A SIMPLE PROCEDURE FOR THE ISOLATION OF LYSOSOMES FROM NORMAL RAT LIVER

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1. Introduction

The isolation of lysosomes from a homogenate of rat liver by conventional differential centrifugation techniques is extremely difficult (see [1]) because of the fact that there are only minor differences in the isopycinc density or sedimentation coefficient of lysosomes and mitochondria [2–4]. The procedure that has been described for the isolation of lysosomes from normal rat liver by isopycnic centrifugation [5,6] is laborious and time-consuming, and damage to the lysosomal membrane may occur during the isolation. Similar objections can be made to the methods recently described in which zonal centrifugation [7,8] or free-flow electrophoresis [9–11] are used.

Other procedures make use of the fact that phagocytosed material enters the lysosomes, so that when appropriate substances are used, an alteration can be brought about of the centrifugal properties of the lysosomes. Thus methods have been described for isolating lysosomes filled with Triton WR 1339 [12,13], Dextran 500 [14] and colloidal iron [15,16]. Apart from the fact that these procedures, too, are laborious and time-consuming, a major objection is that the properties of the lysosomes from animals injected with Triton WR 1339, Dextran 500 or colloidal iron may be profoundly altered.

In this report, a procedure for the isolation of lysosomes from the livers of normal rats is described, in which the isopycnic properties of the mitochondria, rather than the lysosomes, are altered. The procedure is based on the method described by Davis and Bloom [17] for isolating synaptosomes (see also [18,19]). The density of the mitochondria is increased by incubating the organelles with a substrate of a mitochondrial dehydrogenase in the presence of a tetrazolium salt as electron acceptor. Reduction of the tetrazolium salt leads to the formation of an insoluble precipitate in the mitochondria.

2. Materials and methods

2.1. *Procedure for isolation of lysosomes*

Four male rats (Wistar AG strain) weighing 230–250 g each, are decapitated. The livers are removed, chilled in ice-cold 0.25 M sucrose, minced rapidly, and homogenized in a Potter-Elvehjem homogenizer. The homogenate (160 ml) is centrifuged for 10 min at 600 *g*. The pellet is suspended in about 120 ml 0.25 M sucrose and the suspension is centrifuged for 10 min at 600 *g*. The combined supernatants are centrifuged for 20 min at 25 000 *g*. The supernatant is discarded. The pellet (M + L fraction) is suspended in a small vol of 0.25 M sucrose and added to a medium (final vol 200–250 ml) containing 0.25 M sucrose, 40 mM morpholinopropane sulphonic acid (MOPS) buffer (pH 7.0), 80 mM succinate and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT; 0.6 mg per mg protein).

After incubation for 7 min at 20°C, the suspension is centrifuged for 20 min at 25 000 *g*. The supernatant is discarded. The pellet is suspended in 40 ml 0.24 M sucrose containing 5% (w/v) polyvinylpyrrolidone 40 (PVP; Sigma Chemical Company). The Ludox

gradient consists of the following layers: 3%, 2% and 1% (w/v) of Ludox HS 40 (du Pont, Wilmington) from bottom to top, respectively. Each Ludox layer contains 0.24 M sucrose, 5% PVP (w/v), 1 mM EDTA and 40 mM MOPS buffer. The final pH is 7.0. The Ludox solutions are prepared according to Wolff and Pertoft [19]. After layering the tetrazolium-treated M + L fraction on the gradients (using one 50-ml tube/liver), the tubes containing the gradients are centrifuged for 13 min at 3000 *g* in a swing-out rotor (No. 59550; MSE Mistral 6 L centrifuge). The combined 0% Ludox layers are centrifuged for 20 min at 25 000 *g*. The pellet consist of a pink (to red) solid layer at the bottom covered by a red fluffy granular layer. After careful rotation of the tubes, the supernatant with the fluffy layer can be removed. The remaining pellet is suspended in a very small volume of 0.25 M sucrose. The isolation procedure is completed within 4–5 h.

2.2. *Assay methods*

Cytochrome *c* oxidase [20], glucose-6-phosphatase [21] (in the presence of 40 mM tartrate [22]), and D-amino acid oxidase [23] were used as mitochondrial, microsomal and peroxisomal markers, respectively. Acid phosphatase [24], α-galactosidase [25] and N-acetyl-β-glucosaminidase [25] were used as lysosomal markers. In the presence of Ludox-PVP, the phosphate assay could not be carried out, and in the case of glucose-6-phosphatase, glucose formation was measured [26]. In the other enzyme assays there was no significant influence of Ludox-PVP. Protein was measured by a biuret method [27].

