Isolation of pig thyroid lysosomes

Biochemical and morphological characterization

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Open thyroid follicles were prepared by mechanical disruption of pig thyroid fragments through a metal sieve. This procedure allowed preparation of thyroid-cell material depleted of colloid thyroglobulin. Open thyroid follicles were used to prepare a crude particulate fraction, which contained lysosomes, mitochondria and endoplasmic reticulum. These organelles were subfractionated by isopycnic centrifugation on iso-osmotic Percoll gradients. (1) A lysosomal peak was identified by its content of acid hydrolases: acid phosphatase, cathepsin D, β -galactosidase and β -glucuronidase. The lysosomal peak was well separated from mitochondria and endoplasmic reticulum. (2) The lysosomal peak, from which Percoll was removed by centrifugation, was taken as the purified lysosome fraction (L). Lysosomes of fraction L were purified 45–55-fold (as compared with the homogenate) and contained about 5% of the total thyroid acid hydrolase activities. (3) Electron microscopy showed that fraction L was composed of an approx. 90% pure population of lysosomes, with an average diameter of 220 nm. (4) Acid hydrolase activities were almost completely (80–90%) released by an osmotic-pressure-dependent lysis. (5) Thyroglobulin was identified by polyacrylamide-gel electrophoresis as a soluble component of the lysosome fraction. In conclusion, a 50-fold purification of pig thyroid lysosomes was achieved by using a new tissue-disruption procedure and isopycnic centrifugation on Percoll gradient. The presence of thyroglobulin indicates that the lysosome population is probably composed of primary and secondary lysosomes. Isolated thyroid lysosomes should serve as an interesting model to study the reactions whereby thyroid hormones are generated from thyroglobulin and released into the thyroid cells.

INTRODUCTION

It is generally considered that thyroglobulin hydrolysis occurs inside phagolysosomes or secondary lysosomes which result from the fusion of lysosomes with thyroglobulin-containing vesicles or endocytotic vesicles (Van den Hove-Vandenbroucke, 1980). These vesicles are formed at the apical pole of the thyroid cell through different mechanisms: macropinocytosis (Wollman, 1969), micropinocytosis (Seljelid *et al.*, 1970), receptor-mediated endocytosis, fluid pinocytosis (Ericson & Engström, 1978; Van den Hove *et al.*, 1982). Two types of endocytotic structures have been described: colloid droplets of diameter about 1 μ m (Wetzel *et al.*, 1965) and micropinocytotic vesicles ranging in diameter from 0.06 to 0.3 μ m (Engström & Ericson, 1981). Colloid droplet formation occurs only under intense thyroid stimulation (Nadler *et al.*, 1962). Micropinocytosis seems to be the more usual process in basal conditions. None of the thyroglobulin-containing vesicles (endocytotic vesicles, lysosomes) have been isolated in a purified state. Studies of the hydrolysis of thyroglobulin have only been performed on crude fractions containing both acid hydrolases and iodinated proteins (Vandenbroucke *et al.*, 1971, 1972; Itikawa & Kawada, 1974; Lamas & Ingbar, 1978). These tissue fractions, probably contaminated by colloid thyroglobulin and thyroglobulin in endoplasmic-reticulum cisternae, did not allow a precise analysis of the intralysosomal processing of thyroglobulin.

For a successful isolation of lysosomes or other

secretory vesicles from the thyroid, one must overcome two major difficulties, related to (1) the incompatibility between the drastic homogenization required to disrupt this tissue and the lysosome or vesicle fragility and (2) the similarity of sedimentation behaviour of lysosomes and mitochondria.

In this paper we first describe a procedure for the isolation of thyroid lysosomes, based on two new approaches: (1) a mechanical disruption of the tissue before cell homogenization and (2) a subcellular fractionation by a combination of differential centrifugation and isopycnic centrifugation in Percoll gradients.

