A NEW METHOD FOR THE PREPARATION OF RAT LIVER LYSOSOMES

Separation of Cell Organelles of Rat Liver by Carrier-Free Continuous Electrophoresis

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ABSTRACT

A combination of differential centrifugation and carrier-free continuous electrophoresis is introduced as a new method for the isolation of animal cell organelles. Various buffers were systematically checked in order to find the system which preserves the organelles and gives as well a good separation in the free-flow electrophoresis apparatus. Triethanolamine-acetate buffer (10 mM), pH 7.4 was used. The isolated lysosomes were pure according to marker enzymes and electron micrographs. A heterogeneity of the lysosomes in electrophoretic mobility was demonstrated with respect to the marker enzymes arylsulfatase and β-glucuronidase. The lysosomes with higher mobility showed a maximum enrichment of 240-fold with respect to arylsulfatase. The lysosomes with lower electrophoretic mobility showed a 65-fold enrichment with respect to β-glucuronidase. The ratio of β-glucuronidase to arylsulfatase varied from 2:1 to 1:2 in lysosomes of different mobility. The yield amounted to approximately 1 mg of lysosomal protein per gram of liver protein. 5–8 mg of lysosomes can be obtained in one experiment. The electrophoretic separation proves to be an effective tool in obtaining pure and well preserved lysosomes.

INTRODUCTION

The methods which have been applied so far for the preparation of subcellular particles, such as lysosomes, are based upon the well established methods of cell fractionation, i.e. differential centrifugation, density gradient centrifugation, isopycnic centrifugation, and combinations of these methods (1–5).

The property of lysosomes to accumulate dextran and Triton WR-1339 selectively has been utilized for their separation. The loaded lysosomes have a density different from that of the other cell organelles (4, 6). By these methods, the following enrichments in lysosomes have been achieved: 16-fold by centrifugation in a glycogen-sucrose-gradient (4), 20-fold by combination of differential centrifugation and density gradient centrifugation (7), and 80–100-fold by differential centrifugation together with an isopycnic gradient centrifugation in a sucrose-D_2O-gradient. The latter method, however, is difficult to reproduce (8). Several experiments have been carried out which take advantage of other specific properties

of lysosomes for their separation, for example, their protein composition and their ferritin content (2).

Electrophoresis, as a further method for the separation of cell organelles, has not been used very often. It is a method based upon the fact that the membranes of the lysosomes (9) and the other cell organelles have a negative surplus charge at physiological pH. Davenport (10) separated rat liver mitochondria from micorosomes by the use of a density-gradient electrophoresis. The individual cell particles were characterized by their marker enzymes. Plummer (11) was the first to point out that rat liver mitochondria have a defined migration rate in an electric field.

Only after Hannig had described his carrier-free continuous electrophoretic apparatus (12), did it become practicable to apply the electrophoresis as a preparative method. Several authors have reported the efficiency of this apparatus for the separation of cells of different origin and also plant cell organelles (13–15). We, therefore, used this method for the separation of animal cell organelles as described in the following.

MATERIALS AND METHODS

Preparation of the Lysosomal Fraction for Electrophoresis

6–10 Sprague-Dawley rats yielding approximately 60–70 g of liver (wet weight) were killed by cervical dislocation, after having been starved for 48 hr. They were then bled, and the livers quickly removed. The livers were minced, washed twice, and suspended in buffer in the ratio of 1:3 (w/v).

10 mM of triethanolamine-acetate buffer, pH 7.4, was used, containing 1 mM of EDTA.Na_2 and 0.33 M sucrose. The buffer was prepared by adjusting a mixture of 10 mM of triethanolamine and 10 mM of acetic acid to pH 7.4 with NaOH. In the following, this buffer is called basic medium. All buffers and solutions were made with double-distilled water. All reagents used were of analytical grade. Sucrose was "Ampullenqualität" (E. Merck AG., Darmstadt, W. Germany). The minced liver was homogenized with 10 strokes in a 30 ml teflon-glass homogenizer (Arthur H. Thomas Co., Philadelphia, Pa.). The pestle was driven at 150 rpm.

The homogenate was adjusted to a concentration of 10 g of liver (wet weight) per 100 ml by addition of buffer. A lysosomal fraction for electrophoresis was prepared by the differential centrifugation method. The first part of the preparation scheme of Ragab et al. (7) was used, comprising a differential centrifugation which is based upon the scheme of de Duve et al. (1), except that the light mitochondrial fraction was washed four times (instead of once) (Fig. 1). All the steps of the preparation were performed in the cold room and in the MSE-centrifuge at 2°C with the angle-rotor No. 69179.

Electrophoresis

The electrophoretic separation of the lysosomal fraction was performed in the carrier-free continuous electrophoresis apparatus "Model III" (Fa. Desaga, Heidelberg, W. Germany; Brinkmann Instruments, Westbury, N.Y.), which is a refined model of the apparatus described by Hannig (12). The separation chamber is 120 mm broad and the distance between the outlets for the 92 test tubes, in which the separated material is collected, is 1.3 mm.

In this apparatus, a high electric field may be applied, thus achieving sharp separations even at high flow rates. This permits the separation of large amounts of lysosomal fraction in a relatively short time.

The conditions applied were: Buffer for the electrophoretic separation: 10 mM of triethanolamine-acetate, pH 7.4 (basic medium); electrode buffer: 100 mM of triethanolamine-acetate, pH 7.4 (without sucrose and EDTA); field strength: 100 v/cm; chamber temperature: 4°C; buffer flow rate: 200 ml/hr. The amount of homogenate separated per hour corresponds to 10 mg of protein.

Analytical Procedures

Enzyme Assays

For characterization of the individual cell organelles the following marker enzymes were assayed:

MITOCHONDRIA: Monoamine oxidase (Monoamine:O_2 oxidoreductase, EC 1.4.3.4) was assayed as a marker for the outer membrane according to Schnaitman et al. (16), with benzylaminhydrochloride (Schuchardt, Munich, W. Germany) as a substrate. The assay was modified as follows: A sample, containing 1 ml of organelle suspension, 1 ml of 0.2 M phosphate-buffer, pH 7.6, and 1 ml of 0.1 M substrate solution was incubated at 37°C for 60 min. The reaction was stopped by the addition of 0.2 ml of 60% (w/v) perchloric acid. The precipitated protein was spun down, and the clear supernatant was measured at 250 mμ against a reference containing 1 ml of basic medium instead of 1 ml of organelle suspension.

