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Identification of two subpopulations of thyroid lysosomes: relation to the thyroglobulin proteolytic pathway

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Using a combination of differential centrifugation and isopycnic centrifugation in Percoll gradients, we obtained a highly purified preparation of thyroid lysosomes [Alquier, Guenin, Munari-Silem, Audebet & Rousset (1985) Biochem. J. **232**, 529–537] in which we identified thyroglobulin. From this observation, we postulated that the isolated lysosome population could be composed of primary lysosomes and of secondary lysosomes resulting from the fusion of lysosomes with thyroglobulin-containing vesicles. In the present study, we have tried to characterize these lysosome populations by (*a*) subfractionation of purified lysosomes using iterative centrifugation on Percoll gradients and (*b*) by functional studies on cultured thyroid cells. Thyroglobulin analysed by soluble phase radioimmunoassay, Western blotting or immunoprecipitation was used as a marker of secondary lysosomes. The total lysosome population separated from other cell organelles on a first gradient was centrifuged on a second Percoll gradient. Resedimented lysosomes were recovered as a slightly asymmetrical peak under which the distribution patterns of acid hydrolase activities and immunoreactive thyroglobulin did not superimpose. This lysosomal material (L) was separated into two fractions: a light (thyroglobulin-enriched) fraction (L2) and a dense fraction (L1). L1 and L2 subfractions centrifuged on a third series of Percoll gradients were recovered as symmetrical peaks at buoyant densities of 1.12–1.13 and 1.08 g/ml, respectively. In each case, protein and acid hydrolase activities were superimposable. The specific activity of acid phosphatase was slightly lower in L2 than in L1. In contrast, the immunoassayable thyroglobulin content of L2 was about 4-fold higher than that of L1. The overall polypeptide composition of L, L1 and L2 analysed by polyacrylamide-gel electrophoresis was very similar, except for thyroglobulin which was more abundant in L2 than in either L or L1. The functional relationship between L1 and L2 lysosome subpopulations has been studied in cultured thyroid cells reassociated into follicles. Thyroid cells, prelabelled with ^{125}I-iodide to generate ^{125}I-thyroglobulin, were incubated in the absence or in the presence of inhibitors of intralysosomal proteolysis. The fate of ^{125}I-thyroglobulin, and especially its appearance in the lysosomal compartment, was studied by Percoll gradient fractionation and immunoprecipitation. Treatment of prelabelled thyroid cells with chloroquine and leupeptin induced the accumulation of immunoprecipitable ^{125}I-thyroglobulin into a lysosome fraction corresponding to the L2 subpopulation. In control cells, in which intralysosomal proteolysis was not inhibited, ^{125}I-labelled material largely corresponding to degradation products, was found in lysosomes of the L1 subtype. In conclusion, thyroid lysosomes can be subfractionated into two subpopulations which differ in buoyant density, relative abundance and thyroglobulin content. The most abundant denser lysosomes should correspond mostly to primary lysosomes. The thyroglobulin-enriched lighter lysosomes exhibit the properties expected for secondary lysosomes, i.e. for the products of the fusion between dense vesicles (primary lysosomes) and light thyroglobulin-containing vesicles (endocytotic vesicles). Results obtained with the cultured thyroid cell system support these conclusions and give evidence for a relationship between the proteolytic cleavage of thyroglobulin and the transformation of L2 or secondary lysosomes into L1 or primary lysosomes.

INTRODUCTION

Thyroid hormone secretion requires the proteolysis of thyroglobulin, the thyroid prohormone. There is now evidence that lysosomal proteinases play a major role in the generation of free thyroid hormones from the macromolecular precursor (Wollman, 1969; Van den Hove-Vandenbroucke. 1980). Proteolytic cleavage is thought to occur in phagolysosomes or secondary lysosomes, the products of the fusion between primary lysosomes and vesicles which transport thyroglobulin taken up by the cells from the intrafollicular space. This pathway is largely hypothetical and requires experimental substantiation. Using isopycnic centrifugation on Percoll gradients, we have obtained highly purified preparations of lysosomes from pig thyroid glands (Alquier *et al.*, 1985). At the same time, Yoshinari *et al.* (1985) reported the purification of rat thyroid lysosomes by the same method. In the two reports, thyroglobulin was identified as a lysosomal component. During the extraction of

Abbreviations used: TS buffer, 10 mM-Tris/HCl buffer/0.25 M-sucrose; TSH, thyrotropin; SDS/PAGE, SDS/polyacrylamide-gel electrophoresis; LF, lysosome fraction.

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thyroid lysosomes from rats injected with radioactive iodide, Yoshinari *et al.* (1985) observed a certain heterogeneity in the ^{125}I-labelled thyroglobulin content and degradation products among lysosomes. We decided to study the distribution of thyroglobulin by immunochemical detection within the population of isolated pig thyroid lysosomes to identify and characterize vesicles which could correspond to secondary lysosomes, i.e. vesicles containing both acid hydrolases and thyroglobulin, and to try to separate lysosome subpopulations corresponding to primary and secondary lysosomes. Using iterative fractionation steps on Percoll gradients, we have obtained evidence for the existence of lysosome subpopulations in keeping with the concept of primary and secondary lysosomes. In a complementary experimental approach, we have tried to demonstrate a functional relationship between these two lysosome populations using a cultured thyroid cell system, in which we have studied the fate of endogenous ^{125}I-labelled thyroglobulin.

MATERIALS AND METHODS

Materials

p-Nitrophenyl phosphate (disodium salt) was obtained from Sigma Chemical Co. Haemoglobin was from Merck. Percoll and Sephadex G-25 columns were obtained from Pharmacia Fine Chemicals. ^{125}I-Iodide was purchased from Amersham France and Pansorbin from Calbiochem. Nitrocellulose paper HAHY was from Millipore. The products used for electrophoresis were from Bio-Rad Laboratories.