MATERIALS AND METHODS

Materials

NADH (disodium salt, grade III), *p*-nitrophenyl phosphate (disodium salt), cytochrome *c* and guaiacol were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 4-nitrophenyl β -D-galactopyranoside, 4-nitrophenyl β -D-glucuronide, β -glycerophosphate and haemoglobin were from Merck (Darmstadt, Germany). Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Isolation of lysosomes

Pig thyroid glands were collected at the local slaughterhouse, transported on ice to the laboratory and

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Scheme 1. Isolation procedure for thyroid lysosomes

All steps were carried out at 0–4 °C.

processed within 2 h after death of the animal. The complete procedure described below was performed at 0–4 °C. Glands were trimmed free from fat and connective tissue, and chopped into small pieces and washed in 10 mM-Tris/HCl buffer/0.25 M-sucrose, pH 7.4 (TS buffer).

Step 1. Thyroid fragments (about 100 g of tissue) were forced with the bottom of a beaker through a metal sieve (pore size 0.3 mm) and collected in 1 litre of TS buffer. The tissue suspension, taken as the homogenate (H) (samples were homogenized for subsequent assays), was centrifuged at 770 g (r_{av} , 12 cm) for 20 min. The supernatant (S_0), containing most of the colloid thyroglobulin, was eliminated. The resulting pellet was washed by resuspension in TS buffer and centrifugation at 770 g for 20 min. The pellet resuspended in 50 ml of TS buffer represents the 'open follicles' fraction (Scheme 1).

Step 2. Open follicles in TS buffer was homogenized in a glass/Teflon Potter–Elvehjem homogenizer with a tight-fitting Teflon pestle, rotated at 1500 rev./min with six slow up-and-down strokes. The resulting material (P1) was centrifuged at 800 g (r_{av} , 11 cm) for 20 min. The supernatant S2 was collected and the pellet P2 was resuspended in 40 ml of TS buffer, rehomogenized and centrifuged under the same conditions. The final P2 pellet was discarded and the two supernatants S2 (75–80 ml) were pooled and processed by one of two different procedures. In procedure I, S2 was centrifuged at 4000 g (r_{av} , 8 cm) for 20 min to obtain a supernatant S3 and a pellet P3. S3 was further centrifuged at 26000 g (r_{av} , 8 cm)

for 20 min to give fractions P4 and S4. In procedure II, S2 was directly centrifuged at 26000 g for 20 min to obtain the pellet P4 and the supernatant S4. In both cases, P4 resuspended in 15 ml of TS buffer with a syringe was taken as the crude particulate lysosomal fraction.

Step 3. Percoll (20 mosm/kg) was made iso-osmotic by adding 9 vol. of Percoll to 1 vol. of 2.5 M-sucrose/10 mM-Tris/HCl, pH 7.4. This stock solution was diluted to 30% (v/v) Percoll (density 1.067 g/ml) with TS buffer, and 24 ml of this 30% Percoll was mixed with 1.2 ml of P4 suspension and centrifuged in a fixed-angle rotor (Beckman 50.2 Ti) at 60000 g (r_{av} , 8.1 cm) for 45 min. The gradients were collected into 50 fractions by pumping from the top with a Buchler auto-densi-flow apparatus. The fractions corresponding to the lysosomal peak were pooled, diluted 3-fold with TS buffer and centrifuged at 26000 g (r_{av} , 8 cm) for 20 min to remove Percoll. This washing procedure was repeated twice. The final lysosome pellet (L) was resuspended in TS buffer or in 10 mM-Tris/HCl, pH 7.4, and stored at –20 °C. A similar procedure was applied for gradient fractions corresponding to the major protein peak (microsomal fraction + mitochondria), fraction M. In each experiment, the density profile was determined by using density-marker beads (1.036–1.145 g/ml).