Cytochrome *c* oxidase (cytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1), a marker for the inner mitochondrial membrane, was tested by the method of Cooperstein and Lazarow (17), modified by Horie and Morrison (18). The substrate, cytochrome *c* (Boehringer Co., Mannheim, W. Germany), was purified

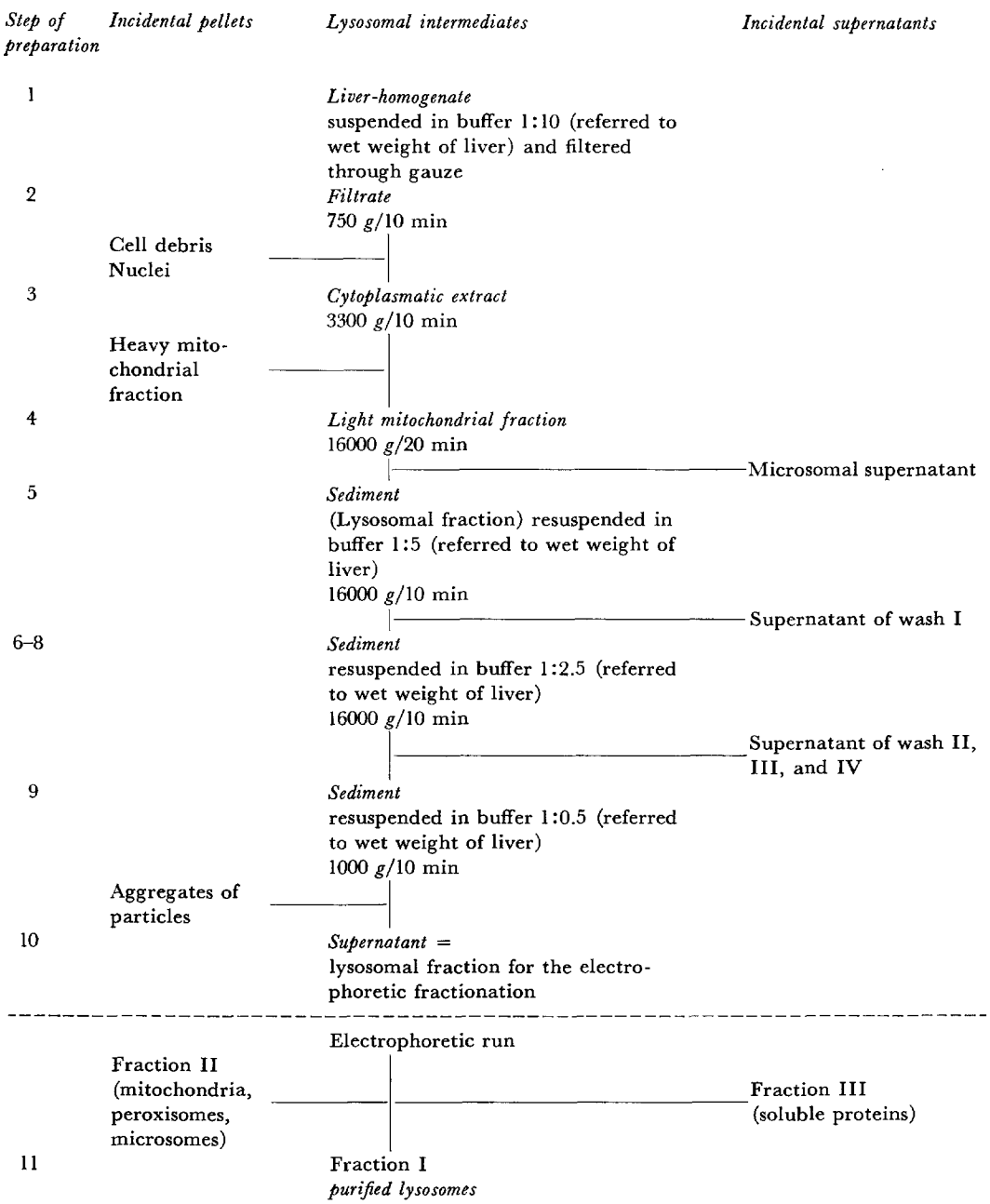

FIGURE 1 Scheme of the lysosomal preparation with differential centrifugation and electrophoresis.

with Dowex (1 × 2, mesh 200-400) after reduction.[1] For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{550} = 27.7 \times 10^3\ M^{-1}\ cm^{-1}$ for cytochrome $c_{red.}$, was used.

Glutamate dehydrogenase (L-glutamate: NAD-oxidoreductase deaminating, EC 1.4.1.2), a marker for the matrix, was determined by the method of Kun

[1] Under these assay conditions, there is linearity between enzyme concentration and substrate turnover to a maximum of 1.1 μM/min (= ΔE 0.03/min).

and Achmatowicz (19), with α-ketoglutaric acid (Boehringer Co.) as a substrate. The enzyme activity was measured in a final volume of 3 ml containing 50 mM of Tris-Cl, 50 mM of $(NH_4)_2SO_4$, 0.01 mM of EDTA, 0.1 mM of NADH, 1 mM of ADP, and 6 mM of α-ketoglutaric acid.

LYSOSOMES: Before testing the lysosomal and peroxisomal marker enzymes, the fractions were sonicated for 15 sec (Measuring & Scientific Equipment, Ltd., London, 100 Watt Ultrasonic Disintegrator, titanium vibrator Nr. 34041, 20 Kc/s, amplitude 6–7 μ). Acid phosphatase (orthophosphoric monoesterphosphohydrolase, EC 3.1.3.2.) was assayed by the method of Bergmeyer (20), with *p*-nitrophenylphosphate (Serva Co., Heidelberg) as a substrate. A sample containing 0.5 ml of buffer-substrate (0.1 M citrate buffer pH 4.8, 1.1×10^{-2} M substrate, 0.5% bovine serum albumin, and 0.5 ml of particle suspension) was incubated for 30 min at 37°C. The reaction was stopped by the addition of 3 ml of 0.03 M NaOH. The yellow color was measured at 405 mμ against a reference containing 0.5 ml of buffer-substrate and 0.5 ml of basic medium. For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{405} = 18.2 \times 10^3\ M^{-1}\ cm^{-1}$ for nitrophenol was used (21).

β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31) was tested by the method of Gianetto and de Duve (22), with phenolphthalein-mono-β-glucuronic acid (Sigma Chemical Co., St. Louis, Mo.) as a substrate. 0.2 M Na_2CO_3 solution was used for stopping the reaction at pH 11.3. When the color intensity of the samples was measured within 20 min, no decrease was observed (as described by Szasz as a source of error [23]). For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{540} = 240\ M^{-1}\ cm^{-1}$ for phenolphthalein at pH 11.3 was used (determined by a calibration curve).

Arylsulfatase (Arylsulfate sulfohydrolase, EC 3.1.6.1.) was tested at pH 5.0 according to the method of Roy (24), with 2-hydroxy 5-nitrophenylsulfate (Sigma Chemical Co.) as a substrate. For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{515} = 11.2 \times 10^3\ M^{-1}\ cm^{-1}$ for 2-hydroxy-5-nitrophenol was used (25).

Before the samples were measured, the precipitated organelles were spun down for 10 min at 2500 *g*.

PEROXISOMES: Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6.) was tested according to Beers et al. (26), with H_2O_2 (Perhydrol, E. Merck AG.) as a substrate.[2] For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{240} = 43.6\ M^{-1}\ cm^{-1}$ for H_2O_2 was used.

