METHOD FOR THE ISOLATION OF IRON-LOADED LYSOSOMES FROM RAT LIVER

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1. Introduction

Following the enunciation of the lysosome concept many attempts to isolate lysosomes from various tissues have been performed (cf. [1]). From a theoretical point of view such attempts could be expected to be successful since the physical properties (median equilibrium density and/or sedimentation coefficient) of lysosomes differ from those of other cell organelles. However, the well established heterogeneity among lysosomes as well as among other populations of subcellular organelles complicates to a great extent the practical procedures of isolating pure lysosomal fractions by use of differential or isopycnic centrifugational techniques.

Isolation of a reasonably pure fraction of liver lysosomes from experimental animals was achieved by administration of Triton WR 1339 [2]; this compound is concentrated in secondary lysosomes, thereby decreasing their density. The two main drawbacks of this method are that the isolated lysosomal particles are larger than normal and that they contain considerable amounts of a detergent known to interfere with lipid metabolism [cf. 1]. Lysosomes loaded with dextran or sucrose are likewise enlarged and accordingly more apt to rupture during isolation procedures than lysosomes of normal size.

Repeated injections of Fe^{3+} as an iron sorbitol citric acid complex to experimental animals result in an accumulation of "iron granules" in secondary lysosomes of liver cells [3]. Based on these findings an investigation was performed to find out whether or not this accumulation causes such an increase in their density as to make it possible to obtain a fraction of lysosomes essentially free of other subcellular organelles.

2. Materials and methods

Jectofer® (an iron–sorbitol–citric acid complex, obtained from AB Astra, Sweden), diluted 1:1 with isotonic saline, was injected intramuscularly into male albino rats weighing 150–200 g. The animals received 15 injections during a period of three weeks in a total amount of 50 mg Fe^{3+} per 100 g of body weight. The rats were starved 15 hr before sacrifice. The blood in the livers was washed out by a short perfusion with 0.30 M sucrose via the portal vein. In order to obtain optimal conditions for preparation without disrupting the lysosomes, homogenization was performed with either a Teflon–glass (Potter–Elvehjem type) homogenizer (maximal clearance 0.23 mm) or a Dounce all glass device with a loosely fitting pestle. The latter was preferred for the isolation of lysosomes from lymphoid tissues [13]. Various sucrose concentrations (0.30–0.75 M) were tested for the initial preparatory steps.

The homogenate was diluted to 10% (w/v). Cell debris and nuclei were sedimented at 600 *g* for 10 min. This procedure was repeated once to increase the yield. The pooled supernatants were exposed to different centrifugational forces (6,000 *g* – 12,500 *g*) during various time periods (6 min–12 min). The resulting pellet (or part of the pellet) was carefully suspended by hand in 0.30 M sucrose (~0.5 g liver/ml) and 2 ml were layered onto continuous sucrose gradients of various ranges. Centrifugation was then performed at 100,000 *g* (maximum RCF) for 1, 2, 4 and 16 hr using a Christ Omega II Ultracentrifuge, Rotor SW-27. Mitochondria and microsomes were prepared as described earlier [4].

Following centrifugation at 100,000 *g*, different

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fractions were assayed for acid phosphatase [5, 13], cathepsin D [6, 13] and aryl sulfatase [7, 13] after complete enzyme activation achieved by repeated freezing and thawing and/or addition of 0.2% Triton X-100 to the incubation medium. The activities of NADPH-cytochrome *c* reductase (marker for microsomes) [9], succinate cytochrome *c* reductase (marker for mitochondria [9] and D-amino acid oxidase (marker for microbodies or peroxisomes [10] as well as the protein content [11] of all fractions were also determined.

For electron microscopical analyses the different fractions obtained after gradient centrifugation were fixed in cacodylate buffered glutaraldehyde (final conc. 2%, 0.1 M buffer, pH 7.2) for 12 hr. After sedimentation of the material at 20,000 *g* for 30 min and postfixation in 2% *s*-collidine-buffered OsO_4 (pH 7.2), dehydration and embedding in Epon were performed. Thin sections from various parts of the embedded material were stained with uranyl acetate and lead citrate and were studied in a Siemens Elmiskop I electron microscope.