Enzyme assays

All assays were performed on fractions stored at –20 °C. The following marker enzymes were assayed: cytochrome *c* oxidase for mitochondria (Hodges & Leonard, 1974), with reduced cytochrome *c* as substrate; NADH-cytochrome *c* reductase for endoplasmic retic-

Table 1. Distribution of protein and marker enzymes in thyroid subcellular fractions obtained by differential centrifugation

Absolute values are given for the homogenate (H) and expressed per g of tissue; enzyme activities are expressed in units and protein in mg. In the other fractions (P₁ to S₄), enzyme activities and protein are expressed as percentages of the values in the homogenate. Recovery is given for procedure II and calculated from fraction P₁ as the ratio $100 \times (P_2 + P_4 + S_4)/P_1$. Results are means $\pm$ S.E.M. ($n = 3$ to 11 experiments).

	Protein	Acid phosphatase	Cathepsin D	β -Galactosidase	Cytochrome <i>c</i> oxidase	Peroxidase	NADH-cytochrome <i>c</i> reductase
No. of expts. ...	11	9	3	3	6	11	3
H	117.7 $\pm$ 11.1	0.66 $\pm$ 0.08	67.3 $\pm$ 6.4	0.53 $\pm$ 0.04	0.19 $\pm$ 0.02	0.59 $\pm$ 0.19	1.3 $\pm$ 0.2
Common steps for procedures I and II	P ₁	3.7 $\pm$ 0.4	34.2 $\pm$ 3.8	42.8 $\pm$ 4.4	—	87.6 $\pm$ 9.1	96.6 $\pm$ 10.1
	P ₂	1.2 $\pm$ 0.1	11.4 $\pm$ 1.8	20.0 $\pm$ 4.4	—	55.0 $\pm$ 6.8	49.2 $\pm$ 17.5
	S ₂	2.6 $\pm$ 0.2	23.0 $\pm$ 2.5	21.2 $\pm$ 2.6	—	17.0 $\pm$ 7.5	37.1 $\pm$ 8.5
Procedure I	P ₃	0.89 $\pm$ 0.09	14.1 $\pm$ 1.6	—	—	14.2 $\pm$ 1.7	10.8 $\pm$ 1.3
	S ₃	1.78 $\pm$ 0.20	11.5 $\pm$ 1.2	—	—	2.5 $\pm$ 0.4	26.6 $\pm$ 7.2
	P ₄	0.55 $\pm$ 0.06	7.4 $\pm$ 0.6	—	—	2.3 $\pm$ 0.4	23.3 $\pm$ 5.1
	S ₄	1.30 $\pm$ 0.10	4.1 $\pm$ 0.4	—	—	0.30 $\pm$ 0.05	3.4 $\pm$ 1.3
Procedure II	P ₄	1.3 $\pm$ 0.1	19.5 $\pm$ 1.6	15.3 $\pm$ 2.4	14.7 $\pm$ 0.4	17.5 $\pm$ 2.2	34.0 $\pm$ 7.2
	S ₄	1.4 $\pm$ 0.2	4.6 $\pm$ 0.4	5.4 $\pm$ 0.3	5.0 $\pm$ 1.5	0.30 $\pm$ 0.05	3.8 $\pm$ 1.2
Recovery (%)	105.4	103.8	94.8	—	83.1	90.1	93.8

ulum, with cytochrome *c* as substrate (de Duve *et al.*, 1955); peroxidase for endoplasmic reticulum, with guaiacol as substrate (Hosoya & Morrison, 1967). Four lysosomal enzymes were assayed: acid phosphatase, with *p*-nitrophenyl phosphate (Lindhardt & Walter, 1963) or β -glycerophosphate (Lisman *et al.*, 1979); cathepsin D, with haemoglobin as substrate (Dunn & Dunn, 1982); β -galactosidase, with *p*-nitrophenyl β -D-galactopyranoside (Distler & Jourdan, 1978); β -glucuronidase, with *p*-nitrophenyl β -D-glucuronide (Fishman, 1974). For acid phosphatase activity measurements with *p*-nitrophenyl phosphate, all fractions were preincubated 5 min at 37 °C to abolish the glucose-6-phosphatase activity, which hydrolyses *p*-nitrophenyl phosphate. The acid phosphatase activity assayed with β -glycerophosphate was named β -glycerophosphatase activity. The unit of enzyme activity is 1 μ mol of substrate transformed/min, except for cathepsin D and peroxidase. A cathepsin D unit represents the amount of enzyme required to produce a change of 1 A_{280} unit/h. For peroxidase, a guaiacol unit is defined as the amount of enzyme causing a change of 1 A_{470} unit/min.