MICROSOMES: Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) was tested by the method of de Duve et al. (1), with glucose-6-phosphate (disodium salt, Boehringer Co.) as a substrate. Determination of inorganic phosphate was carried out according to Lowry and Lopez (27). For the expression of the enzyme activity in international units, a molar extinction coefficient of $\epsilon_{700} = 5.3 \times 10^3\ M^{-1}\ cm^{-1}$ for phosphate determined according to Lowry and Lopez (27) was used.

Determination of Soluble Enzyme Activities

For determination of dissolved marker enzymes of the cell particles, the electrophoretically obtained fractions were spun down immediately after electrophoretic separation in the Spinco Model L, Rotor 50 (50,000 *g*) for 15 min. The supernatants thus obtained were used for determination of the nonsedimentable enzyme activities.

Protein Content

Protein was determined according to the method of Lowry et al. (28). Bovine serum albumin, Fraction V (Serva Co.,) was used as a standard. After precipitation of the protein, it is absolutely necessary to remove carefully all traces of basic medium (by wiping out the test tubes), since the smallest amount of triethanolamine disturbs the reaction strongly by forming a nonprotein-specific blue-green color.

Optical Control

PHASE-CONTRAST MICROSCOPY: During the preparation the homogenate was examined for agglutination of cell organelles, in the phase-contrast microscope (Carl Zeiss, Oberkochen, W. Germany) at a magnification of 500–1000.

ELECTRON MICROSCOPY: Lysosomal fraction and individual electrophoretically obtained fractions were spun down in the Spinco-L-50-centrifuge at 30,000 rpm for 20 min. The pellets were fixed with 2–4% glutaraldehyde (2 hr). The glutaraldehyde was removed by washing with basic medium for 12 hr; the pellets were fixed again with 1% osmium tetroxide, and dehydrated by washing with alcohols of increasing concentration. The pellets were placed in propylene oxide for 30 min and afterwards in a mixture of propylene oxide and Epon (1 : 1) for 12 hr. Thereafter, the pellets were transferred into pure Epon which was polymerized for 40 hr in an oven at 60°C. After at least 5 days of storage, the capsules were sectioned. The sections were cut with an LKB-Ultrotome (LKB, Stockholm, Type 4802A) and examined in the JEM-T 7 electron microscope (Jeol Co., Tokyo), after being contrasted with uranyl acetate and stained

[2] Under these assay conditions, there is linearity between enzyme concentration and substrate turnover to a maximum of 2.75×10^3 μM/min (= ΔE 0.12/min).

with alkaline lead citrate. Two to three specimens of each pellet were cut at different levels.

RESULTS

Buffer Conditions and Preparation of the Lysosomal Fraction for an Efficient Electrophoretic Separation of the Cell Organelles

A buffer was needed which satisfies the following conditions:

(*a*) Cell particle agglutination should be prevented during the preparation of the lysosomal fraction and in the course of the electrophoretic separation.

(*b*) The normal morphological features of the cell particles should be maintained.

(*c*) Proteins absorbed by the particle surface should be removed during the washing of the homogenate in order to uncover the specific charge patterns of the particles.

(*d*) The buffer should meet the requirements of the electrophoretic setup.

Systematic variations of buffer conditions (pH, different ions, ionic strength, nonionic additives) were carried out. It was found that agglutination increased with increasing ionic strength.

A particularly large agglutination (which exceeds the usual effect of ionic strength) is observed when divalent ions such as Mg^{++} or Ca^{++} are added. In addition, the permeability of the lysosomal membrane is increased by the binding of such ions especially at slightly alkaline pH (9). The ionic strength, therefore, was kept as low as possible, and divalent ions were trapped by a chelating agent such as EDTA (1 mM). A lower limit of concentration (approximately 0.01 M for the triethanolamine-acetate buffer used) is set for the buffer by its capacity, which is necessary to maintain the pH at 7.4 in the organelle suspension and during the electrophoresis. By adding 0.33 M sucrose, the buffer solutions were made slightly hypertonic.

The physiological pH of 7.4 was found to be optimal. Buffer substances which are disintegrated by electrolysis (e.g., buffers containing chloride ions) should be excluded. Therefore, the tested 25 mM Tris-Cl buffer could not be used in spite of morphologically well-preserved organelles. Tris-citrate (25 mM) could be used in the electrophoresis, but it caused morphological alteration of the organelles: the matrix of the mitochondria became very much shrunken and the intracristal space expanded greatly. Besides this, both Tris buffers caused more or less agglutination of the organelles, and the electrophoretic separation of the lysosomal fraction prepared with Tris-citrate was unsatisfactory. Triethanolamine-acetate buffer proved to be the best in all the above-mentioned respects. However, with this buffer sufficient removal of proteins bound to the particle surface can only be achieved by washing the lysosomal fraction four times.

With the aim of reducing the number of washing steps, each of which involves a loss of material and time, the use of a phosphate buffer (0.1 M, pH 7.4) known to extract proteins from the outer membranes of the mitochondria (29) was examined. It caused, however, strong agglutination of the particles and had a strong destructive effect on the membranes (especially on the outer membrane of the mitochondria). This caused a change in the result of the electrophoretic separation (30).

Fig. 2 shows the recovery of marker enzymes obtained in the individual preparation steps (Fig. 1) with reference to the crude homogenate. The purification factors are shown in Table I. Values of six preparations were averaged.

About a threefold increase in the activity of catalase, the marker enzyme for peroxisomes, is observed during the course of the lysosomal preparation (steps 1–10 in Fig. 1). The contents of mitochondria and microsomes remain almost constant according to the activities of the cytochrome *c* oxidase (marker enzyme for mitochondria) and glucose-6-phosphatase (marker enzyme for microsomes). In contrast to these findings, the lysosome content increased significantly as indicated by a 7-8-fold increase in acid phosphatase and β-glucuronidase activities and a 14-fold increase in arylsulfatase activity.

The yields of lysosomal and mitochondrial marker enzymes (shown in Fig. 2) demonstrate the effectiveness of the preparation scheme used. Fig. 3 shows the electron micrograph of a section of the lysosomal fraction. Despite the 8-fold increase in lysosomal enzyme activities, only a small number of lysosomes was found. The bulk of the lysosomal fraction consisted of mitochondria.

Electrophoresis

General Separation Results

The purified and washed lysosomal fraction was fractionated by electrophoresis. The fractions

TABLE I

Enrichment of the Marker Enzymes of the Cell Organelles during the Preparation of the Lysosomal Fraction

The specific activities of the enzymes are given with respect to the specific activities of these enzymes in the crude homogenate (step 1) which were set at 1.00.