Isolation of thyroid lysosomes

Pig thyroid glands were obtained from the local slaughterhouse, transported on ice to the laboratory and processed within 2 h after death of the animal. All further purification steps were carried out at 0–4 °C. Lysosomes were isolated from pig thyroid fragments using a three-step procedure as previously described (Alquier *et al.*, 1985). All fractionation steps were carried out in a Tris/sucrose buffer:10 mM-Tris/HCl buffer/0.25 M-sucrose, pH 7.4 (TS buffer). Open thyroid follicles were prepared by mechanical disruption of thyroid fragments through a metal sieve. This thyroid cell material depleted in colloid thyroglobulin was then homogenized and submitted to differential centrifugation at 26000 *g* ($r_{av.}$ 8 cm) for 20 min to obtain a crude particulate fraction containing lysosomes, mitochondria and endoplasmic reticulum. Lysosomes were separated from other cell organelles by isopycnic centrifugation on iso-osmotical Percoll gradients. 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 (stock solution). The stock solution was diluted to 30% (v/v) Percoll with TS buffer; 23 ml of the 30% Percoll solution was mixed with 1.5 ml of crude thyroid particulate fraction and centrifuged in a fixed-angle rotor (Beckman 50.2 Ti) at 60000 *g* ($r_{av.}$ 8.1 cm) for 25 min. The gradients were collected into 25 fractions by pumping from the top with a Buchler auto-densiflow apparatus. The peak of material with a buoyant density of 1.13 g/ml corresponds to lysosomes from biochemical and morphological criteria. The specific activities of acid phosphatase, cathepsin D, glucuronidase and β-galactosidase in this vesicle fraction were increased 45–55-fold compared with the homogenate. The gradient 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 pellet resuspended in TS buffer corresponded to the total population of thyroid lysosomes.

Subfractionation of thyroid lysosomes

Thyroid lysosomes in TS buffer were submitted to a second centrifugation on 30% (v/v) Percoll gradients using the same conditions as those mentioned above. The resulting lysosome peak (L) was divided into two fractions corresponding to light (L2) and dense (L1) lysosomes, respectively (see Results section). After washing to remove Percoll, L1 and L2 lysosomal subfractions were centrifuged on a third series of 30% (v/v) Percoll gradients. At each of the three gradient steps, fractions of the gradients were analysed for protein, acid hydrolase activity and thyroglobulin. The density profile of the Percoll gradients was determined using density marker beads (1.036–1.145 g/ml).

Culture of thyroid cells

Pig thyroid cells were prepared by a discontinuous trypsinization procedure as previously described (Rousset *et al.*, 1976). Freshly dispersed cells were cultured as reported (Munari-Silem *et al.*, 1986). Briefly, cells were suspended in F12 medium containing 10% fetal (v/v) calf serum, penicillin (100 units/ml), streptomycin (50 μg/ml) and thyrotropin (TSH) (0.5 munit/ml). Samples of 10 ml were introduced in 10 cm Falcon Petri dishes. Cell cultures were performed at 37 °C under air/CO_2 (95:5) for 2 days. Under these conditions, thyroid cells reassociated into follicle-like structures.

Labelling and incubation of thyroid cells

At the end of the culture period, cells were washed with F12 medium and preincubated in the same medium with ^{125}I-iodide (10 μCi) for 90 min at 37 °C. After the labelling period, cells were washed with F12 medium and incubated in the complete culture medium supplemented with 1 mM-methimazole (to block the incorporation of residual ^{125}I-iodide into proteins) and 1 mM-sodium perchlorate (to release residual intracellular ^{125}I-iodide into the medium) for 15 h at 37 °C. Half of the Petri dishes were maintained in control conditions and the other half were treated with 0.1 mM-chloroquine and leupeptin (50 μg/ml). These substances were used to inhibit lysosomal proteolytic activities and therefore the degradation of ^{125}I-thyroglobulin formed during the labelling period, which could reach the lysosomal compartment. At the end of the incubation period, cells were washed with F12 medium, scraped off the dishes using a rubber 'policeman' and collected by centrifugation at 80–100 *g* for 7 min at 4 °C. Cell pellets were resuspended in TS buffer mixed with thyroid fragments (the open thyroid follicle fraction described in the section 'Isolation of thyroid lysosomes') as carrier material, homogenized and fractionated on two sequential series of Percoll gradients as described above. Fractions of the Percoll gradients were counted for radioactivity and analysed for their content of immunoprecipitable ^{125}I-thyroglobulin (thyroglobulin labelled in the cultured thyroid cell system).

Radioimmunoassay of thyroglobulin

Radioiodination of thyroglobulin. Pig thyroglobulin purified by velocity sedimentation on sucrose gradient (Rousset *et al.*, 1980) from the colloid thyroglobulin solution obtained after mechanical disruption of follicles on the metal sieve (iodine content: ~ 40 iodine atoms/mol) was labelled with ^{125}I-iodide by chemical oxidation. Thyroglobulin (5 μg) was incubated with 0.5 mCi of Na^{125}I in the presence of chloramine T (20 μg) in a total volume of 65 μl of 0.2 M-Na_2HPO_4/NaH_2PO_4 buffer, pH 7.4. The reaction was stopped after 1 min at room temperature by addition of 100 μl of sodium metabisulphite (2.5 mg/ml). ^{125}I-Thyroglobulin was separated from residual free ^{125}I-iodide by filtration on a Sephadex G-25 column pre-equilibrated with 10 mM-Na_2HPO_4/NaH_2PO_4 buffer/0.15 M-NaCl/10% (w/v) bovine serum albumin, pH 7.4 (buffer A). ^{125}I-Thyroglobulin (recovered in the void volume had a specific radioactivity of 20–25 μCi/μg of protein.

Preparation of anti-thyroglobulin antibodies. Two male rabbits (albinos hybrids) were injected subcutaneously on days 1 and 21 with 2 ml of a suspension containing 1 mg of pure pig thyroglobulin in 1 ml of 0.15 M-NaCl and 1 ml of complete Freund's adjuvant. Rabbits were bled on day 26. Serum was stored at −20 °C. The antibody titre of the serum of the immunized animals (expressed as the highest dilution which gives a significant response in the radioimmunoassay) was higher than 10^6.

Radioimmunoassay procedures. To test antisera, 1–2 ng (30000–40000 c.p.m.) of ^{125}I-thyroglobulin in 50 μl of buffer A was mixed with 200 μl of diluted rabbit immune serum (dilution ranging from 1/3000 to 1/300000) in a total volume of 400 μl of buffer A. After 18 h at 4 °C, 1 mg of protein A adsorbent (Pansorbin) in 20 μl of buffer A was added and incubation was continued for 30 min at room temperature. The suspension was then centrifuged at 700 *g* ($r_{av.}$ 10 cm) for 15 min. Pellets were washed in 0.2 M-Na_2HPO_4/NaH_2PO_4 buffer/25 mM-EDTA, pH 7.2, centrifuged as above and counted for radioactivity in a Packard gamma counter At dilutions ranging from 1/100000 to 1/200000, the two sera were able to immunoprecipitate 30–40% of ^{125}I-thyroglobulin. These serum dilutions were selected for the radioimmunoassay.

Thyroglobulin was assayed in lysosomal fractions as follows: ^{125}I-thyroglobulin (30000–40000 c.p.m.) was incubated with unlabelled thyroglobulin (1–300 ng) or the lysosomal subfraction in the presence of the anti-thyroglobulin immune serum (final dilution: 1/160000) in a total volume of 400 μl of buffer A for 18 h at 4 °C. Immune complexes were collected using protein A adsorbent as indicated above in the procedure for testing the anti-thyroglobulin antibody activity of immune sera. Each determination was made in triplicate.