Electron microscopy

Lysosome fractions were fixed with 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4, for 2 h at 4 °C, washed twice in the same buffer and post-fixed with 1% OsO₄ in 0.15 M-cacodylate buffer, pH 7.4, for 2 h at 4 °C. After dehydration, pellets were embedded in Epon 812 epoxy resin. Ultrathin sections (70 nm) were post-stained with uranyl acetate and lead citrate and examined in a Jeol 1200 EX electron microscope at 80 kV (Centre de Microscopie électronique et Pathologie ultrastructurale, Faculté de Médecine Alexis Carrel, Lyon). Observations were made on sections corresponding to different parts of the lysosome pellet.

Other methods

Protein was assayed (Lowry *et al.*, 1951) after solubilization with 1% deoxycholate, with bovine serum

albumin as standard. Since high concentrations of Percoll can interfere in the protein assay, gradient fractions without protein were used as blanks. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Rousset & Wolff, 1980) was performed on slab gels.

RESULTS

Isolation of thyroid lysosomes

Preparation of a crude particulate fraction which contains lysosomes. The first step in the isolation procedure led to (a) the separation of thyroid epithelium from the connective framework and (b) the preparation of open follicles or cell sheets or strips. The overall organization of the thyroid cells was maintained. The nuclei appeared in the basal region of the cells. Cells were attached side-by-side through junctional complexes, but were devoid of basal plasma membrane. The disruption of the follicular structure resulted in the release of colloid thyroglobulin into the medium. Most of the thyroglobulin was separated from the cellular compartment by a low-speed centrifugation. The thyroglobulin/cell-protein ratio, which was about 8 in intact thyroid tissue, was decreased to 0.13 in P1 material (values determined by thyroglobulin radioimmunoassay; B. Rousset & R. Rabilloud, unpublished work). After the two steps of homogenization (sieve + Potter), 20–25% of homogenate acid hydrolase activities were present in S2 fraction. This fraction also contained mitochondria and endoplasmic reticulum. Some 60% of acid hydrolase activities were found in S₀. Two different procedures (Table 1) were then tested to prepare a crude lysosomal fraction (P4) from S2. When prepared by procedure I, pellet P4 contained 32% of acid phosphatase and 13% of cytochrome *c* oxidase of the S supernatant. In contrast, P4 obtained directly from S2 (procedure II) contained 100% of cytochrome *c* oxidase and 85% of acid phosphatase of S2. It appeared therefore that 80% of mitochondria or mitochondrial

Fig. 1. Distribution profiles of acid phosphatase and cytochrome *c* oxidase of P4 material fractionated on 30% iso-osmotic Percoll gradients

(a) P4 fraction prepared by procedure I; (b) P4 fraction prepared by procedure II. ●, Acid phosphatase; ▲, cytochrome *c* oxidase. The size of the fractions was 0.5 ml.