Enzyme		Preparation step									
As marker for	Name	1	2	3	4	5	6	7	8	9	10
Mitochondria	Cytochrome *c* oxidase	1.00	0.84	0.44	0.12	0.50	0.71	0.70	0.92	2.16	1.45
Microsomes	Glucose-6-phosphatase	1.00	1.03	0.97	0.98	1.45	1.54	1.06	0.99	0.42	0.53
Peroxisomes	Catalase	1.00	0.93	1.32	1.38	2.41	4.46	4.40	4.92	3.35	3.10
Lysosomes	Acid phosphatase	1.00	1.08	1.20	1.10	4.90	8.22	8.80	8.25	6.70	7.53
	β-Glucuronidase	1.00	0.98	1.20	1.00	5.60	8.90	9.45	10.53	7.60	8.20
	Arylsulfatase	1.00	1.01	1.20	1.03	5.65	11.25	12.50	13.40	12.12	13.85
		Protein yield									
	Yield (%)	100	90	58	46	5.6	3.2	2.25	1.30	1.20	1.10

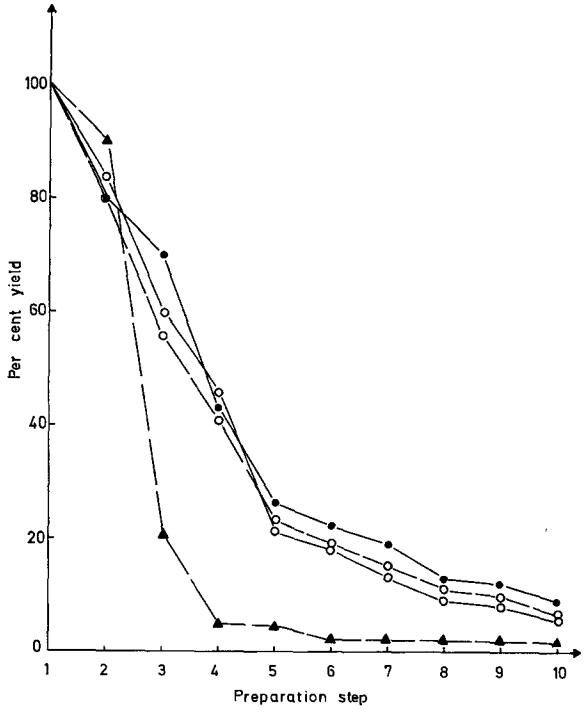

FIGURE 2 Yield of lysosomal and mitochondrial enzymes during the preparation of the lysosomal fraction. —○——○—, acid phosphatase; —○— – –○– –, β-glucuronidase; —●——●—, arylsulfatase; —▲——▲—, cytochrome *c* oxidase. A yield of the lysosomal enzymes after the differential centrifugations in the range of 20–25% is shown (step 5). By the four washings this will be reduced to 6–9%.

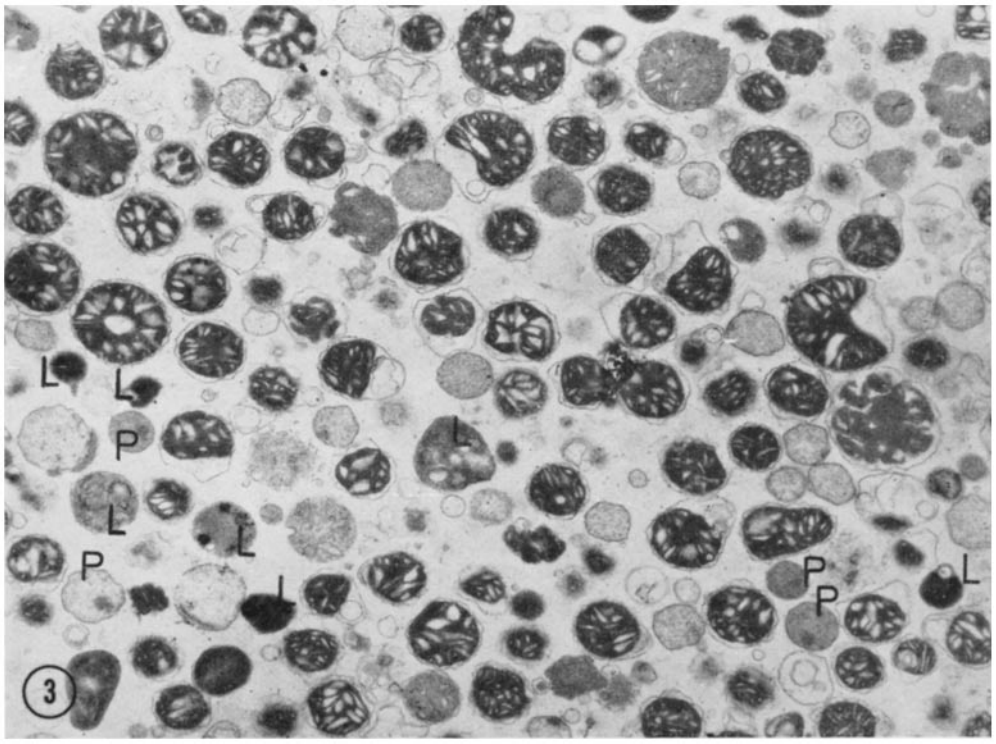

FIGURE 3 Section of the lysosomal fraction (step 10). The bulk of the fraction is made up of mitochondria. Despite the 8-fold increase of lysosomal enzyme activities, only a small number of lysosomes (*L*) is found. *P* indicates peroxisomes. × 8500.

were assayed by the marker enzymes. In order to obtain comparable graphs, the activity distribution curves were normalized by setting the maximal value at 100. The result of the electrophoresis is shown in Fig. 4 by a graph of the separation obtained in the electrophoretic chamber, and in Fig. 5 by the distribution of the various marker enzymes found in the individual fractions.

Lysosomes (*I*) (assayed by β-glucuronidase and arylsulfatase) are clearly separated from the other organelles (*II*) (mitochondria, peroxisomes, and microsomes, assayed by cytochrome *c* oxidase, catalase, and glucose-6-phosphatase). The mitochondria-peroxisomes fraction (*II*) has the lowest electrophoretic mobility towards the anode. It appears in the center of the distribution curve obtained by the assay of the protein content (Fig. 4). A slightly higher mobility is found for the microsomes. The lysosomes have the highest anodic mobility of all particles.

THE LYSOSOMAL ENZYME ACTIVITIES (PEAKS I AND III)

ENRICHMENT OF LYSOSOMAL ENZYMES (PEAK I): Different enrichment factors for the three marker enzymes of the lysosomes (*I*, Fig. 5), compared with their activities in the crude homogenate (step 1), were obtained (Fig. 6): With respect to the specific activity of acid phosphatase, the maximal value was 40-fold, with respect to the specific activity of β-glucuronidase, 65-fold, and with respect to the specific activity of arylsulfatase, 240-fold.

MORPHOLOGY AND PURITY OF THE LYSOSOMES STUDIED IN THE ELECTRON MICROSCOPE: Lysosomes from the lysosomal peak I, which did not overlap with peak II (left part), were examined in the electron microscope (see Fig. 7). The lysosome fractions were very pure (only a few mitochondria could be detected as contaminants), and the particles had a completely normal and intact appearance.