2.3. *Determination of latency*

The latency of the lysosomal enzymes was calculated with the aid of the following formula:

$$\text{Latency} = \frac{\text{act}_{max} - \text{act}_{free}}{\text{act}_{max}} \times 100\%$$

where act_{max} = activity after sonication of the fractions for 1 min at 2200 kHz and 2 μm peak to peak, and act_{free} = activity before sonication.

2.4. *Assessment of purity of fractions*

A biochemical assessment of the purity of the final fractions was obtained by the method described by Leighton et al. [28]. Assuming that the homogenate contains 22% microsomal [29], 20% mitochondrial [1], 2.5% peroxisomal [28] and 1% lysosomal protein (see [1]), and that only these components are present in the ML fraction, the following relationship holds (see [1,28,30] for a discussion):

$$c = 22\,(P_{microsomes}) + 20\,(P_{mitochondria}) + 2.5\,(P_{peroxisomes}) + 1\,(P_{lysosomes})$$

where c = composition of the fraction,

and $P = \frac{\text{specific activity of marker enzyme in fraction}}{\text{specific activity of marker enzyme in homogenat}}$

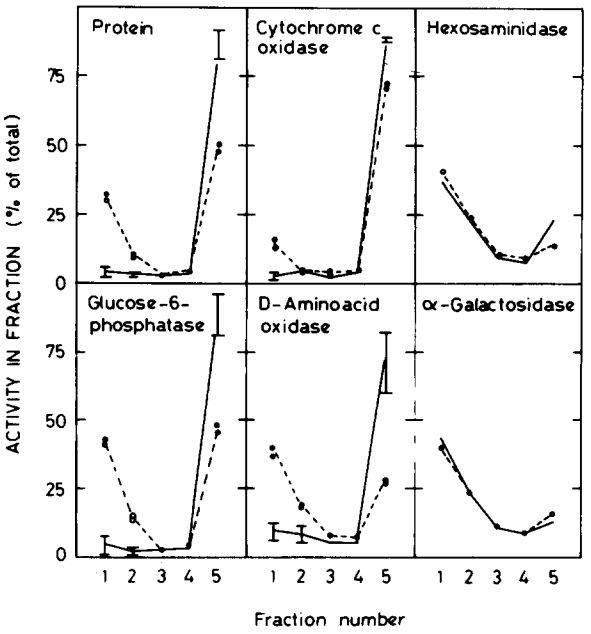

Fig.1. Distribution of protein and marker enzymes in the fractions obtained after centrifugation on a Ludox-PVP gradient of a mitochondrial-lysosomal fraction. For details, see text. Fractions 1–4 are the 0, 1, 2 and 3% Ludox layers and fraction 5 is the pellet. The recoveries with respect to the mitochondrial-lysosomal fraction were 90–110%. (•——•) Mitochondrial-lysosomal fraction preincubated with succinate and INT; the vertical bars give the means ± SEM (10 experiments) of values that differ significantly from those obtained without INT preincubation (o—— o) No preincubation (2 experiments). Act_{max} = activity after sonication.

3. Results

In fig.1, the distribution of total protein and total activity of different marker enzymes in the fractions obtained after centrifugation of a mitochondrial-lysosomal fraction on a Ludox-PVP density gradient is shown. Preincubation of the mitochondrial-lysosomal fraction with succinate and INT leads to a decrease in the amount of protein and a decrease in total activity of cytochrome *c* oxidase, glucose 6-phosphatase and D-alanine oxidase in the lighter Ludox layers, and an increase in the pellet. The pretreatment with INT and succinate had little effect on the lysosomal enzymes in the different fractions.

The data on the lysosomal enzymes are presented in the form of a De Duve plot in fig.2. It is clear that pretreatment of the mitochondrial-lysosomal fraction leads to a marked increase of the relative specific activity of the lysosomal enzymes in the 0%

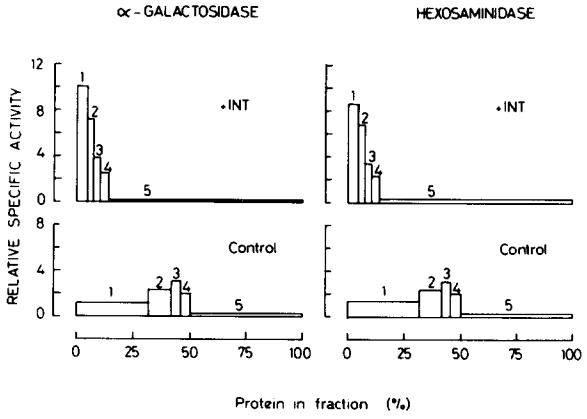

Fig.2. De Duve plots of α-galactosidase and *N*-acetyl-β-glucosaminidase in fractions obtained after centrifugation on a Ludox-PVP gradient of a mitochondrial-lysosomal fraction. For details, see text. + INT, mitochondrial-lysosomal fraction preincubated with succinate and INT (mean values obtained from 10 experiments; Control, no preincubation (mean of 2 experiments).