particles could be removed by centrifugation at 4000 *g*, but the removal of mitochondria was accompanied by a marked decrease in the recovery of lysosomes (acid hydrolase activities) in the 26000 *g* pellet. To determine which of the two P4-preparation procedures was the most suitable for lysosome purification, we have examined the ability of Percoll gradients to separate lysosomes from mitochondria. Acid phosphatase and cytochrome *c* activity profiles obtained by centrifugation on 30% Percoll gradients are shown in Fig. 1. Whatever the procedure (I or II) for P4 preparation, there were two peaks of acid phosphatase and one peak of cytochrome *c* oxidase. In both cases, the heavy peak (density 1.10 g/ml) of acid phosphatase was clearly separated from the peak of cytochrome *c* oxidase; the light peak (density 1.035 g/ml) of acid phosphatase partially overlapped the peak of cytochrome *c* oxidase. As expected, the enzyme activities were higher (2–3-fold) in gradients corresponding to P4 prepared directly from S2. Since the method of P4 preparation did not affect the separation on Percoll gradients, and since the yield of lysosomal enzyme markers (especially in the heavy peak) was higher with procedure II, this procedure was selected for subsequent studies.

Isopycnic centrifugation on Percoll gradient. The general distribution profiles of protein, lysosomal and non-lysosomal enzyme markers on Percoll gradient are reported in Fig. 2. Material from P4 sedimented as a large

white fluffy band in the top half of the gradient and a narrow yellow-to-brown band in the lower part of the gradient. In agreement, the protein profile exhibited a large peak with a median density of 1.06 g/ml and a small peak or a shoulder in the region 1.08–1.14 g/ml. Enzyme markers for endoplasmic reticulum (NADH-cytochrome *c* reductase and peroxidase) appeared as a single peak with a median density of 1.06 g/ml, which overlapped the cytochrome *c* oxidase peak. Plasma membranes, detected by 5'-nucleotidase activity, were found in the very first fraction of the gradients (result not shown). The four lysosomal markers (acid phosphatase, cathepsin D, β -galactosidase and β -glucuronidase) were mainly found in a heavy peak (density 1.14 g/ml) and to a variable extent in a light peak (density 1.035 g/ml). The light peak contained about 26, 16, 10 and 10% of total acid phosphatase, β -glucuronidase, β -galactosidase and cathepsin D respectively. To characterize further the nature of the two peaks of acid-hydrolase-containing organelles, we measured the enzyme (acid phosphatase) release resulting from the osmotic-pressure-induced lysis (Fig. 3). When the sucrose concentration was decreased from 0.25 M to 0, the non-sedimentable (at 26000 *g*) acid phosphatase activity of P4 increased from 10 to about 70% (insert of Fig. 3). Peroxidase (a membrane-bound enzyme) activity of P4 remained in the sedimentable fraction whatever the treatment. Sonication or three successive freezing/thawing cycles led to the release of about 80% of the total P4 acid phosphatase activity (measured in the presence of 0.04% Triton X-100). The analysis on a 30% Percoll gradient showed that the pretreatment of P4 in 0.125 M- and 0.062 M-sucrose caused a progressive disappearance of the heavy peak of acid phosphatase. The light peak of acid phosphatase as well as the peroxidase peak were not affected by the treatment. Thyroid organelles with a density higher than 1.08 g/ml on Percoll gradients, identified by their content of acid hydrolases and their sensitivity to osmotic shock, seemed to represent lysosomes. This was further ascertained by morphological and biochemical criteria.

Morphological and biochemical characterization of isolated thyroid lysosomes

Acid-hydrolase-containing organelles (fractions 38–44 on the Percoll gradient) were collected and separated from Percoll by three washing cycles in TS buffer; the final pellet was taken as the purified lysosome fraction, L, and used in the subsequent studies. Protein in fraction L represented 0.095% of the total homogenate protein. Starting from 100 g of thyroid tissue, 8–12 mg of protein of L material was obtained (Table 2). The specific activity of acid hydrolases in fraction L was increased about 50-fold as compared with the homogenate. The contamination of fraction L by endoplasmic reticulum was very low; no peroxidase activity could be detected, and the relative specific activity of NADH-cytochrome *c* reductase in fraction L was 1.5. The contamination of fraction L by mitochondria was more significant, since the relative specific activity of cytochrome *c* oxidase in fraction L was 5.6. Nevertheless, the ratio of the relative specific activity of acid hydrolase to that of cytochrome *c* oxidase was close to 10.