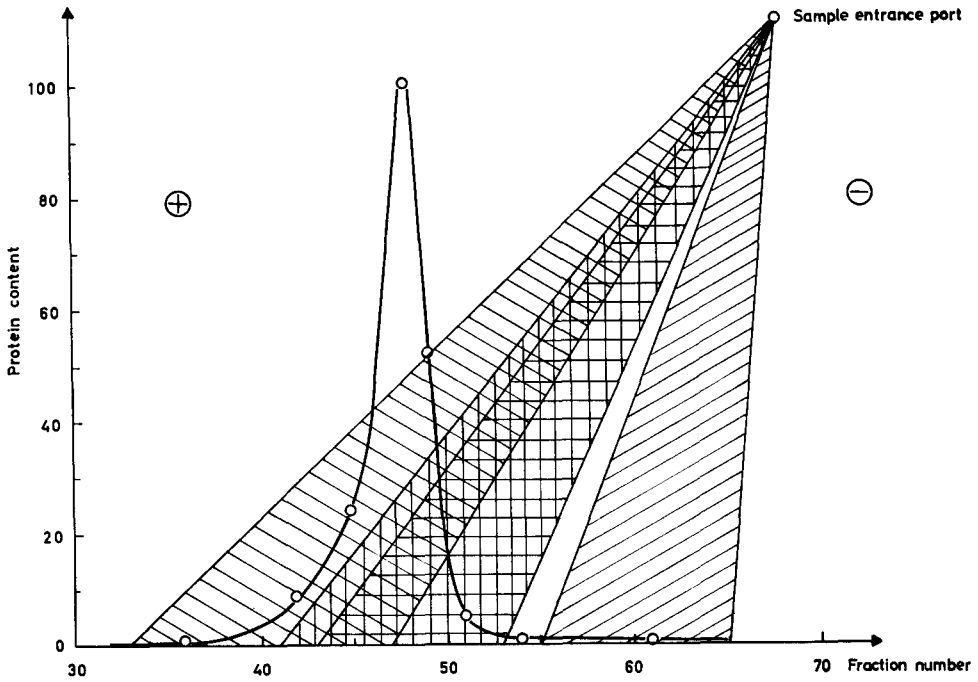

FIGURE 4 Separation in the electrophoretic chamber (reconstructed by means of the distribution of the enzyme activities in the individual fractions). The various cell components show different electrophoretic migration rates: ▧, lysosomes; ▥, microsomes; ≣, mitochondria and peroxisomes; ▨, dissolved proteins. The curve shows the protein distribution on the bottom of the separation chamber.

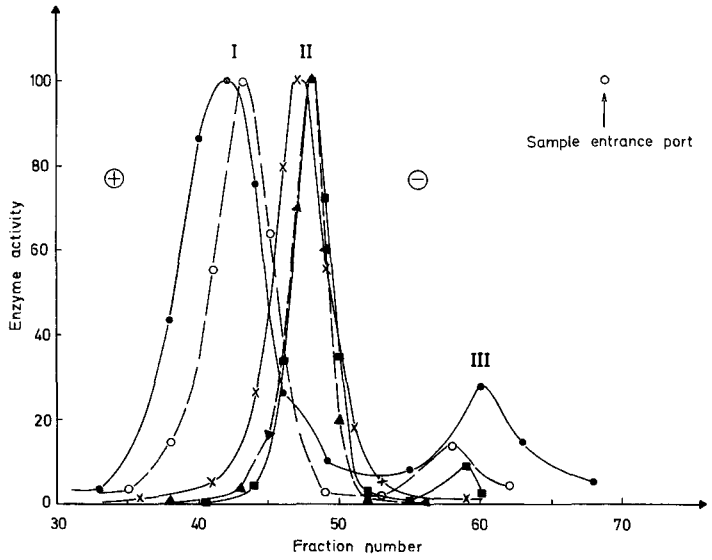

FIGURE 5 Distribution of the various marker enzymes in the individual electrophoretic fractions: *I*, lysosomal activities; *II*, microsomal, mitochondrial and peroxisomal activities; *III*, free activities. —●——●—, arylsulfatase; —○- - -○—, β-glucuronidase; —×——×—, glucose-6-phosphatase; —▲- - -▲—, cytochrome *c* oxidase; —■——■—, catalase.

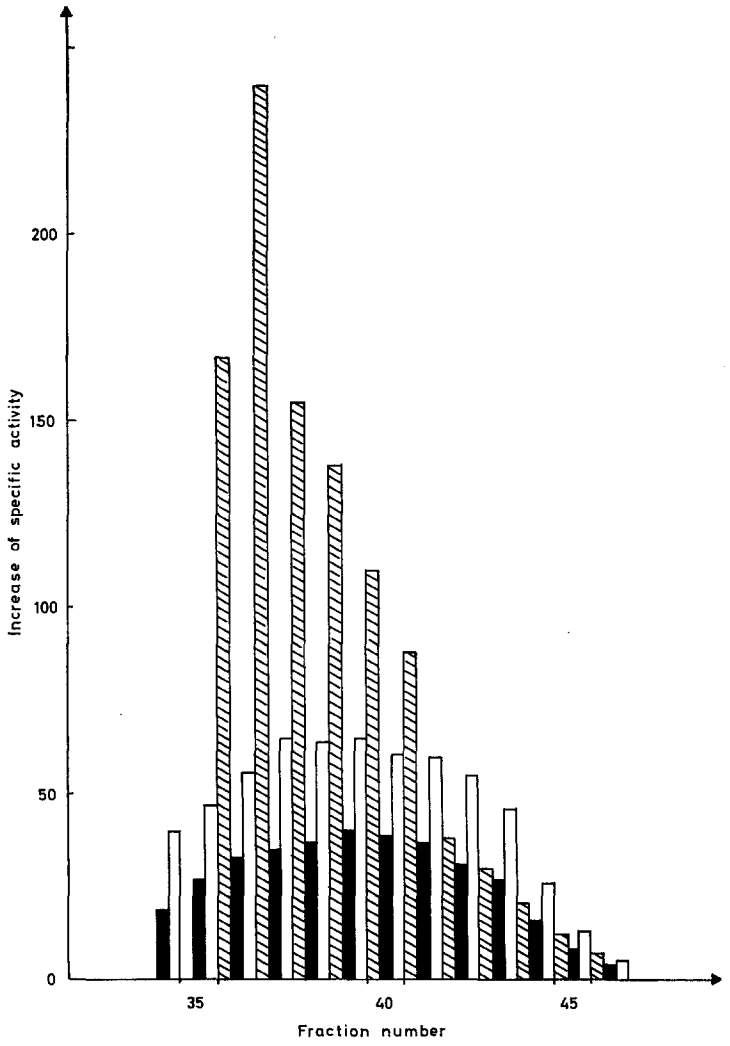

FIGURE 6 Enrichment of lysosomal enzymes in the electrophoretic fractions with respect to the specific activities in the crude homogenate. ■, acid phosphatase; □, β-glucuronidase; ▨, arylsulfatase.

HETEROGENEITY OF THE LYSOSOMES: There is some heterogeneity of the lysosomal fraction. This follows from the observation that two of the three marker enzymes give identical distribution curves, whereas one (arylsulfatase) indicates a type of lysosome with a higher anodic mobility and a higher content of arylsulfatase than the other lysosomes. The estimated ratio of β-glucuronidase:arylsulfatase is 2:1 in the cathodic side and 1:2 in the anodic side of peak I (see Fig. 5).