3. Results and discussion

When homogenization was performed in 0.30 M sucrose, the Teflon homogenizer seemed to bring about a more effective grinding than the Dounce homogenizing device since the enzyme content in the 600 *g* supernatant was higher after using the Teflon homogenizer (table 1). This effect was not accompanied by a higher degree of lysosome rupture with the Teflon device, as indicated by the similar proportion between sedimentable and unsedimentable enzyme activity following centrifugation at 10,000 *g* for 20 min. For these reasons the Teflon homogenizer was preferred. Homogenization in 0.50–0.75 M sucrose did not increase the ratio between sedimentable and unsedimentable activity of acid phosphatase. Furthermore, high sucrose concentrations interfered with the formation of the two-layered pellet described beneath.

The pellet obtained after centrifugation of the 600 *g* supernatants contained considerable amounts of non-lysosomal protein which interfered with the subsequent gradient centrifugation. Since in some of the experiments the pellet after differential centrifugation was two-layered – consisting of a brown bottom layer and a pale top layer – enzyme analyses of these two parts were performed. The upper part of the pellet showed relatively high levels of mitochondrial enzyme activity while the relative specific activities of acid hydrolases were low. Considerably higher relative specific activities of acid hydrolases were found in the lower, tightly packed part of the pellet, which consequently was considered to be useful as a crude lysosomal fraction for subsequent gradient centrifugations. The most useful method to separate the two layers of the pellet was to sediment the 600 *g* supernatant at 6,000 *g* for 7.5 min. After this centrifugation the pellet was constantly two-layered and the supernatant together with the pale loosely packed upper pellet could easily be sucked off. Higher centrifugational forces and longer centrifugation times resulted in a less sharp separation of the two components of the pellet.

Table 1
Comparison of Teflon and Dounce homogenizers.

Homogenizer	600 *g* × 10 min		10,000 *g* × 20 min	
	Pellet	Supernatant	Pellet	Supernatant
Dounce	10.2	8.7	5.7	2.9
Teflon	6.9	11.0	6.9	3.1

Homogenization was performed in 0.30 M ice-cold sucrose at 205 rpm with four up-and-down strokes (Teflon) or with eight strokes using the Dounce device. In order to distinguish between sedimentable and unsedimentable activity the first supernatant was pelleted at 10,000 *g* for 20 min. The fractions were assayed for *p*-nitrophenyl phosphatase [8]. The values are given an μmole *p*-nitrophenol released/min/g liver. The experiment is typical of four.

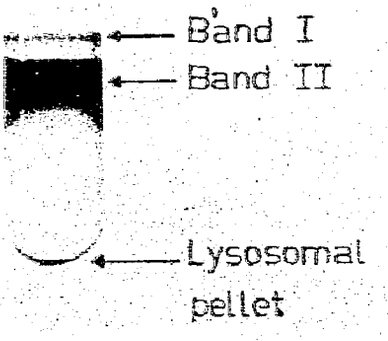

Fig. 1. Picture illustrating appearance of tube after 2 hr centrifugation at 100,00 *g*. The gradient was ranging between 1.4 and 2.2 M sucrose.