Electron-microscopic examination confirmed the high degree of purity of fraction L. Only a few mitochondria or mitochondrial particles were observed (Fig. 4). The micrograph presented in Fig. 4 is representative of the

Fig. 2. Protein and marker-enzyme distributions after isopycnic centrifugation on 30% Percoll gradients of the P4 fraction (procedure II)

The values for distributions are the percentages of total activity recovered in each fraction of the gradient. The hatched area indicates the region of the gradient that was taken as the lysosome fraction. (a) Protein; (b) acid phosphatase; (c) cathepsin D; (d) cytochrome *c* oxidase; (e) NADH-cytochrome *c* reductase; (f) peroxidase; (g) density curve; (h) β -galactosidase; (i) β -glucuronidase.

composition of the mass of the lysosome pellet. A thin layer (representing few per cent of the total depth of the pellet) composed of membrane material (probably mitochondrial and/or endoplasmic-reticulum membranes, membranes of lysed lysosomes) was observed on the top side of the lysosome pellet. More than 90% of the vesicles corresponded to lysosomes. The lysosome population exhibited some heterogeneity in terms of content. Lysosomes contained an electron-dense core, sometimes interrupted by vacuoles. The measured diameter varied from 80 to about 500 nm, but 60% of the isolated thyroid lysosomes had a diameter between 180 and 260 nm.

The density of lysosomes was determined on gradients of different Percoll concentrations (25–40%) by using density marker beads of densities 1.133 and 1.145 g/ml. The lysosome peak sedimented between the two density markers under all the conditions tested. Therefore the buoyant density of isolated thyroid lysosomes was close to 1.14 g/ml.

Soluble and membrane components of thyroid lysosomes were separated by centrifugation at 105000 *g* for 60 min after osmotic lysis in 0.05 M-sucrose. The soluble fraction contained more than 80% of the acid hydrolase activities and about 70% of the lysosomal proteins.

The soluble and the membrane fractions from lysosomes were analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 5). The polypeptide composition of the two fractions appeared very different. The soluble lysosomal fraction (lane 5 of Fig. 5) contained two main components with apparent M_r 50000 and 55000 and a high- M_r species with a relative mobility similar to that of the thyroglobulin subunit (apparent M_r 280000). The lysosomal membrane fraction (lanes 4 and 8 of Fig. 5) contained many polypeptides; the most prominent corresponded to the main components of the P4 fraction. Thyroglobulin was found in the soluble lysosomal compartment, but also in the membrane fraction. Results reported in Fig. 5 show that (a) the polypeptide composition of the intralysosomal soluble material was markedly different from that of lysosomal membranes and (b) the protein distribution patterns were reproducible from one lysosome preparation to another.

DISCUSSION

The results reported in this paper indicate that significant quantities of thyroid lysosomes can be isolated with relatively high purity. This has been made possible

Fig. 3. Effect of the pretreatment of the crude lysosomal fraction (P4) in decreasing sucrose concentration on the distribution profile of acid phosphatase and peroxidase on 30%-Percoll gradients

Pellets (procedure II) were resuspended in 10 mM-Tris/HCl buffer containing decreasing concentrations of sucrose (0.25 M to 0). After 15 min at 4 °C, the suspension was centrifuged at 26000 *g* (r_{av} , 8 cm) for 20 min. Supernatants were assayed for their acid phosphatase content. Pellets were suspended in TS buffer and fractionated on 30%-Percoll gradients. Gradient fractions were assayed for acid phosphatase (—) and peroxidase (····). The sucrose concentration during the pretreatment was: 0.25 M (Δ , $\blacktriangle$); 0.125 M ($\square$, $\blacksquare$); 0.063 M ($\circ$, $\bullet$). Insert: acid phosphatase (A.P.) content of the post-treatment 26000 *g* supernatant of P4 fraction (expressed as percentage of total P4 activity measured in the presence of 0.04% Triton X-100) as a function of the sucrose concentration.