SEDIMENTATION BEHAVIOR OF THE LYSOSOMAL ENZYMES: The sedimentation behavior of the lysosomal enzymes (Fig. 8) was investigated as a test for the integrity of the separated lysosomes in peak I. The presence of nonsedimenting enzymes may be due to a release of enzymes from the cell particles either before or after the separation. The presence of nonparticle-bound enzymes may indicate a damage of the lysosomal membrane.

In the lysosomal fraction (peak I), 15–30% of the enzymes are free (peak I). They were released in the collecting tubes after separation. A comparable loss of enzymes is known from preparation methods without electrophoresis (2).

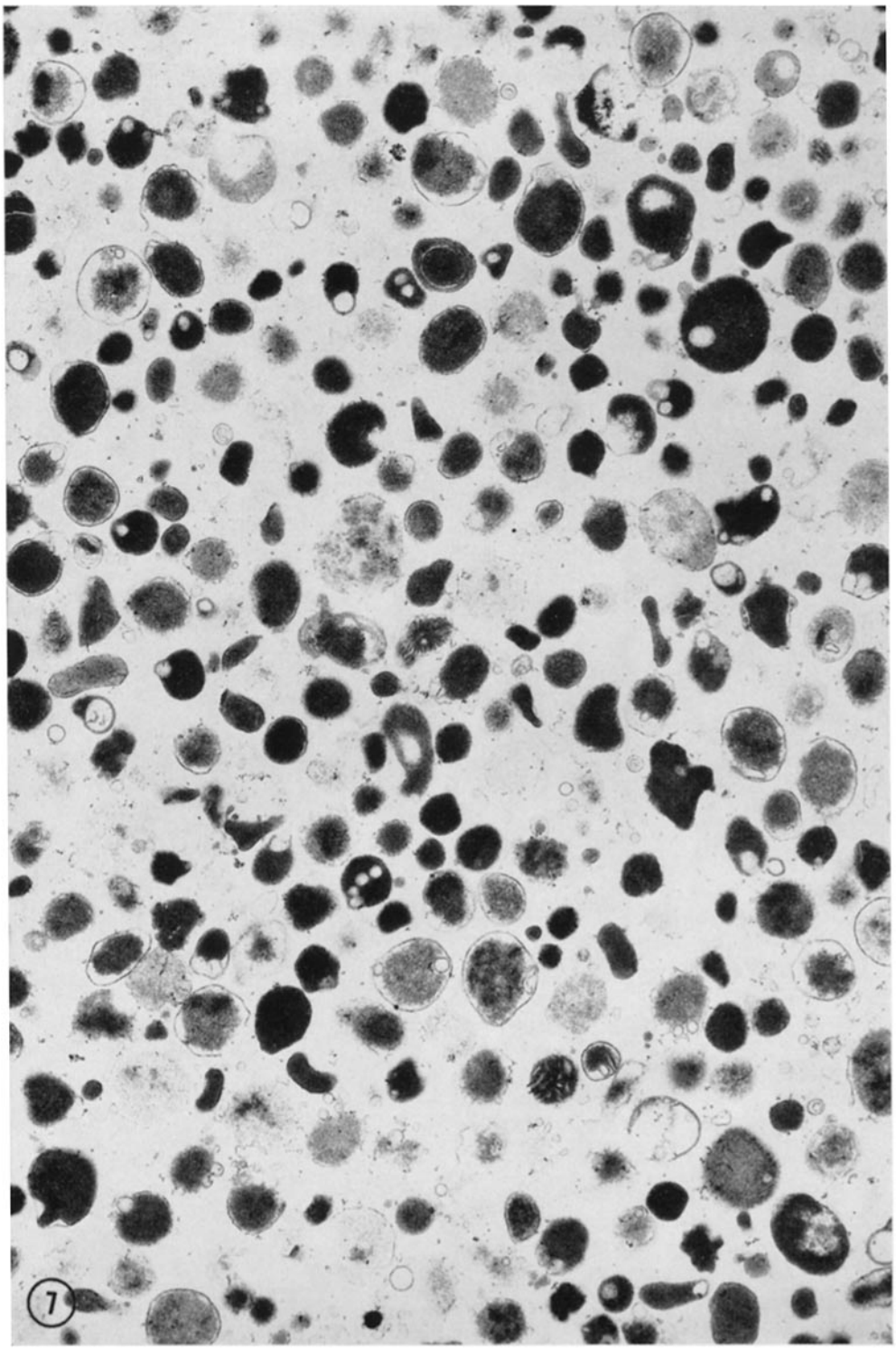

FIGURE 7 Lysosomes from lysosomal peak I which did not overlap with mitochondrial peak II. The lysosomes have a completely normal and intact appearance. Only a few mitochondria can be detected as contaminants. × 12,000.

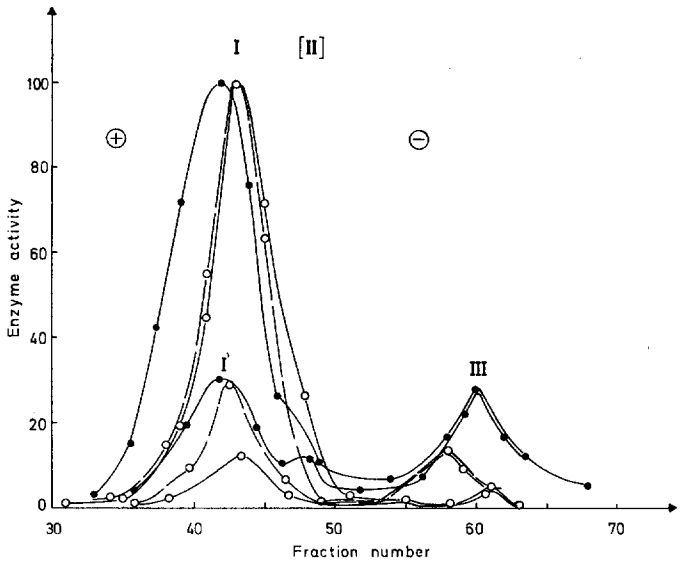

FIGURE 8 Distribution of the lysosomal marker enzymes in the electrophoretic pattern and their sedimentation behavior. Peak *I:* Acid phosphatase (○——○) and β-glucuronidase (—○- - -○—) show an identical distribution. The arylsulfatase maximum (—●——●—) is shifted towards the anode. The nonsedimentable portions (15–30%; 54000 *g*/15 min) of the enzyme activities are represented in peak *I'*. Peak *III:* The activities in peak *III* are completely nonsedimentable by the conditions mentioned above. This means that they are not related to any subcellular structure.

Several authors (31, 32) have pointed out sucrose as a labilizing reagent for lysosomes.

According to the sedimentation behavior, peak III consists of free enzymes which have been separated electrophoretically from the lysosomes. Therefore, they must have been released before electrophoresis.

The small shoulder at the cathodic side of the distribution curve of the arylsulfatase activity (see Fig. 8) can easily be explained by the fact that the enzyme arylsulfatase exists in two forms of different electrophoretic mobility. Therefore, in the nonparticle-bound state the activity of this enzyme appears in two places on the electrophoretic pattern. The main part is in peak III, and the other part makes up the shoulder which is discussed here.