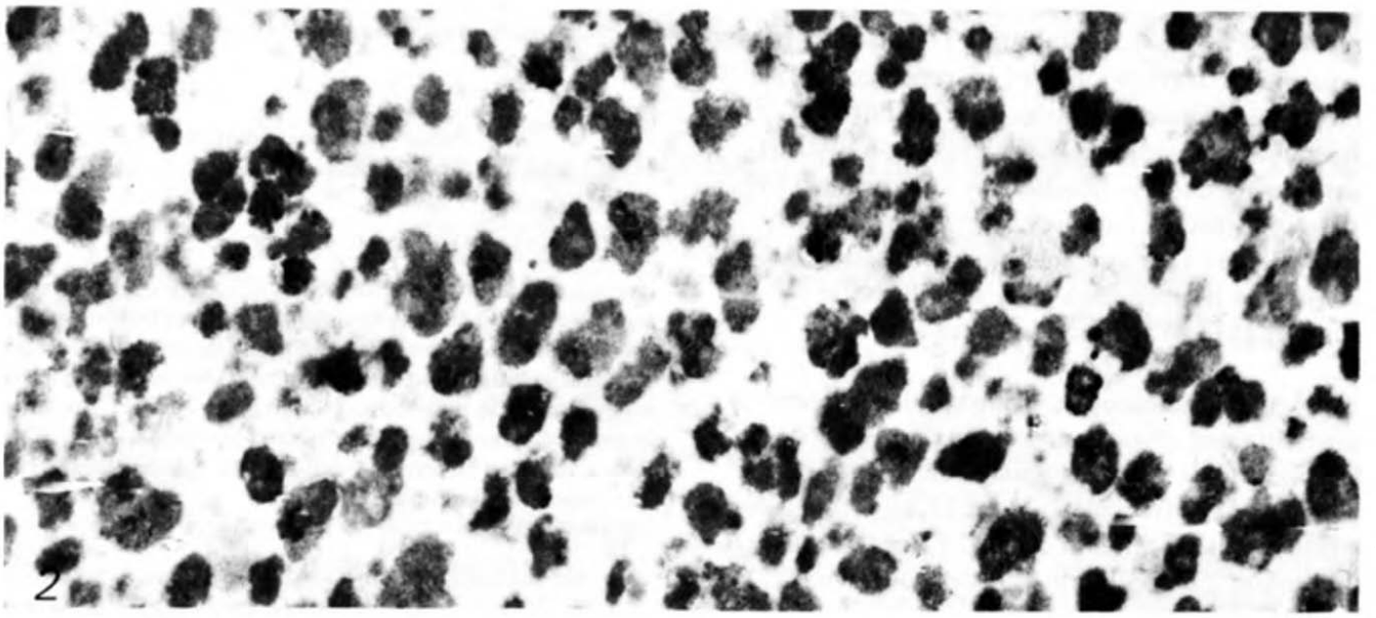

Fig. 2. Electron microscopic picture of isolated pellet consisting entirely of iron-loaded lysosomes in the area illustrated. × 16,500.

The resuspended, dark brown pellet was used for density gradient centrifugation and was layered onto continuous sucrose gradients of various ranges (1.0 – 1.3 M at the top and 1.8 – 2.4 M at the bottom). Centrifugation resulted in the formation of an upper light brown band (band 1) with a broad white band underneath (band 2) and at the bottom of the gradient a thin, dark brown pellet (fig. 1). Enzyme assays showed that the pellet contained high relative specific activities of acid hydrolases. The specific activity reached its highest values when the density of the lower part of the gradient was increased above 1.28 g/cm^3. Concomitantly the activity of mitochondrial enzyme in the pellet decreased. The specific activities of lysosomal enzymes were lower in bands 1 and 2. By electron microscopy, band 2 contained an abundance of mitochondria and few lysosomes. There were also many mitochondria in band 1. The pellet was composed of tightly packed iron granule-containing lysosomes, with occasional mitochondria in ter-