Immunoprecipitation of ^{125}I-thyroglobulin in lysosomal fractions from cultured thyroid cells

Percoll fractions (0.8 ml) containing lysosomal material from labelled thyroid cells were mixed with 500 μl of the anti-thyroglobulin immune serum diluted to 1/25 (by vol.) in buffer A and incubated for 18 h at 4 °C. Protein A adsorbent (30 mg/tube) was then added and the mixture was processed as described for the radioimmunoassay. Controls were performed using normal rabbit serum instead of the anti-thyroglobulin immune serum.

Polyacrylamide-gel electropheresis and Western blot analysis

SDS/polyacrylamide-gel electrophoresis (PAGE) was performed on slab gels (Rousset & Wolff, 1980). Identification of thyroglobulin by Western blot analysis was performed using the anti-thyroglobulin antibodies mentioned above according to a procedure previously reported (Durrieu *et al.*, 1987). Thyroglobulin–anti-thyroglobulin antibody complexes on nitrocellulose sheets were detected using a goat anti-(rabbit Ig) antibody (Antibodies Inc., Davis, CA, U.S.A.) as second antibody and ^{125}I-protein A. Autoradiography was carried out using X Omat AR Kodak film.

Other methods

Acid phosphatase (EC 3.1.3.2) and cathepsin D (EC 3.4.23.5) were assayed as previously reported (Alquier *et al.*, 1985) using *p*-nitrophenylphosphate and haemoglobin as substrates, respectively.

Protein was assayed according to Lowry *et al.* (1951) after treatment of the samples with 1% (w/v) sodium deoxycholate to solubilize membrane protein. Samples of bovine serum albumin were submitted to the same detergent treatment to generate the standard curve. Since Percoll interferes in the assay, control Percoll gradient fractions were used as blanks in the protein assay.

RESULTS

Qualitative and quantitative analysis of lysosomal thyroglobulin

Purified thyroid lysosomes were submitted to an osmotic-pressure-dependent lysis (by decreasing the sucrose concentration from 0.25 M to 0) and the intralysosomal soluble protein fraction, representing about 80% of total lysosomal proteins, was analysed for its thyroglobulin content. Preparations of purified thyroid lysosomes from pigs contained a high molecular mass protein with an electrophoretic mobility identical to pure native pig thyroglobulin on SDS/polyacrylamide gels (Fig. 1). The amount of this high molecular mass lysosomal protein was estimated by densitometric analysis of polyacryamide gels using a standard curve generated with pure thyroglobulin. It represented between 5 and 10% (depending on the lysosome preparation) of total intralysosomal soluble proteins. The identity of this molecular species was confirmed by Western blot analysis (Fig. 1). Anti-(pig thyroglobulin) antibodies stained the lysosomal protein having the mobility of pure native thyroglobulin and did not react with any thyroglobulin fragments. Results shown by Fig. 2 indicate that lysosomal thyroglobulin exhibited immunological reactivities similar to those of pure native thyroglobulin in the soluble-phase radioimmunoassay. Serial dilutions of soluble lysosomal extracts induced an inhibition of the binding of labelled thyroglobulin to anti-thyroglobulin antibodies (Fig. 2). The inhibition curve was parallel to that obtained with unlabelled pure native thyroglobulin. It was therefore possible to estimate the amount of immunoassayable thyroglobulin in lysosomes using the native thyroglobulin displacement curve

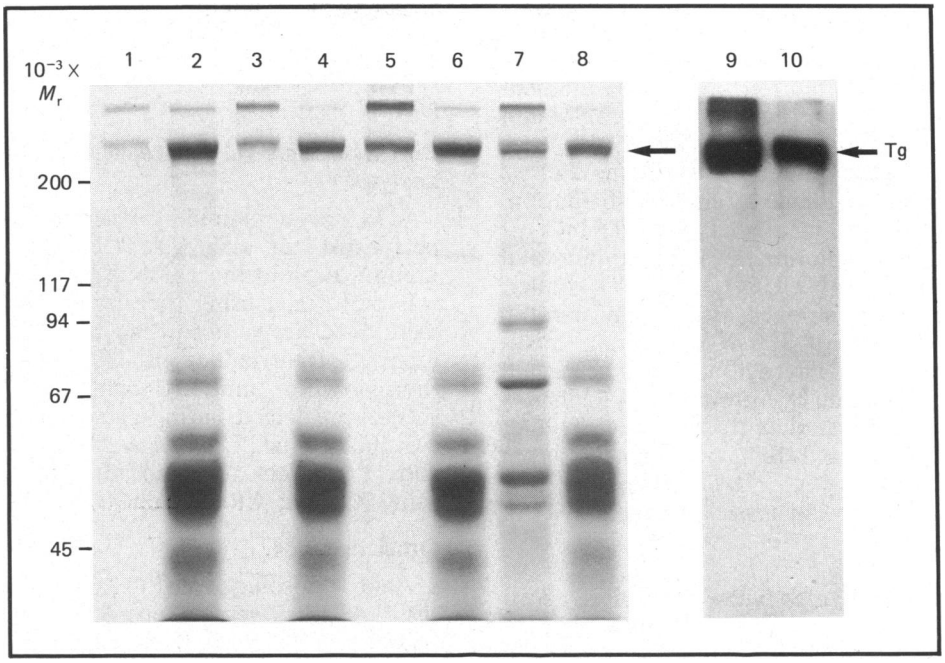

Fig. 1. Characterization of thyroglobulin as a thyroid lysosomal component: analysis by SDS/PAGE and Western blotting.

Purified lysosomes (after the first Percoll gradient) were suspended in an hypo-osmotic medium (10 mM-Tris/HCl buffer, pH 7.4) and centrifuged at 100000 *g* ($r_{av.}$ 8.1 cm) for 40 min to obtain the soluble fraction. Soluble lysosomal protein (90 μg) from four different preparations (lanes 2, 4, 6 and 8) were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue. The thyroglobulin content of these soluble extracts was estimated by comparison with different amounts of pure thyroglobulin: 2.5 μg (lane 1), 5 μg (lane 3) and 10 μg (lane 5). The crude postnuclear particulate fraction (P4 pellet from which lysosomes were purified on Percoll gradients) was analysed in lane 7. Western blot analysis of pure thyroglobulin (lane 9) and soluble lysosomal proteins (lane 10) using polyclonal anti-(pig thyroglobulin) antibodies is shown. The arrows indicate the position of thyroglobulin (Tg).

as a standard curve. The thyroglobulin content of different preparations of purified thyroid lysosomes is reported in Table 1. It appears that immunoassayable thyroglobulin represented about 7% of total intralysosomal soluble protein.