Table 1
Purification of lysosomes by density-gradient centrifugation on a Ludox gradient after treatment of a mitochondrial-lysosomal fraction with succinate and a tetrazolium salt

	0% Ludox fraction from gradient[a]			Precipitate from 0% Ludox fraction[b]		
	Specific activity (mU/mg protein)	Purification factor	Yield (% of activity in homogenate)	Specific activity (mU/mg protein)	Purification factor	Yield (% of activity in homogenate)
Protein		1.0	0.97 ± 0.42		1.0	0.195 ± 0.056
α-Galactosidase	47.1 ± 4.2	26.6 ± 6.8	24.4 ± 7.7	88.5 ± 20.0	60.3 ± 17.3	11.1 ± 2.6
N-acetyl-β-glucosaminidase	238.7 ± 20.3	26.1 ± 7.1	21.5 ± 4.6	685 ± 69	62.1 ± 13.8	12.3 ± 2.6
Cytochrome *c* oxidase	–[c]	1.05 ± 0.35	1.01 ± 0.59	–[c]	0.29 ± 0.14	0.059 ± 0.037
Glucose 6-phosphatase	0.121 ± 0.013	1.47 ± 0.35	1.29 ± 0.47	0.064 ± 0.011	0.82 ± 0.16	0.16 ± 0.075
D-amino acid oxidase	65.6 ± 12.5	3.58 ± 1.56	3.20 ± 1.24	38.9 ± 26.6	1.94 ± 1.51	0.36 ± 0.21

[a] Values are means ± SEM of 10 experiments.
[b] Values are means ± SEM of 7 experiments.
[c] Since the substrate concentration varied in different experiments no mean value can be given.
One mU = 1 μmole/min.

Table 2
Composition of 0% Ludox fraction and of purified lysosomal fraction after density gradient centrifugation of mitochondrial-lysosomal fraction

Fraction	No of expts	Percentage			
		Microsomes	Mitochondria	Peroxisomes	Lysosomes
0% Ludox fraction; no tetrazolium salt pretreatment (A)	2	49.9 (49.0, 50.8)	16.0 (11.3, 20.7) [1]	5.3 (4.8, 5.8)	3.0 (2.8, 3.2)
0% Ludox fraction; with tetrazolium salt pretreatment (B)	10	32.3 ± 7.7	21.0 ± 7.0	9.0 ± 3.9	26.4 ± 6.9
Purified lysosomal fraction (precipitate from B)	7	18.1 ± 3.5	5.7 ± 2.6	4.9 ± 3.8	61.2 ± 15.6

For experimental details see text. The composition of the fractions was estimated by the procedure of Beaufay [1] as described in the text. Values are means ± SEM.

Ludox fraction. The properties of the 0% Ludox fraction obtained after pretreatment of the mitochondrial-lysosomal fraction with succinate plus INT, and of the purified lysosomal fractions are shown in table 1. There is an increase in specific activity of the lysosomal enzymes and a decrease in that of the mitochondrial, peroxisomal and microsomal markers in the final purified lysosomal fractions. The yield of lysosomal enzymes is 11–12%.

The composition of the 0% Ludox fraction and of the purified lysosomal fraction obtained as described above are shown in table 2. The purified lysosomal fraction contains about 61% lysosomes, 6% mitochondria, 5% peroxisomes and 18% microsomes.

The lysosomes remain intact during the isolation procedure, as judged from the fact that the latency of *N*-acetyl-β-glucosaminidase decreased only very slightly in the final preparations (table 3). In the electron microscope, intact vesicular structures could be seen, together with a small proportion of mitochondrial profiles (P. J. Weijers, unpublished observations).

Table 3
Latency of *N*-acetyl-β-glucosaminidase and acid phosphatase

Fraction	No. of expts	Latency (%)	
		N-acetyl-β-glucosaminidase	Acid phosphatase
Mitochondrial lysosomal fraction	10	43.2 ± 4.3	68.0 ± 4.2
0% Ludox fraction (tetrazolium salt pretreatment)	10	43.2 ± 4.0	–
Purified lysosomal fraction	7	38.0 ± 3.9	–

For experimental details see text. Values are means ± SEM.

4. Discussion

The results show that the method can be confidently applied for the isolation of a purified lysosomal preparation from normal rat liver. Since such preparations consist of lysosomes whose physical properties would not be expected to be altered, in contrast to lysosomes isolated from rats injected with Triton WR 1339, Dextran 500 or colloidal iron, they should be particularly useful for investigating such problems as the permeability of the lysosomal membrane and the antigenic properties of the surface of the membrane. Furthermore, the method described could in principle be applied to all tissues in which separation of lysosomes from mitochondria presents a problem.

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