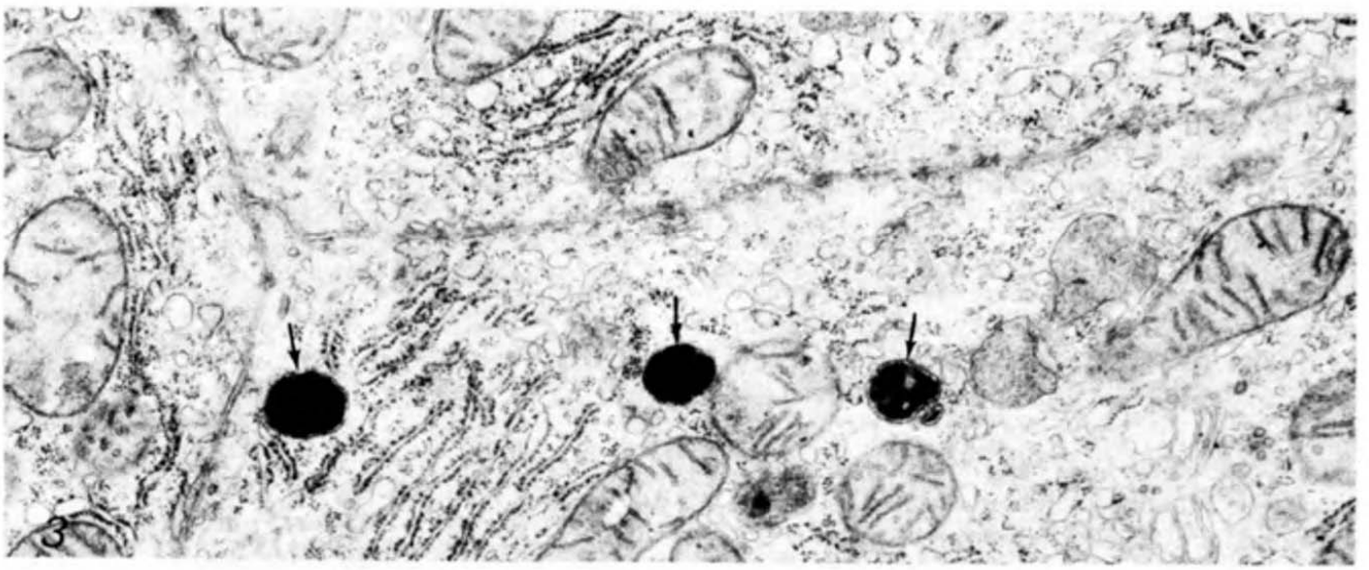

Fig. 3. Portion of hepatic parenchymal cells containing 3 iron-loaded secondary lysosomes (*arrow*). × 16,500.

Table 2
Distribution of NADPH-cytochrome *c* reductase, succinate cytochrome *c* reductase and D-amino acid oxidase in various subcellular fractions after Jectofer® administration.

	NADPH-cytochrome *c* reductase*		Succinate cytochrome *c* reductase*		D-amino acid oxidase**	
	per g liver	per mg protein	per g liver	per mg protein	per g liver	per mg protein
Homogenate	8.3 ±2.1	0.046 ±0.014	29.5 ±5.1	0.164±0.028	60.1 ±9.1	0.39 ±0.042
Microsomes	3.3 ±0.4	0.186 ±0.020	0.54±0.12	0.031±0.007	–	–
Mitochondria	0.19±0.04	0.008 ±0.002	9.1 ±1.5	0.394±0.066	6.0 ±0.9	0.270±0.035
Origin	0.03±0.01	0.0023±0.001	2.2 ±0.3	0.179±0.028	2.2 ±0.3	0.182±0.021
Lysosomal fraction	0.0006	0.001	0.01±0.005	0.015±0.009	0.14±0.02	0.240±0.031

* μmole cytochrome *c* reduced/min.
** $Ext_{440} \times 10^3/2$ hr.
All values are expressed as means ± SEM, of at least five experiments.
"Origin" denotes the suspension layered on top of the continuous sucrose gradient.

spersed (fig. 3). These lysosomes had an appearance inseparable from that of lysosomes in liver cells (parenchymal and Kupffer cells alike) *in situ* after administration of Jectofer® (fig. 3). Measurements of the lysosomes in normal livers and in livers of rats given Jectofer® did not reveal any significant difference in size between these lysosomes.

Experiments with different centrifugation times revealed that after 1 hr the bulk of acid hydrolase-containing particles was not yet pelleted; however, this was the case after 2 hr. Longer centrifugation times resulted in a decrease of the specific activity of lysosomal enzymes.

The final recommended fractionation procedure is summarized in fig. 4. Following this preparation scheme the protein content of the lysosomal pellet was about 0.7 mg/g liver. The activities of mitochondrial and microsomal enzymes are shown in table 2. The low specific activities of NADPH-cytochrome *c* reductase and succinate cytochrome *c* reductase in the lysosomal fraction demonstrate that it can only contain negligible amounts of enzymically active endoplasmic reticulum fragments and mitochondria. Similar conclusions can be drawn concerning peroxisomes as is evident from the distribution of D-amino acid oxidase activity (table 2).