The release of thyroglobulin from lysosomes exposed to decreasing osmotic pressure is reported in Fig. 3. Aliquots of purified thyroid lysosomes were suspended in 10 mM-Tris/HCl-buffered medium, the sucrose concentration of which varied from 0.25 M to 0. Released material, i.e. the material which no longer sedimented at 26000 *g* ($r_{av.}$ 8 cm) after 20 min increased when the sucrose concentration decreased. There was a difference between the acid hydrolase or protein release curves and the thyroglobulin release curve. At a concentration of sucrose of 0.19–0.20 M, 50% of the acid phosphatase release was obtained, whereas the half-maximum release of immunoassayable thyroglobulin only occurred at 0.14–0.15 M-sucrose.

Subfractionation of thyroid lysosomes

We have studied the distribution of protein, acid hydrolase activities (acid phosphatase and/or cathepsin D) and thyroglobulin immunoreactivity in vesicle fractions resulting from iterative isopycnic centrifugation on Percoll gradients. Fig. 4(*a*) shows the four parameter profiles after the first Percoll gradient used to obtain the total thyroid lysosome population from the crude thyroid particulate fraction. Lysosomes were found between gradient fraction 14 and gradient fraction 24. The maximum acid hydrolase activity was in gradient fractions 20 and 21. Below the lysosomal enzyme peak, there was a small peak of immunoassayable thyroglobulin, the top of which was located around gradient fraction no. 18. The bulk of thyroglobulin was associated with the major protein peak (fraction 3–9) containing other cell organelles, especially endoplasmic reticulum. The thyroglobulin in this peak was found to be uniodinated (our unpublished work). Gradient fractions 15–24 were pooled and submitted to the washing procedure to remove Percoll. The resulting lysosome fraction corresponding to the total thyroid lysosome population was centrifuged on a second 30% (v/v) Percoll gradient, the parameter profiles of which are shown in Fig. 4(*b*). It is clearly apparent that the lysosomal enzyme peaks did not superimpose with the thyroglobulin peak, maximum values were found in gradient fraction 20 and gradient fraction 18, respectively. Thyroglobulin-containing vesicles appeared to have an apparent buoyant density slightly lower than that of the majority of lysosomes. It should be noticed that lysosomal enzyme peaks were asymmetrical (especially the cathepsin D peak) and the asymmetry was on the side of the thyroglobulin peak. This argues in favour of the existence of a non-homogeneous lysosome population. To test whether a lysosome subpopulation containing both acid hydrolases and thyroglobulin could be separated from the bulk of lysosomes on the basis of their density, the Percoll gradient subfractionation was pursued one step further; results of a representative

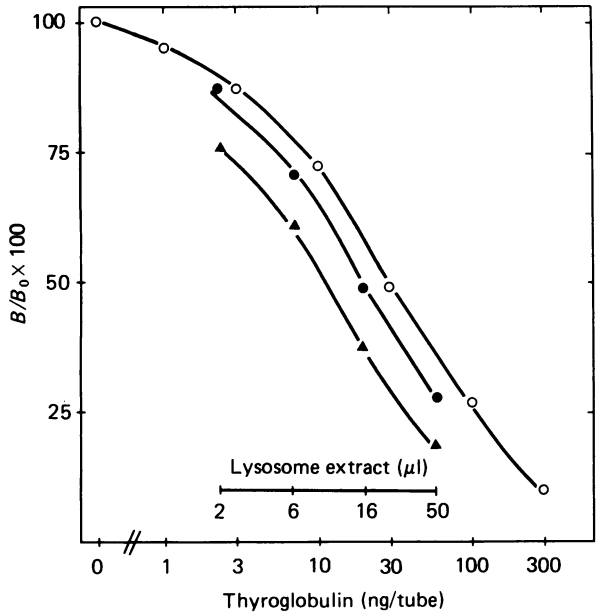

Fig. 2. Identification of immunoreactive thyroglobulin in purified thyroid lysosomes

Competition curves between ^{125}I-thyroglobulin and unlabelled pure thyroglobulin or soluble lysosomal extracts. Labelled thyroglobulin (~ 30000 c.p.m.) was incubated with unlabelled thyroglobulin (○) or lysosome extract (12–50 μl) from two different preparations (●, ▲). Samples were incubated and processed as reported in the Materials and methods section. Binding values (B) obtained in the presence of various amounts of unlabelled thyroglobulin or lysosome extracts are expressed as the percentage of the maximal binding (B_0) obtained in the absence of competitor. Each point represents the mean of three determinations.

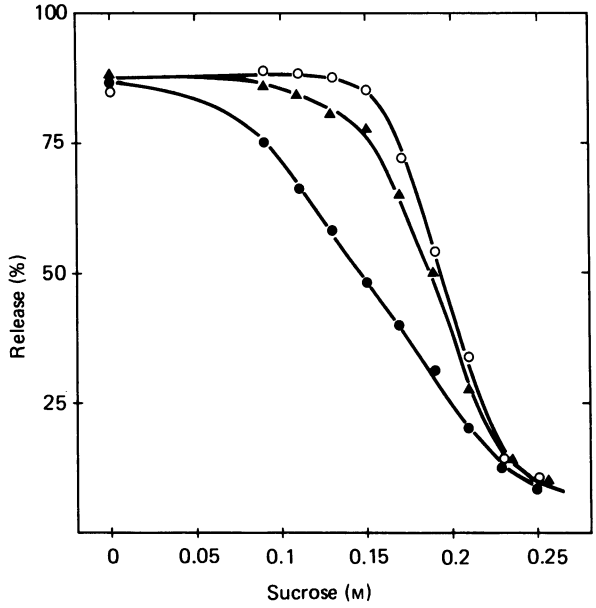

Fig. 3. Release of acid phosphatase, protein and immunoreactive thyroglobulin from purified lysosomes as a function of sucrose concentration

Aliquots of intact lysosomes were suspended in 10 mM-Tris/HCl buffer containing decreasing concentrations of sucrose (0.25 M to 0). After 10 min at 4 °C, the suspensions were centrifuged at 26000 g ($r_{av.}$ 8 cm) for 20 min. Supernatants were analysed for protein (○), acid phosphatase (▲) and immunoreactive thyroglobulin (●). Results are expressed as the percentage of the total content measured on 0.1% (v/v) Triton X-100-treated lysosomes. Each point represents the mean of three determinations.

Table 1. Thyroglobulin content of purified thyroid lysosomes

Results from six different preparations. Lysosomal thyroglobulin determined by soluble-phase radioimmunoassay was expressed as a proportion of total intralysosomal soluble protein.