Table 2. Isolation of pig thyroid lysosomes on Percoll gradients

Yields from the homogenate and the particulate fraction P4 are given for protein and marker enzymes. Relative specific activity (RSA) for a given enzyme activity is the ratio of the percentage of activity of this enzyme to the percentage of protein in the lysosomal fraction. Values represent means $\pm$ S.E.M. for three preparations, except for β -glucuronidase, which was assayed in one preparation only.

	Lysosome purification from:			
	Particulate fraction (P4)		Homogenate	
	Yield (%)	RSA	Yield (%)	RSA
Protein	7.3 $\pm$ 0.6	—	0.095 $\pm$ 0.008	—
Acid phosphatase	25.1 $\pm$ 0.2	3.8 $\pm$ 0.2	4.9 $\pm$ 0.4	52.1 $\pm$ 3.5
β -Glycerophosphatase	30.0 $\pm$ 2.3	4.8 $\pm$ 0.6	5.1 $\pm$ 0.2	50.5 $\pm$ 4.5
Cathepsin D	29.4 $\pm$ 1.9	4.0 $\pm$ 0.3	4.5 $\pm$ 0.3	47.8 $\pm$ 2.9
β -Galactosidase	30.6 $\pm$ 0.8	4.7 $\pm$ 0.8	5.7 $\pm$ 1.4	44.5 $\pm$ 7.5
β -Glucuronidase	—	—	6.5	43.2
Cytochrome <i>c</i> oxidase	3.1 $\pm$ 0.2	0.40 $\pm$ 0.02	0.55 $\pm$ 0.03	5.6 $\pm$ 0.2
NADH-cytochrome <i>c</i> reductase	1.4 $\pm$ 0.4	0.19 $\pm$ 0.03	0.15 $\pm$ 0.04	1.5 $\pm$ 0.2

by introducing a new method of disruption of the thyroid tissue which allows one first to separate thyroid-cell epithelium from the connective tissue, and therefore makes the homogenization easier, and second to eliminate thyroglobulin stores, which contaminate intracellular organelles in conventional approaches. Percoll

appears to be a convenient centrifugation medium to separate lysosomes from other thyroid particles; it was particularly effective in separating mitochondria from the lysosomal fraction. It must be noted that such a good separation does not seem to be possible in the liver without an alteration of the sedimentation behaviour of

Fig. 4. Electron-microscopic analysis of purified thyroid lysosomes

(a) Micrograph of a random section through the lysosome pellet recovered after Percoll removal; bar represents 2 μm. (c) Higher magnification of lysosomes with homogeneous electron-dense core and lysosomes which contain clear vacuoles; bar represents 200 nm. (b) Size distribution of thyroid lysosomes. The diameters of 1400 particles were measured on micrographs (magnification $\times 40000$). Mean frequencies ($\pm$ S.E.M., $n = 4$) are given for each size class from 60 to 500 nm.

mitochondria (Lardeux *et al.*, 1963) or lysosomes (Trouet, 1964; Leighton *et al.*, 1968; Arborgh *et al.*, 1975). By using Percoll, thyroid lysosomes were purified 50-fold with a yield of about 5%. It is noteworthy that the specific activity of four acid hydrolases (acid

phosphatase or β -glycerophosphatase, cathepsin D, β -galactosidase and β -glucuronidase) increased to the same extent. Despite the efficiency of the separation between lysosomes and mitochondria on Percoll gradients, mitochondria seem to represent the major

Fig. 5. Analysis by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of the polypeptide composition of soluble and membrane fractions of isolated thyroid lysosomes

