vesicles (16). Fraction 3 is relatively heterogeneous: tubular structures are frequent.

Acid phosphatase cytochemical tests were car-

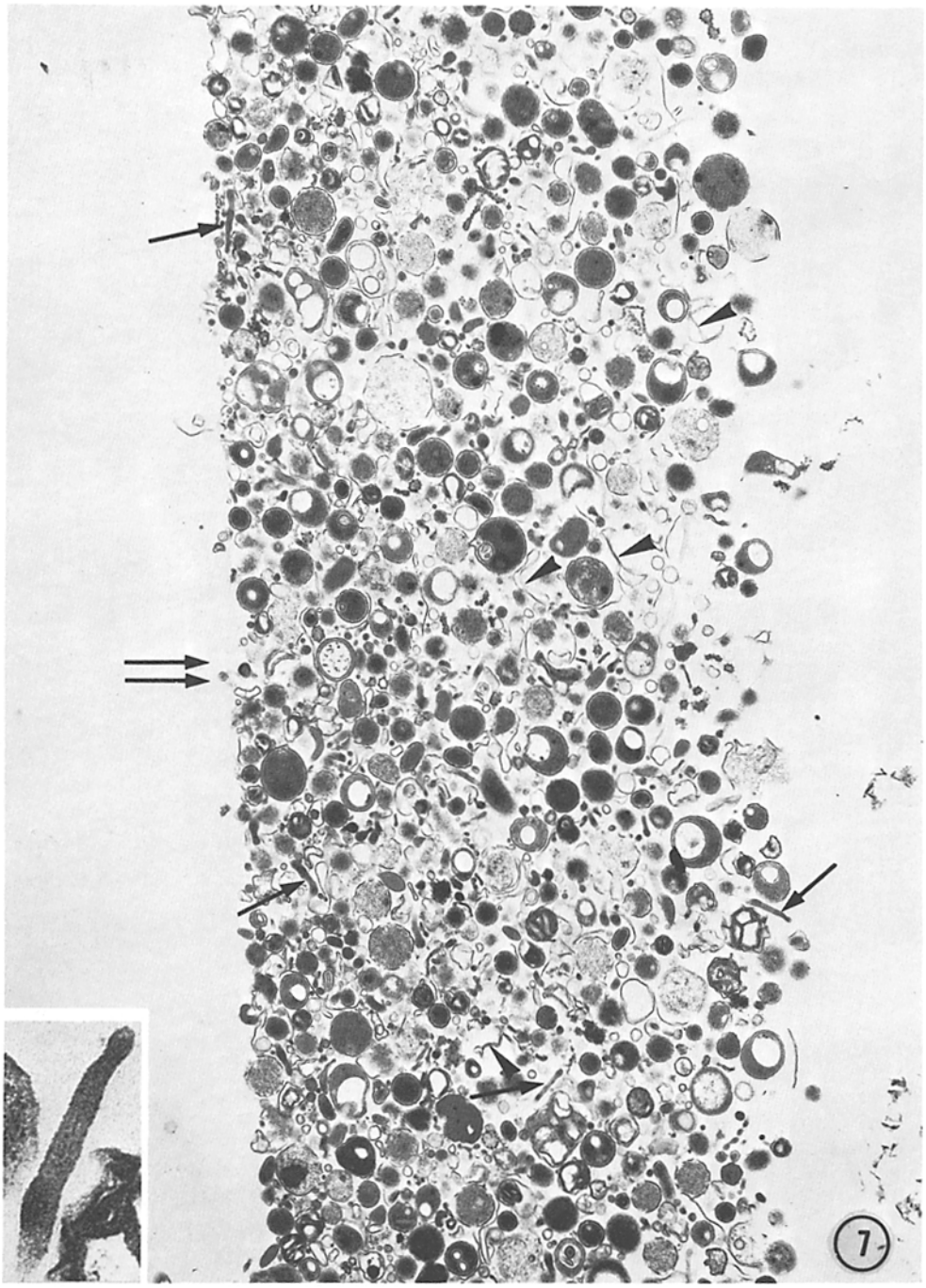

FIGURE 7 Random sample through the depth of the pellicle of fraction 3. The main constituents seen in fraction 2 are present, together with membrane fragments (arrowheads) and numerous tubular components (singel arrows and *inset*). Double arrow: surface contact with the Millipore filter. × 14,200; *inset*, × 72,700.