Lysosome preparation	Protein (mg/ml)	Immunoassayable thyroglobulin (μg/ml)	$\frac{\text{Thyroglobulin}}{\text{Protein}} \times 100$
I	1.0	65	6.5
II	3.0	171	5.7
III	0.5	35	7.0
IV	1.4	125	8.9
V	1.4	102	7.3
VI	1.1	58	5.3

experiment are shown on Fig. 5. The parameter profiles of the second Percoll gradient are reported in Fig. 5(*a*). Fig. 5(*a*) and Fig. 4(*b*) illustrate the same fractionation step on two different preparations; the difference in amplitude in the thyroglobulin peak between the two experiments is solely due to a difference in the graphical scale. The lysosome material after the second Percoll gradient (Fig. 5*a*) was divided into two portions: the first one (L1) corresponding to the pool of the gradient fractions 20–24 and the second (L2) to the pool of the gradient fractions 13–19. The pooled L1 and L2 lysosomal fractions were submitted to the washing procedure to remove Percoll and then centrifuged on a third 30% (v/v) Percoll gradient. Since L2 represented a lower amount of material than L1, L2 used for the third centrifugation step was derived from several identical second-step gradients. The parameter profiles corresponding to the centrifugation of L2 and L1 are illustrated in Fig. 5(*b*) and 5(*c*), respectively. Material from L1 and L2 was recovered at buoyant densities of 1.12–1.13 and 1.08 g/ml, respectively. The L1 lysosome subpopulation had the same buoyant density as the unfractionated lysosomes; this population represented the majority of thyroid lysosomes. As expected, the L2 lysosome subpopulation had a higher thyroglobulin content than the L1 subpopulation. In Fig. 5(*b*), there was a low-density sedimenting peak which could correspond to lysosomal membrane or altered lysosomes. Indeed, between the two Percoll gradient centrifugations, lysosomes were submitted to three successive centrifugations (to remove Percoll) which could lead to the breakdown of part of the vesicles. The appearance of light lysosomal material was usually observed both between step 1 and step 2 and between step 2 and step 3 of the subfractionation on Percoll gradients.

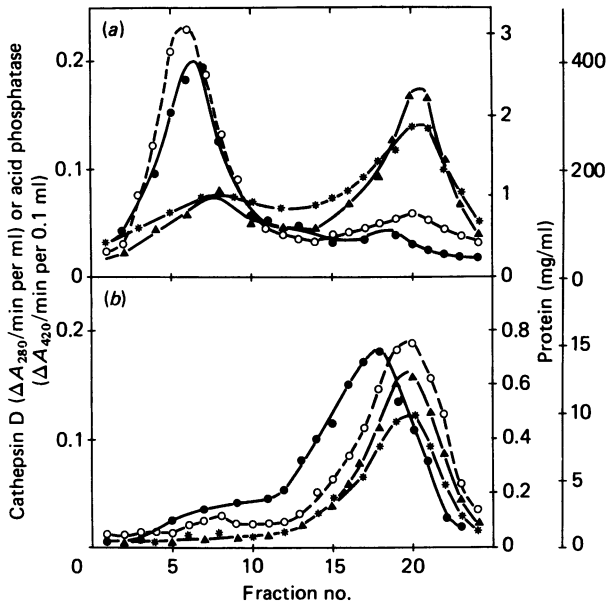

Fig. 4. Localization of thyroglobulin-containing lysosomes on Percoll gradients

(*a*) The crude thyroid postnuclear particulate fraction (P4) was homogenized in TS buffer. Aliquots of 1.5 ml (about 8 mg of protein) were mixed with 23 ml of 30% (v/v) Percoll and centrifuged at 60000 *g* ($r_{av.}$ 8.1 cm) for 25 min. Fractions of 1 ml were collected from the top to the bottom and assayed for protein (○), acid phosphatase (▲), cathepsin D (✱) and immunoassayable thyroglobulin (●). (*b*) Purified lysosomes (~ 5 mg of protein), after the Percoll washing procedure, were submitted to a second centrifugation on 30% (v/v) Percoll gradient using the same conditions as those mentioned above.

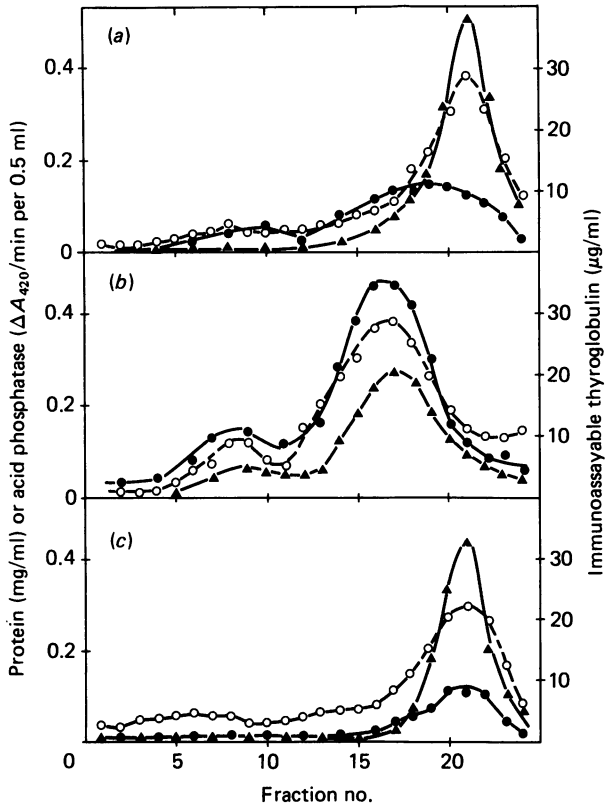

Fig. 5. Separation of two subpopulations of thyroid lysosomes

(*a*) Purified lysosomes were submitted to a second centrifugation on Percoll gradients. Gradient fractions were analysed for protein (○), acid phosphatase (▲), and thyroglobulin (●). The lysosome peak was divided into two fractions corresponding to light (L2) and dense (L1) lysosomes (see Results). After washing to remove Percoll, L2 and L1 lysosomal subfractions (about 2 mg of protein) were centrifuged on a third series of 30% (v/v) Percoll gradients (*b* and *c*, respectively). Gradient fractions were analysed as described above.