THE MITOCHONDRIAL FRACTION (PEAK II)

The mitochondria and peroxisomes, which migrate together, appeared in the main protein peak. The assay of this fraction with three mitochondrial marker enzymes (monoamine oxidase as a marker for the outer membrane, cytochrome *c* oxidase for the inner membrane, and glutamate dehydrogenase for the matrix) yielded the same distribution curve (Fig. 9).

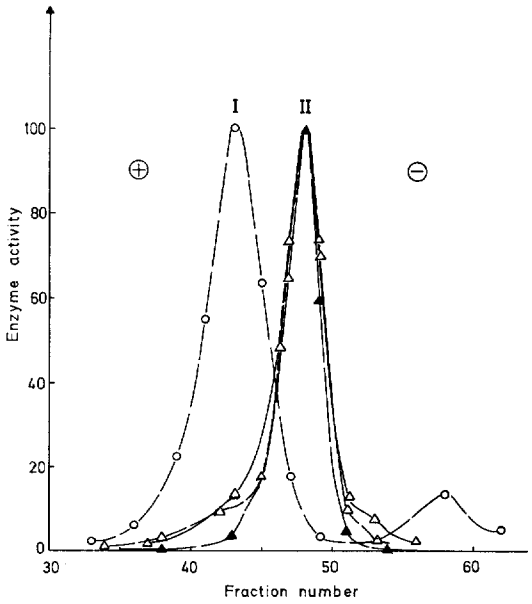

FIGURE 9 The three mitochondrial marker enzmes, appear in peak *II* in identical distribution (-△- - -△- glutamate dehydrogenase; —▲——▲—, cytochrome *c* oxidase; —△——△-, monoamine oxidase). The distribution of β-glucuronidase (—○——○-) is given as a marker for the lysosomal position.

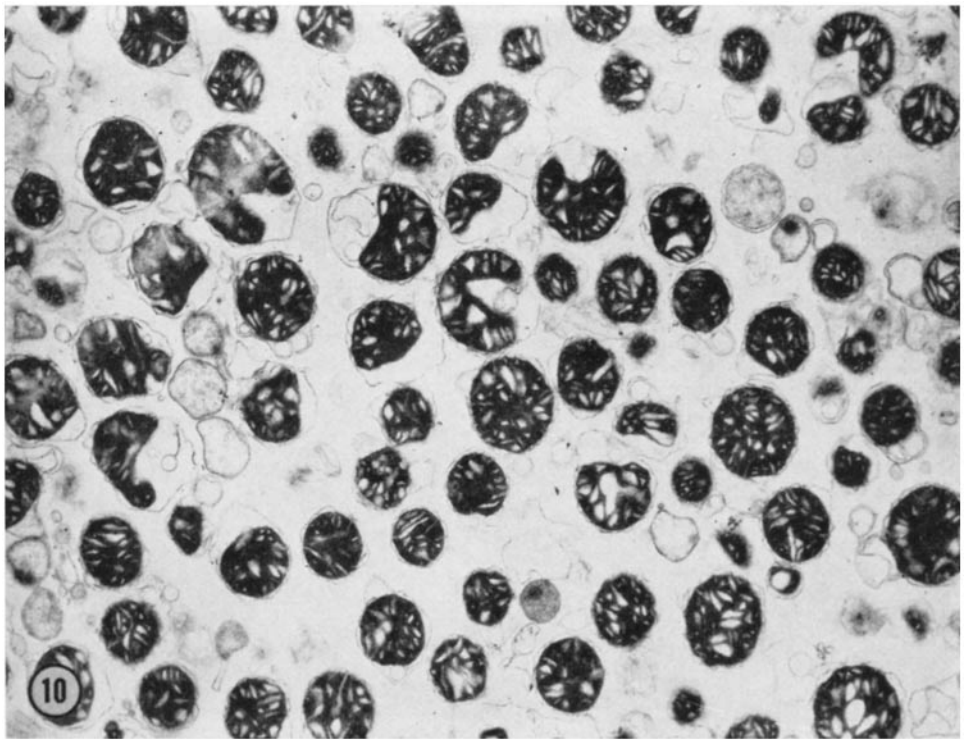

FIGURE 10 Well preserved mitochondria and some peroxisomes are found to be components of the cathodic part from the "mitochondrial" fraction. × 15,000.

Microsomes assayed for glucose-6-phosphatase were found in the same region of the electrophoretic pattern as mitochondria, but, with respect to the mitochondrial activity maximum, the maximum of glucose-6-phosphatase activity is shifted towards the anode (Fig. 5). Therefore, it is concluded that the anodic part of the "mitochondrial" fraction is enriched in microsomes.

ELECTRON MICROSCOPIC CONTROL: The electron micrographs confirm the biochemical findings. Fig. 10 demonstrates that the cathodic part of the mitochondrial fractions contains mainly well-preserved mitochondria and some peroxisomes. Electron micrographs of the anodic part with the highest activity of glucose-6-phosphatase show a high number of empty membrane vesicles (Fig. 11). These may be identified as microsomes.

Tabular Summary of the Results from the Lysosomal Preparation

The yields and enrichments of the lysosomal enzyme activities obtained by lysosomal preparation and electrophoresis are shown in Table II. Table III demonstrates the efficiency of the preparation in removing nonlysosomal activities. In the preparation of the lysosomal fraction, only the microsomal content decreased; the content of the other particles increased slightly. In contrast, the electrophoresis showed a very effective removal of nonlysosomal activities.

In one experiment, 5–8 mg of lysosomes can be obtained from 70 g of rat liver (wet weight).

DISCUSSION

The successful separation of lysosomes from rat liver by differential centrifugation and electrophoresis depends primarily on the buffer used. The agglutination of cell organelles can be prevented during the preparation of homogenates by a low buffer concentration. However, in many buffer systems that permit preparation of the homogenate with very little agglutination, much agglutination occurs during the electrophoretic separation. If prepared and separed in 10 mM of triethanolamine-acetate buffer, pH 7.4, however, these lysosomal fractions do not show ag-