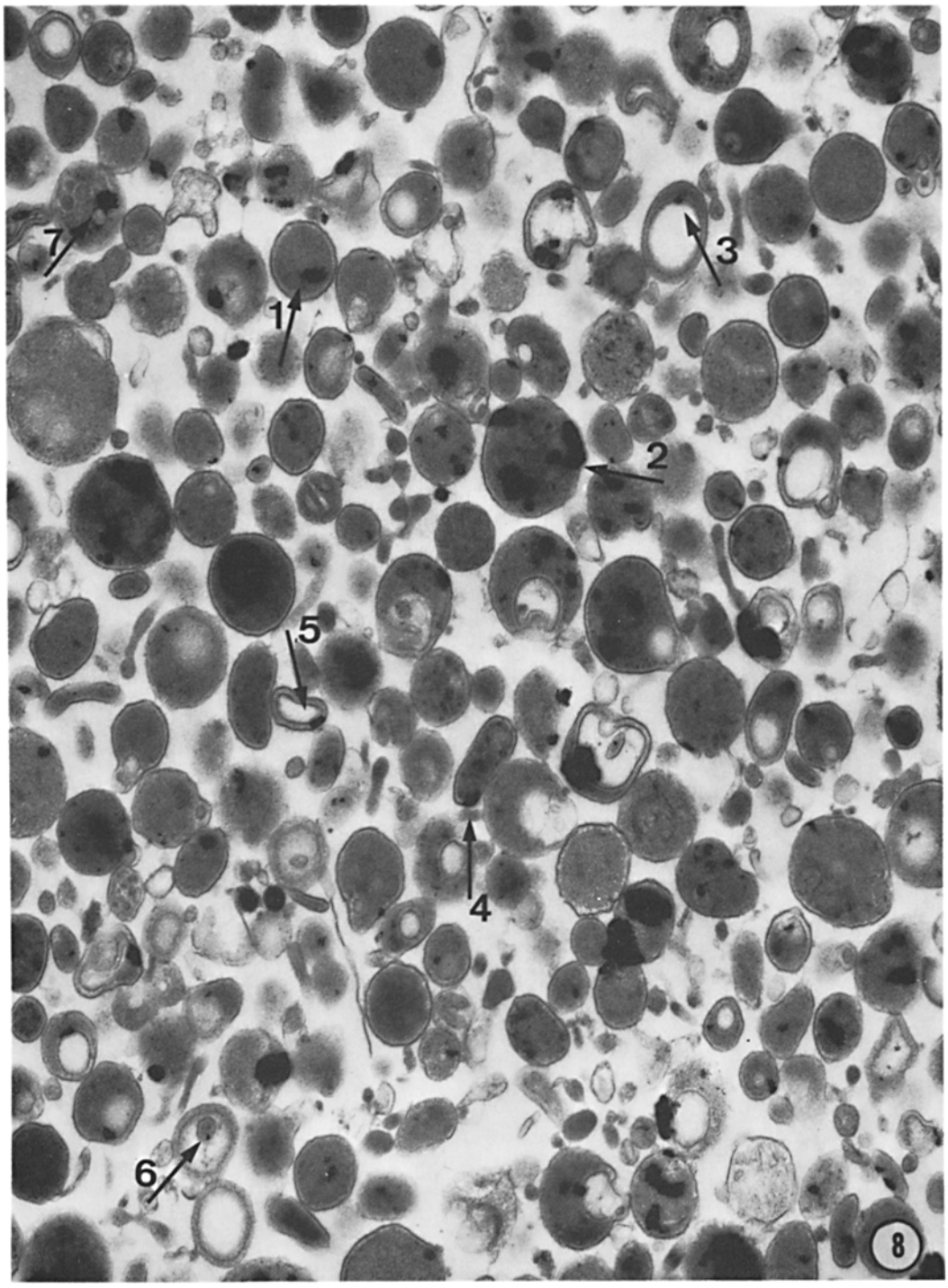

FIGURE 8 Acid phosphatase cytochemical test on fraction 2. The test shows that the different kinds of components may contain the reaction product: rounded (*1*), residual (*2*), ring-shaped (*3*), elongated (*4*) dense bodies, annular profile (*5*), coated vesicle (*6*), and body containing vesicles (*7*). × 35,500.