Composition of L1 and L2 lysosome subpopulations

Vesicles from L1 and L2 lysosome peaks were washed to remove Percoll and analysed (*a*) for their content of acid phosphatase and thyroglobulin; (*b*) for their sensitivity to osmotic pressure and (*c*) for their polypeptide composition. The specific activity of acid phosphatase was slightly decreased in L2 and increased in L1 as compared to L: values obtained from two experiments were 1.7 ± 0.3, 2.0 ± 0.4 and 1.3 ± 0.2 ΔA_{420}/min per mg (mean ± S.D.) in L, L1 and L2, respectively. In contrast, immunoassayable thyroglobulin expressed as a percentage of total protein was decreased in L1 and increased in L2 compared with L (Fig. 6). Accordingly, the ratio between immunoassayable thyroglobulin and acid phosphatase was decreased in L1 and increased in L2 compared with the value obtained for unfractionated lysosomes (L fraction). Isolated subpopulations of lysosomes (L1 and L2) were tested for their sensitivity to decreased osmotic pressure using the same protocol as in Fig. 3. The acid phosphatase release curves for L1 and L2 were very similar. The same was true for the thyroglobulin release curves (Fig. 7). As observed for unfractionated lysosomes, there was a marked difference between the sucrose concentrations required to release 50% of acid phosphatase and those required to release 50% of thyroglobulin. Despite the difference in thyroglobulin content, L1 and L2 lysosome subpopulations did not exibit a marked difference in osmotic properties. These data indicate that the various centrifugation steps did not alter the properties of lysosomes. The small proportion of lysosomes which break during this complex fractionation procedure did not sediment with intact lysosomes.

Analysis of the protein content of L, L1 and L2 lysosome fractions by SDS/PAGE is shown by Fig. 8. It is apparent that thyroglobulin (subunit of M_r 280000) was in higher proportion in L2 than in L and in lower proportion in L1 than in L. Almost all the proteins present in L were recovered in L1 and L2, however, there was one exception, a polypeptide in the region M_r 50000–54000 was more abundant in L2 than in either L or L1 or only present in L2.

Functional properties of L1 and L2 lysosome subpopulations

Thyroid cells cultured in the presence of TSH reassociate in follicle-like structures. This is illustrated in Fig. 9. In this system *in vitro*, thyroglobulin has been shown to be released and iodinated in newly formed intrafollicular spaces (Lissitzky *et al.*, 1971; Fayet *et al.*, 1971) as in intact thyroid follicles. We have used this cell

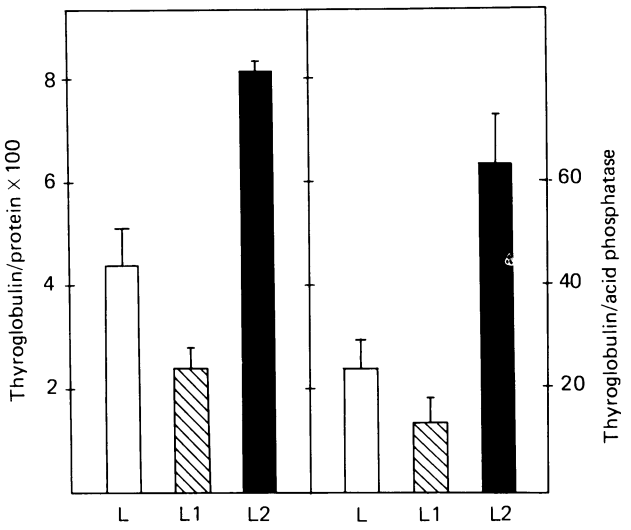

Fig. 6. Analysis of the immunoassayable thyroglobulin content of L1 and L2 lysosome subpopulations

Lysosomal fractions (L, the total lysosome population after the second Percoll gradient: L1 and L2, lysosome subpopulations obtained after the third Percoll gradient) were suspended in an hypo-osomotic medium (10 mM-Tris/HCl buffer, pH 7.4) and assayed for thyroglobulin by radioimmunoassay. The amount of immunoassayable thyroglobulin in each fraction was expressed as a percentage of the total protein of the fraction (left panel) or as the ratio between immunoassayable thyroglobulin (μg/ml) and acid phosphatase activity (ΔA_{420}/min per ml) (right panel). Columns and vertical bars represent the mean ± S.D. of three determinations.

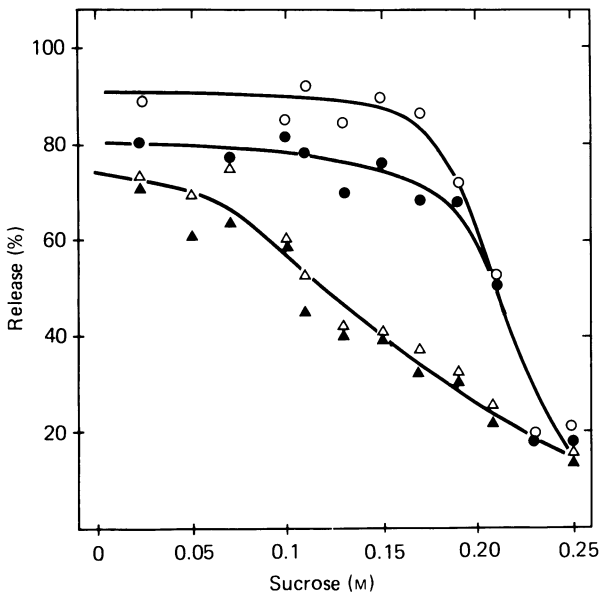

Fig. 7. Osmotic properties of L1 and L2 lysosome sub-populations

Release of acid phosphatase (○, ●) and immunoassayable thyroglobulin (△, ▲) as a function of sucrose concentration. Conditions are the same as in Fig. 3. Open symbols, L1 lysosomes; closed symbols, L2 lysosomes.

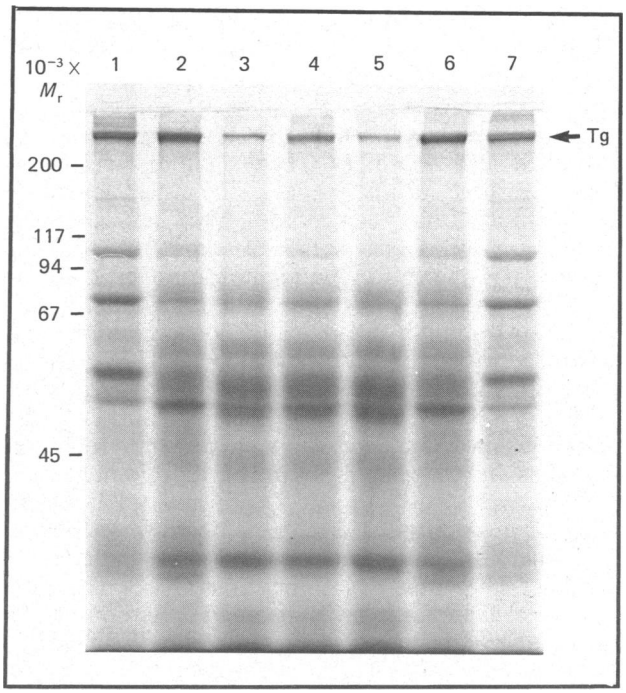

Fig. 8. Polypeptide composition of soluble fractions from thyroid lysosome subpopulations: analysis by SDS/PAGE