Lysosomes were suspended in an hypo-osmotic medium (10 mM-Tris/HCl buffer, pH 7.4) and centrifuged at 100000 *g* for 45 min to prepare the soluble and membrane fractions. Samples contained 10 μ g (lane 1), 20 μ g (lane 9) or 100 μ g (lanes 2–8) of protein. The 9%-acrylamide gel was stained with Coomassie Blue. Arrows indicate the mobility of M_r markers, the following standard proteins: myosin heavy chain, β -galactosidase, phosphorylase *b*, bovine serum albumin, tubulin and ovalbumin. Samples were: lanes 1 and 9, thyroglobulin (TG); lane 2, P4 pellet; lane 3, unfractionated lysosomes; lanes 4 and 8, lysosomal membranes from two separate preparations; lanes 5 and 7, soluble lysosomal material from two separate preparations; lane 6, fraction M.

contaminant of the thyroid lysosome preparation. Few mitochondria were observed on electron micrographs, and the relative specific activity of cytochrome *c* oxidase in fraction L was 5. However, the latter value seems to overestimate the contamination. This could be due to the underestimation of the low cytochrome *c* oxidase activity of the thyroid homogenate. A thyroid lysosome purification of 4-fold with a yield of 8–10% on metrizamide gradients has been reported (Van den Hove-Vandenbroucke & de Nayer, 1976). Our data may be compared with those obtained in liver. Purification factors of 37–80-fold have been obtained for rat liver lysosomes. However, the yield in liver is generally higher (6–25%). The degree of purification achieved for lysosomes of other tissues, such as kidney (Maunsbach, 1969), lymphoid tissues (Bowers, 1974) or brain (Lisman *et al.*, 1979), is markedly lower (6–17-fold), the yield being 4–6%. As mentioned above, the yield of our purification procedure is relatively low; this is mainly due to a considerable loss of lysosomes during the first step, the opening of follicles. Indeed, supernatant S_0 contained 65% of the total acid hydrolase activities, among which 20% was in a latent form. When collected by

centrifugation at 26000 *g*, lysosomes present in the S_0 supernatant exhibited the same density on Percoll gradients as did those prepared from the open-follicle fraction (results not shown). The lysosomes from fraction S_0 were discarded, since they had been in contact with a high concentration of colloid thyroglobulin. The yield of lysosomes, when calculated from the open-follicle fraction, approaches 15%, a value within the range of values reported for liver.

The bimodal distribution of acid phosphatase and β -glucuronidase on the Percoll gradients does not mean that thyroid lysosomes can be separated into two populations of different densities. Indeed, acid hydrolase activities associated with the low-density particles were not released by lowering the osmolality of the medium; these enzyme markers behave as membrane-bound enzymes. The low-density particles may represent particles deriving from compartments in continuity with lysosomes (Muller *et al.*, 1980; Rome *et al.*, 1979).

The buoyant density of the purified thyroid lysosomes determined in different gradient conditions was 1.14 g/ml, a value similar to that obtained for rat liver lysosomes (Dobrota & Hinton, 1980; Solheim & Seglen, 1983).

Secondary thyroid lysosomes are supposed to contain thyroglobulin, the macromolecular precursor of thyroid hormones (Van de Hove-Vandenbroucke, 1980). We have identified thyroglobulin in the high-speed supernatant of lysed lysosomes. Therefore our thyroid lysosome preparations should contain both primary and secondary lysosomes. Our work has not determined whether all isolated lysosomes contain thyroglobulin and whether all releasable thyroglobulin originates from lysosomes. Indeed, the presence of secretory vesicles as contaminants of the purified lysosome fraction cannot be excluded. Other approaches are required to characterize lysosomal thyroglobulin further and to identify thyroglobulin degradation products.

Obtaining purified thyroid lysosomes should represent an important step in the study of still-open questions on the thyroid-hormone-secretory pathway, among which are the sequence of proteolytic cleavages of thyroglobulin which leads to the release of hormone residues, and the process by which free thyroid hormones leak from the lysosomes.

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