TABLE VI

Enzyme Content of Fractions Examined in the Electron Microscope

Enzyme	Fraction 1		Fraction 2		Fraction 3	
	(a)	(b)	(a)	(b)	(a)	(b)
Acid phosphatase	3.8	40.7	9.3	81.7	4.5	63.8
Cytochrome oxidase	0.018	0.18	0.014	0.12	0.024	0.34
Monoamine oxidase	0.049	0.5	0.036	0.32	0.11	1.61
Glucose-6-phosphatase	0.021	0.21	0.029	0.25	0.024	0.34
NADPH cytochrome *c* reductase	0.079	0.81	0.050	0.44	0.070	0.99
Alkaline phosphodiesterase	1.14	11.6	1.03	9.04	0.48	6.76
Proteins	0.098		0.114		0.071	

The fractions have been obtained after centrifugation in a discontinuous metrizamide gradient as described in the text. (a) Percentage values with respect to whole liver; (b) relative specific activity.

ried out on fraction 2; a large proportion but not all of the organelles was labeled. However, each kind of organelle quoted above may give a positive reaction as shown in Fig. 8.

Taking into account the possible contamination of the lysosome preparation by plasma membrane components, 5'AMPase cytochemical tests were performed on fraction 2 in the presence of fluoride, to inhibit acid phosphatase. As shown in Fig. 9, many structures which were acid phosphatase-positive were labeled after the 5'AMPase cytochemical test. The reaction product is located mainly on the outside (cytoplasmic) face of the membrane. Thus, a high proportion of the 5'AMPase found in fraction 2 seems to be associated with the membrane of structures which, according to the acid phosphatase cytochemical test, may be considered as lysosomes.

DISCUSSION

The problem of liver lysosome isolation has been thoroughly discussed by Beaufay (7). He noticed that the distribution curve of sedimentation coefficients of mitochondria and peroxisomes overlaps that of the lysosomes too much to expect that a good separation of lysosomes from the mitochondria and the peroxisomes could be achieved by differential centrifugation in sucrose. Nevertheless, Ragab et al. (20) have published a method based on differential centrifugation, that leads to a 24 times purification of acid phosphatase with a yield of 6.7%. However, as stated by Beaufay (7), such preparations are probably heavily contaminated with peroxisomes.

Stahn et al. (24) combined differential centrifugation and carrier-free continuous electrophoresis to isolate rat liver lysosomes. There are two drawbacks to this method. Firstly, the relative specific activities of the lysosomal enzymes measured considerably differ from one enzyme to another, and thus it is difficult to truly appreciate the extent of purification. Secondly, the yield is rather poor, only about 4%.

Complete purification of normal lysosomes by isopycnic centrifugation in sucrose and in glycogen gradients seems also impossible, owing chiefly to an inevitable contamination by peroxisomes. The isolation of liver lysosomes by density equilibration in sucrose is possible only if the density of these granules has been selectively changed by treatment of the animal. If a compound reaches the lysosomes but cannot be digested at a sufficiently fast rate, it accumulates in the organelles. The latter increase in size, and their density changes. The density modification may be sufficient to allow lysosomes to migrate, in a sucrose gradient, far from their normal equilibrium density and, thus, far from the site where contaminating particles, chiefly peroxisomes, equilibrate. The method that is most widely used is that of Trouet (26) which has been refined by Leighton et al. (18). It is founded on the accumulation of Triton WR 1339, a nonionic detergent in lysosomes (28, 30). Trouet's method allows a purification of about 30 with a yield of 20%; the Leighton method allows a purification that approaches 50 with a yield of 8%. Amongst the other methods based on lysosome density changes, let us quote one where lysosomes are loaded with dextran (7) or with iron (2). In the dextran method, the lysosomes are purified 43 times, with a yield of 12%; in the Arborgh method (2), the yield is 11 and the purification, 33. Obviously, a major drawback to these meth-