Lysosomal fractions (L, L1, L2) were suspended in an hypo-osmotic medium (10 mM-Tris/HCl buffer, pH 7.4) and centrifuged at 100000 *g* ($r_{av.}$ 8.1 cm) for 40 min to prepare the soluble fractions. Samples contained 100 μg of protein: lanes 1 and 7, P4 particulate fractions; lanes 2 and 6, L2 lysosomes; lanes 3 and 5, L1 lysosomes; lane 4, total thyroid lysosomes (L). Numbers on the right side of the Figure indicate the mobility of marker proteins (expressed as M_r). The arrow indicates the thyroglobulin band.

culture system to study the appearance of newly formed ^{125}I-thyroglobulin in the lysosome compartment. After a period of labelling with ^{125}I-iodide, reassociated thyroid cells were incubated in either the basal conditions or in the presence of pharmacological agents which inhibit intralysosomal protein degradation: chloroquine by increasing the intralysosomal pH and leupeptin by inhibiting thiol-proteinases. By preventing intralysosomal proteolysis, these inhibitors were expected to induce the accumulation of internalized ^{125}I-thyroglobulin in secondary lysosomes. Results of such an experiment are illustrated in Fig. 10. The Percoll gradient profile of Fig. 10(*a*) shows that, in control conditions, about 35% of ^{125}I-labelled material present in thyroid cell vesicles is recovered in the lysosome fraction (LF). In that fraction the peak of radioactivity coincides with the peak of acid phosphatase activity. The Percoll gradient radioactivity profile corresponding to chloroquine- and leupeptin-treated cells (Fig. 10*b*) appears very different: (i) there was an increase in the radioactivity associated with low density particles (d = 1.035–1.060 g/ml); (ii) the proportion of radioactivity found in LF was decreased to 20% and (iii) the distribution of the lysosome-associated radioactivity no longer coincided with that of acid phosphatase activity. It should be noticed that more than 90% of the radioactivity present on the gradients corresponded to protein-bound ^{125}I-thyroglobulin or related polypeptides.

The sedimentation properties of lysosomes which contain ^{125}I-labelled material as well as the nature of these ^{125}I-labelled components were further analysed by

centrifuging LF from control cells and treated cells on a second series of Percoll gradients. ^{125}I-Labelled compounds present in lysosomes from untreated cells sedimented at the density of L1 lysosomes (Fig. 10*c*), whereas the majority of the ^{125}I-labelled material of chloroquine- plus leupeptin-treated cell lysosomes was found at a lower density corresponding to that of the L2 lysosome sub-population (Fig. 10*d*). The proportion of the radioactivity immunoprecipitable by anti-thyroglobulin antibodies was low (30%) in lysosomes from control cells and high (about 80%) in lysosomes from cells treated by the inhibitors of the lysosomal proteolysis. These differences are in agreement with the expected inhibitory effect of chloroquine and leupeptin, since we know that anti-thyroglobulin antibodies used in these experiments either did not react or reacted weakly with thyroglobulin degradation products (B. Rousset, R. Rabilloud & Y. Munari-Silem, unpublished work). Besides lysosomes of the L2 subtype, LF from treated cells contained a small peak of vesicles with a density close to 1.06 g/ml. This peak was not observed in control conditions.

DISCUSSION

In addition to their role in the biochemical machinery of the cell (Barrett, 1984), lysosomes of the thyroid cell have a key function in the thyroid differentiated metabolism. The lysosomes involved in the thyroid hormone biosynthetic pathway could be theoretically identified by their content of thyroglobulin (still undegraded or very partially degraded), thyroglobulin fragments, free thyroid hormones or iodotyrosines. By h.p.l.c. analysis we have been unable to identify free iodotyrosines or thyroid hormones as soluble components in purified thyroid lysosomes. These small molecules probably leave the lysosomes rapidly after their generation. Thyroglobulin, the intact molecule and/or rather large degradation products, seem to represent the only available parameter for the identification of secondary lysosomes. At a given time, the thyroid lysosome populations should theoretically be composed of (*a*) lysosomes which do not participate (if any) in the thyroglobulin hydrolytic process or lysosomes in which the thyroglobulin proteolytic cycle is finished; (*b*) a continuous spectrum of lysosomes in which thyroglobulin hydrolysis and the generation of free thyroid hormone is in progress and at different stages and (*c*) lysosomes which have recently fused with endocytotic vesicles and which therefore contain native or almost intact thyroglobulin. The first lysosome fraction is generally considered as the primary lysosome population and the third, the secondary lysosome population. We have been able to distinguish experimentally two populations of acid hydrolase-containing vesicles which are either depleted or enriched in immunoreactive thyroglobulin. According to this property, lysosome subfractions have been called primary and secondary lysosomes.

The lysosome subpopulations were differentiated using polyclonal anti-(pig thyroglobulin) antibodies which strongly react with high molecular mass thyroglobulin (with the apparent mobility of the intact thyroglobulin subunit) and little or not at all with thyroglobulin fragments. The thyroglobulin-depleted primary lysosomes (L1) and thyroglobulin-enriched secondary lysosomes (L2) differ in relative abundance and buoyant density. From the location of the immunoreactive thyroglobulin peak and acid hydrolase activity peaks of Fig. 4(*b*) (second Percoll gradient step), one can see that thyroglobulin-containing lysosomes (L2) were in lower proportion than L1 lysosomes. From the amount of material recovered as L1 and L2 in Fig. 5, we have estimated that L2 lysosomes could account at the very most for 20–30% of total lysosomes. The thyroglobulin-enriched lysosomes had a density clearly lower than the thyroglobulin-depleted lysosomes: 1.08 versus 1.13 g/ml. Such a difference of density between light lysosomes which contains an undegraded endocytosed ligand and dense lysosomes which contain ligand degradation products has been previously reported for liver lysosomes (Berg *et al.*, 1985). It should be noticed that thyroglobulin-enriched lysosomes or secondary lysosomes differed from acid hydrolase-containing vesicles recovered at a density of 1.05 g/ml (Pertoft & Wärmegärd, 1978) which did not correspond to osmotically active vesicles (Alquier *et al.*, 1985) and represented vesicles from a prolysosomal compartment of the Golgi–endoplasmic reticulum–lysosome (Rome *et al.*, 1979; Yoshinari *et al.*, 1985) or lysosomal membranes or particles corresponding to broken lysosomes. Percoll gradient fractions with a density ranging from 1.04 to