In table 3 the activities of three acid hydrolases are given for the original homogenate, the material layered on top of the gradient (= origin), and the lysosomal fraction (represented by the final pellet). All three enzymes are concentrated approx. 35-fold over the homogenate. If one assumes that the acid hydrolases are exclusively distributed in monomodal fashion, the lysosomal recovery would amount to approx. 12%. On the other hand, if the distribution of

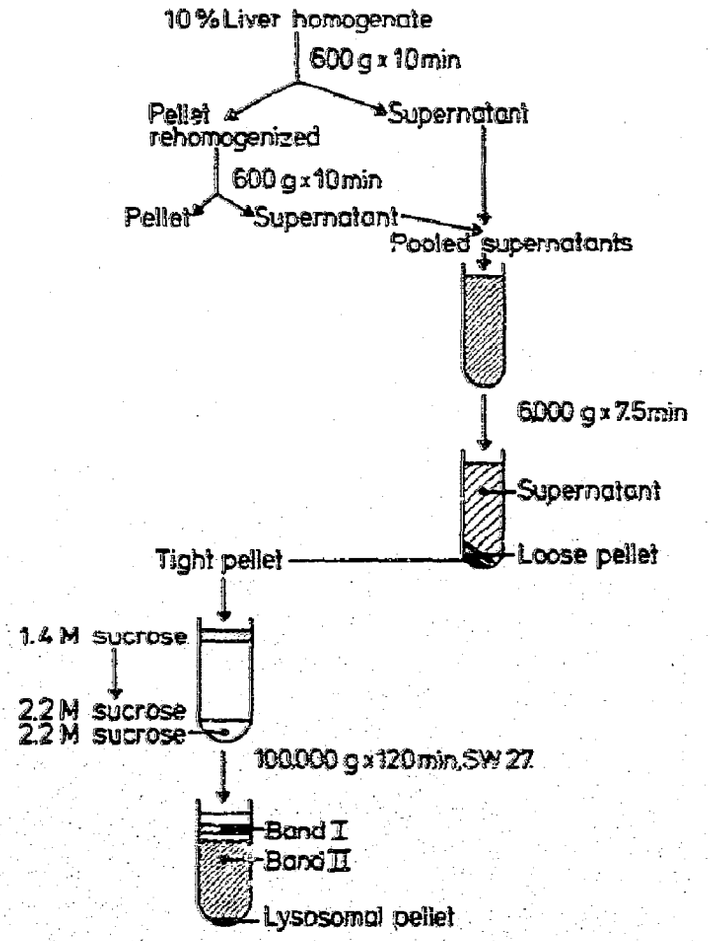

Fig. 4. Summary of recommended fractionation procedure

Table 3
Distribution of acid hydrolases in homogenate, "origin", and lysosomal fraction.

Fraction	Aryl sulfatase		Acid phosphatase		Cathepsin D	
	Specific activity[1]	Total activity[2]	Specific activity[1]	Total activity[2]	Specific activity[1]	Total activity[2]
Homogenate	1	100	1	100	1	100
Origin	4.0±0.29	34±3.9	4.4±0.5	32±1.1	3.5±0.36	26±1.3
Lysosomal fraction	33 ±2.7	12±1.0	33 ±1.8	11±0.66	35 ±1.6	13±0.67

[1] Enzyme activity/mg protein in relation to the homogenate activity (= 1).
[2] Enzyme activity/g liver as % of the homogenate activity (= 100).
Absolute values for the enzyme activities in the homogenate were the following: Acid phosphatase (μmole PO_4^{2-} released/min): specific activity 0.025 ± 0.002; total activity 5.0 ± 0.3. Aryl sulfatase (μmole 4-NC released/min): specific activity 0.0086 ± 0.0009; total activity 1.45 ± 0.09. Cathepsin D (μmole tyrosine released/min): specific activity 0.0058 ± 0.001; total activity 0.92 ± 0.1. All values are expressed as means ± SEM, of at least five experients.
"Origin" denotes the suspension layered on top of the continuous sucrose gradient.

acid hydrolases is multimodal – and there are some data which indicate that certain acid hydrolases are also present in the ER (cf. [12]) – the calculated recovery as well as the given degree of purification are underestimated. Purification of the same order of magnitude can be obtained by using Triton WR 1339. However, this is gained at the expense of the yield, which with the Triton method usually does not exceed 8% (cf. [1]).

4. Conclusion

The data presented indicate that the accumulation of iron granules in secondary lysosomes of rat liver following repeated injections of Fe^{3+} is attended by an increase in their density (equilibrium density in sucrose > 1.28 g/cm^3). This change makes it possible to isolate a highly purified lysosomal fraction by means of ultracentrifugation in sucrose gradients.

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