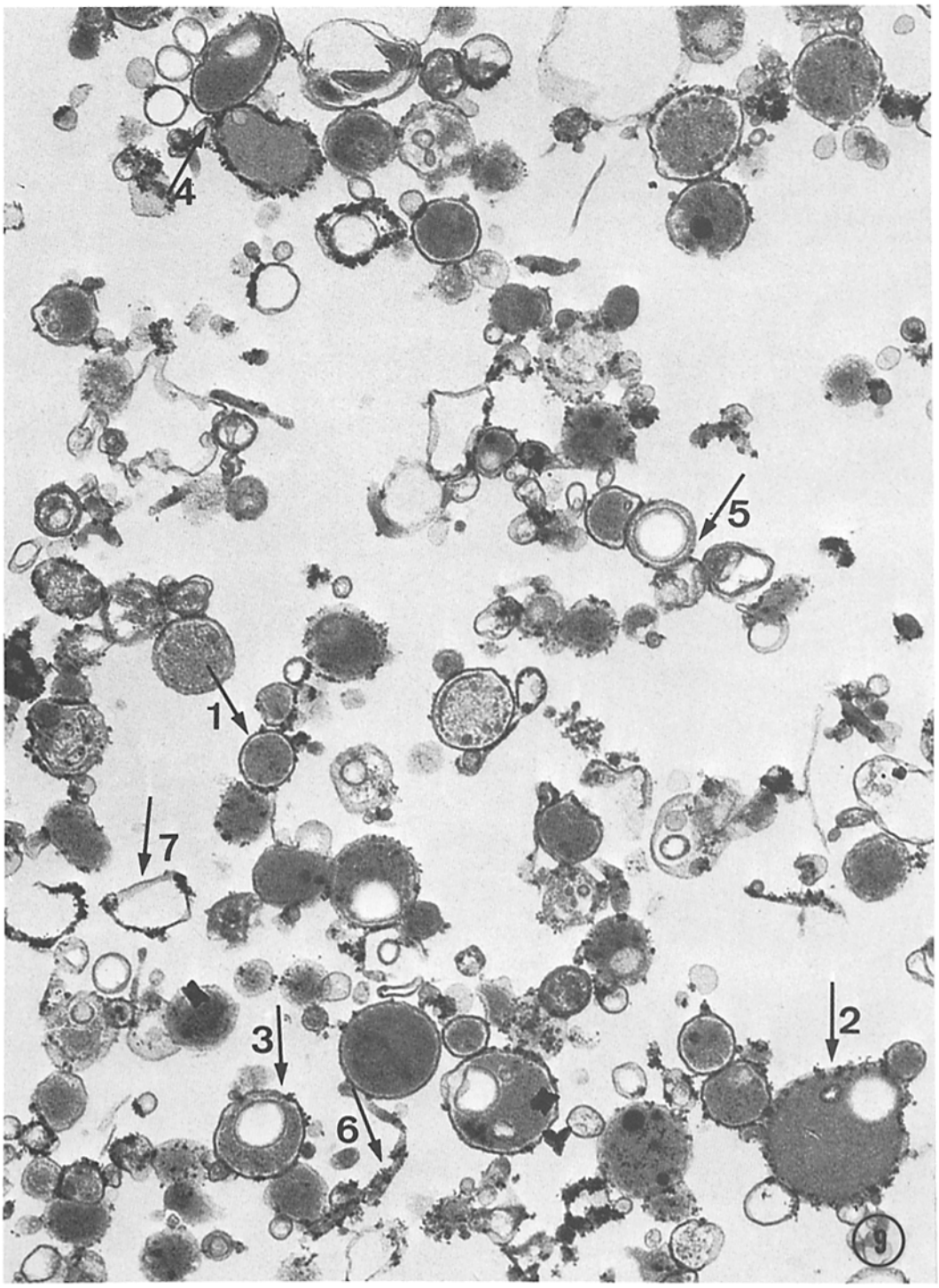

FIGURE 9 5'AMPase cytochemical test on fraction 2. The test was performed in the presence of 10 mM NaF to inhibit acid phosphatase. Most of the structures that, according to Fig. 8, were acid phosphatase-positive are 5'AMPase-positive. The reaction product is chiefly located at the outside face of the membrane. Rounded (*1*), residual (*2*), ring-shaped (*3*), elongated (*4*) dense bodies, and annular profile (*5*). In addition, the reaction product is found on tubular structures (*6*) and empty vacuoles (*7*) of undetermined origin (empty lysosomes?, plasma membrane vesicles?). × 35,500.

ods is that they require treatment of the animals and that the lysosomes are abnormal. For example, the stability in vitro of lysosomes loaded with Triton WR 1339 differs markedly from that of normal lysosomes (28).