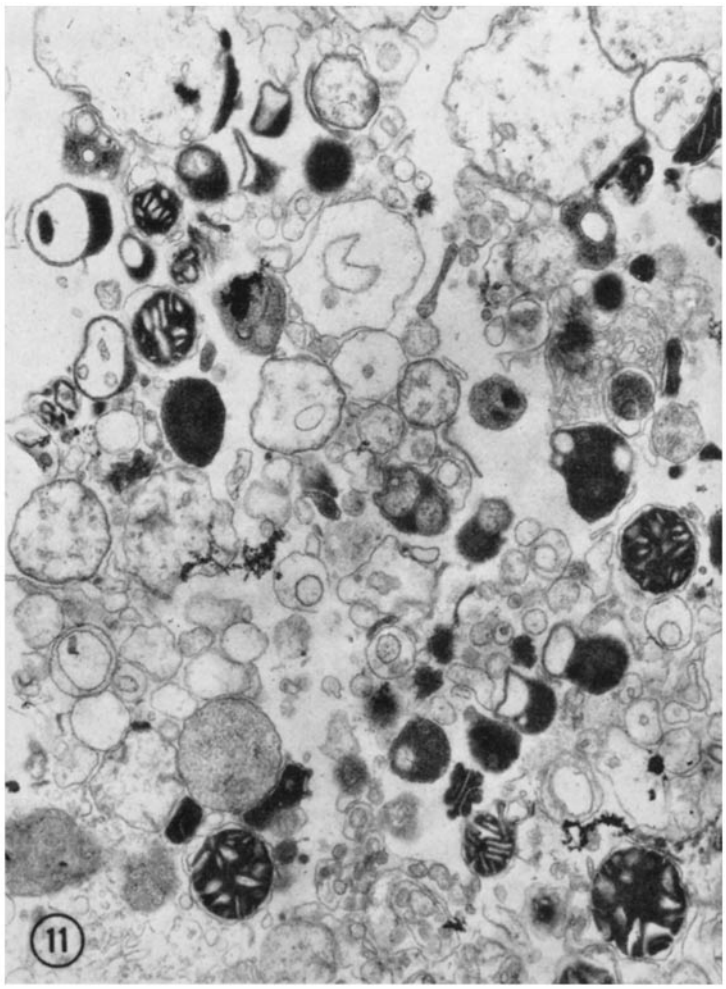

FIGURE 11 Electron micrograph of a fraction with high glucose-6-phosphatase activity from the anodic part of the mitochondrial fraction. It shows a high number of empty membrane vesicles which may be identified as microsomes. × 15,000.

glutination. In addition, it was found that the lysosomal fraction had to be extensively washed. In this way, a differentiation of the negative surface charges of the organelles was achieved, and this was necessary for successful electrophoretic separation. Of the various buffers tried, the above buffer system was shown to be very suitable for this critical washing step; also, in this buffer the morphology of the organelles was not changed. It, therefore, meets all necessary requirements. A yield of 20% and an 8- to 10-fold enrichment of the lysosomal enzymes were achieved in the first part of the preparation (differential centrifugation). This is in agreement with the data reported by de Duve et al. (1). The extensive washing which was applied in our method decreased the yield to between 6 and 9%. The result of the electrophoretic separation of the lysosomal fraction prepared in this way shows the lysosomal enzyme activities to be clearly separated from the mitochondrial, peroxisomal, and microsomal activities. The over-all enrichment for acid phosphatase was 40-fold, for β-glucuronidase 65-fold, and for arylsulfatase 240-fold.

The yield of the particle-bound lysosomal enzyme activities was different for the three measured marker enzymes: 90% for β-glucuronidase, 75% for acid phosphatase, and 75% for arylsulfatase.

TABLE II

Yield and Enrichment of Lysosomal Enzyme Activities by Preparation of the Lysosomal Fraction and in the Electrophoresis

Preparation step	Acid phosphatase			β-Glucuronidase			Arylsulfatase		
	Yield	Enrichment Spec. act.*	Factor	Yield	Enrichment Spec. act.*	Factor	Yield	Enrichment Spec. act.*	Factor
Crude homogenate (Step 1)	100	0.288×10^3	1	100	4.15×10^3	1	100	90	1
Lysosomal fraction (Step 10)	5.8	2.16×10^3	7.5	6.5	34.2×10^3	8.2	9	1.23×10^3	13.8
Electrophoresis	4.2	11.6×10^3	40‡	5.8	271×10^3	65‡	6.5	21.4×10^3	240‡

* Specific activity = μmole/min and mg protein.
‡ Value from the fraction with highest specific activity.

TABLE III

Efficiency of the Preparation Scheme and the Electrophoresis with Respect to the Purification of the Lysosomes from Nonlysosomal Enzyme Activities

Preparation step	Cytochrome *c* oxidase Spec. act.*	Catalase Spec. act.*	Glucose-6-phosphatase Spec. act.*
Crude homogenate (Step 1)	58	2.6×10^5	20.4
Lysosomal fraction (Step 10)	83	8.2×10^5	10.8
Purified lysosomes (Electrophoretic fraction 36-41)	0.8	0.065×10^5	0.34

* Specific activity = μmole/min and mg protein.

The loss of 10% in β-glucuronidase and part of the losses of acid phosphatase and arylsulfatase may be explained by a release of these enzymes from the particles. The released enzymes appear in the electrophoretic fractions which contain the soluble proteins (*III*, Fig. 8). The additional loss of the acid phosphatase and the arylsulfatase activities may be explained by the instability of these enzymes at pH 7.4.

A smaller part of the loss of activity of acid phosphatase may be explained by the separation of microsomal *p*-nitrophenylphosphate–splitting enzymes. This is clearly shown by a shoulder of acid phosphatase activity in the position of the glucose-6-phosphatase maximum (Figs. 5 and 8, fraction 46).

The enrichment of the various lysosomal marker enzymes is strikingly different. Since the activity of β-glucuronidase may be traced through the preparation and separation without losses, it is reasonable to use the activity of this enzyme as an internal standard for the enrichment of the other enzymes. The ratio of the enrichment of β-glucuronidase:acid phosphatase is 1:0.7, which may easily be explained by the causes mentioned above. In contrast, the ratio of the enrichment of β-glucuronidase:arylsulfatase is 1:4. This may be explained by the presence of high amounts of arylsulfatase in the lysosomes with the higher mobility.

In addition, the electrophoretic separation is very efficient for the removal of nonlysosomal enzymes. In fractions 36–41, the region in which lysosomal and mitochondrial activities do not overlap, the specific activities of nonlysosomal enzymes were only 0.8–1.7% of those of the starting material (Table III).

The lysosomes were not only heterogeneous with respect to their morphology but also to their enzyme pattern. This enzymatic heterogeneity has already been discussed by de Duve and coworkers (5) at the time of the discovery (1) of these organelles. Subsequently, other authors have reported on the enzymatic heterogeneity of lysosomes from various tissues (33–35). The heterogeneity of lysosomes with respect to their arylsulfatase content has been shown for lysosomes from brain (36), mouse kidney, and mouse liver

(37). Our results show the same for the lysosomes of rat liver. The electrophoretic method used by us shows that lysosomes with different enzyme patterns are also distinguished by a different electrophoretic mobility. This finding corresponds well with the work of Tappel et al. (38) and Treadwell and Santos-Buch (39) who have shown that parts of the lysosomal enzymes are incorporated in the membrane.

In conclusion, it may be said that the combination of differential centrifugation and electrophoresis is a simple and nondestructive method for the preparation of lysosomes from rat liver.

However, the substitution of differential centrifugation, which implies high losses of material and very impure lysosomal preparations (about 90% of the protein content consists of nonlysosomal material), by zonal centrifugation in a Ficoll gradient, which improves yield as well as purity of the preparation (40), is suggested. We would expect that the use of a lysosomal fraction prepared by this method would help to reduce the overlapping of mitochondrial and lysosomal enzyme activities in the electrophoretic pattern.

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