Taking into account the purification and the yield, the method we describe in this paper is superior to the other published methods for the purification of rat liver lysosomes that do not need any treatment of the animal. In fraction 2, recovered between densities 1.109 and 1.135, acid hydrolases are purified from 68 to 80 times with a yield of 10–12%. This is at least as good as the method of Leighton et al. (18) which makes use of Triton WR 1339-injected rats. Moreover, the method has the great advantage that it does not require a preliminary treatment of the animals; thus lysosomes are definitely normal.

A relatively high amount of plasma membrane marker enzymes is found in our lysosome preparation. According to Thinès-Sempoux (25), alkaline phosphodiesterase in an amount similar to what we found is associated with lysosomes isolated after the injection of rats with Triton WR 1339. (Leighton et al. [18] did not consider the possible contamination of lysosomes with plasma membranes.) As we have already stated, a significant part of alkaline phosphodiesterase, alkaline phosphatase, and 5'AMPase originates from enzymes present in the lysosome matrix and thus cannot be considered as membrane markers. On the other hand, when 5' AMPase is cytochemically localized in fraction 2, it is mainly associated with the membrane of organelles which are acid phosphatase-positive and may thus be considered as lysosomes. This result suggests that most of the plasma membrane enzyme activity we have determined is not located in the contaminating plasma membrane fragments, but in the lysosomal membrane. Obviously, this observation compells us to reconsider the amount of plasma membrane protein that could be present in the lysosome preparation. It must be markedly lower than one can estimate by supposing that the alkaline hydrolase activity is associated only with the contaminating plasma membrane fragments.

The data listed in Table V estimate the lysosomal protein content of the liver. In the most purified fraction, fraction 2, the percentage of the lysosomal and of the unassigned protein is 68.1. However, as stated above, this amount is underestimated since a high proportion of the plasma marker enzymes is in fact associated with lysosomal structures. It is probable that the lysosomal and the unassigned protein represent 80–90% of the protein associated with fraction 2. If we suppose that the amount of the unassigned protein is negligible, we may deduce that lysosomal proteins represent 1–1.4% of the total liver proteins, according to whether acid phosphatase, β-galactosidase, or *N*-acetylglucosaminidase is taken as reference enzyme. Such a value is lower than that (2%) given by Leighton et al. (18), whose method is based on lysosomes purified after injection of Triton WR 1339. As suggested by these authors and by Baudhuin et al. (5), this could be explained if the Triton WR 1339 treatment causes an increase of the lysosomal membranes and an accumulation of plasma lipoproteins in lysosomes. On the other hand, it is significantly higher than the amount (0.8%) that Baudhuin proposes according to his morphometric data (4). There are two possible interpretations to this discrepancy: First, our supposition that unassigned protein in fraction 2 is negligible could be incorrect. Our marker enzyme determinations would not give full information on all the membrane consitituents, or the enzymes would not exhibit the same specific activity in each component of the organelles with which they are associated. A second possibility is that, in the morphometric determinations, some components of the lysosomal system were not considered as a consequence of the pleomorphic aspect of lysosomes.

Examination of the fractions in the electron microscope illustrates the morphological heterogeneity of lysosomes. The results are in agreement with numerous cytochemical observations *in situ* that show the various forms that acid phosphatase-positive structures can take.

To conclude, we will make a brief comment on the possible use of the metrizamide gradient to purify peroxisomes. Indeed, as shown by catalase distribution, after centrifugation in a linear density gradient, peroxisomes are recovered far from the lysosomes and the mitochondria at the bottom of the gradient. The relative specific activity of catalase is as high as the activity that can be obtained by the method of Leighton et al. (18), the best method at present for purifying rat liver peroxisomes. Up to now, we have not extensively investigated the question.

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