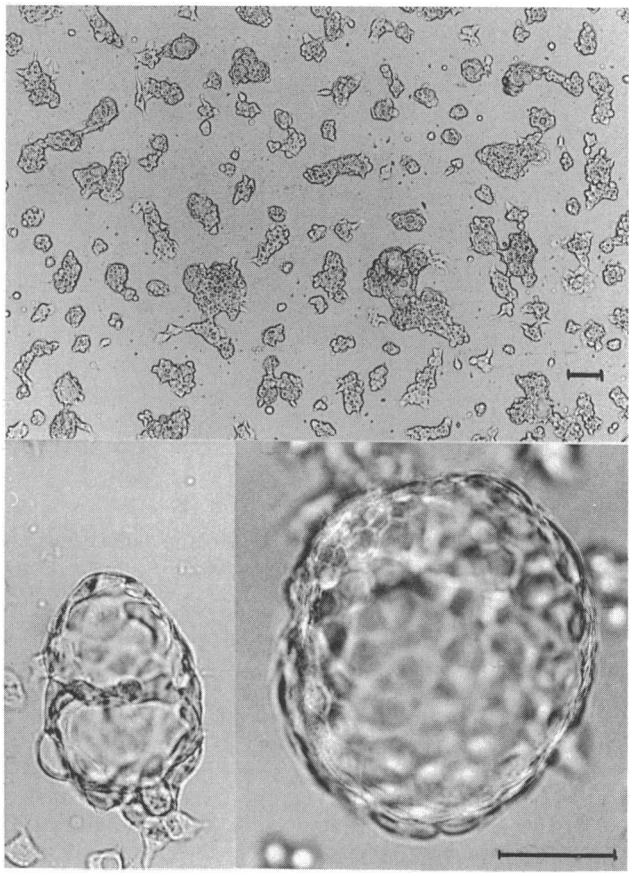

Fig. 9. Photomicrographs of cultured thyroid cells reorganized into follicles

Pig thyroid cells were cultured for 2 days in the presence of TSH (0.5 unit/ml). Top panel: phase contrast microscopy of thyroid cells reassociated into follicle-like structures at low magnification. Bottom panels: differential interference contrast microscopy. Higher magnification (× 500) of follicle structures. Bars represent 50 μm.

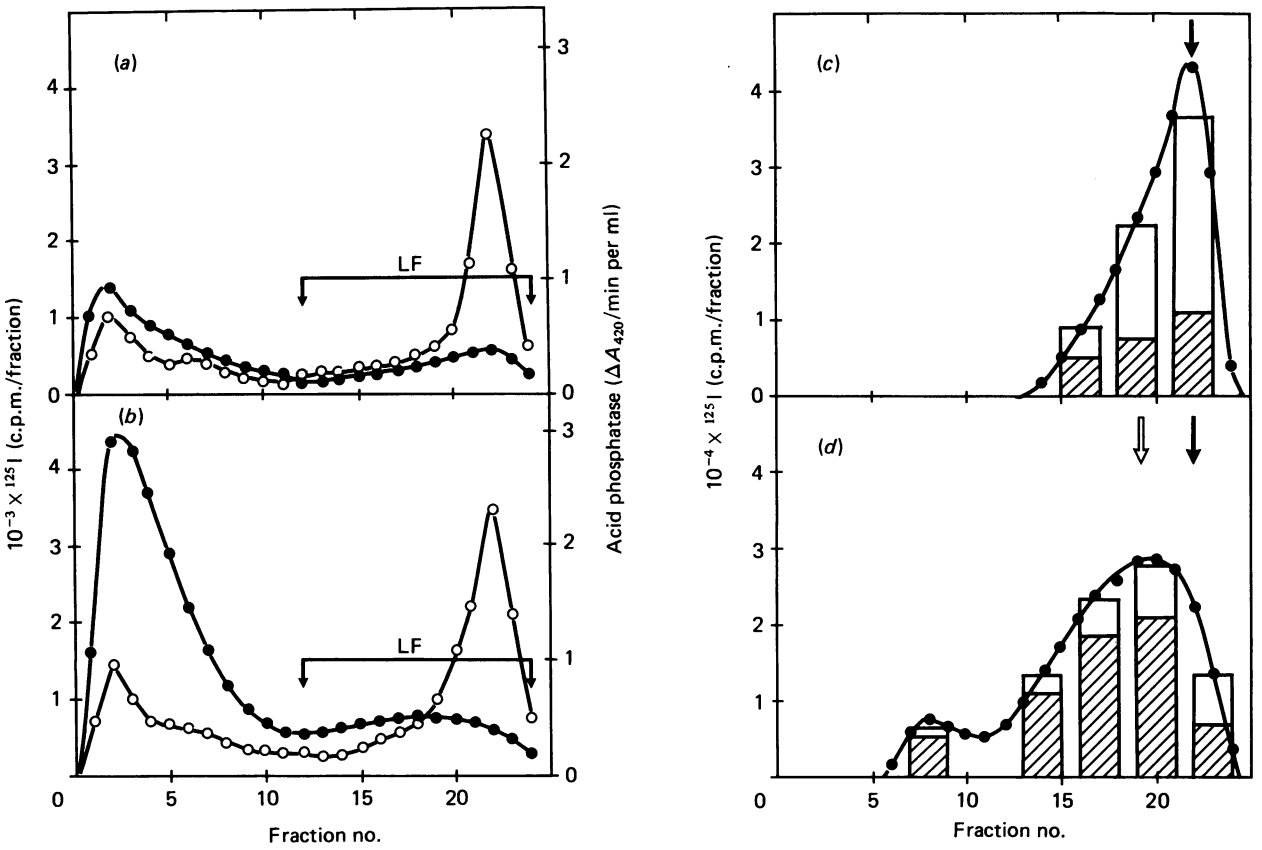

Fig. 10. Percoll gradient fractionation of lysosomes from ^{125}I-prelabelled thyroid cells

Crude particulate fractions (obtained by centrifugation at 26000 *g*, $r_{av.}$ 8 cm, for 20 min) from ^{125}I-prelabelled thyroid cells were fractionated on a first series of Percoll gradients (*a* and *b*). The resulting lysosomal fractions (LF) were sedimented on a second series of Percoll gradients (*c* and *d*). Conditions were: (*a* and *c*) control cells; (*b* and *d*) cells treated with 0.1 mM-chloroquine and leupeptin (50 μg/ml); ●, ^{125}I radioactivity measurements; ○, acid phosphatase activity. Columns represented in (*c*) and (*d*) correspond to the results of the thyroglobulin immunoprecipitation experiment. Fractions of the lysosome peaks were pooled three by three and submitted to the immunoprecipitation protocol described in the Materials and methods section. Open columns, total radioactivity of the pooled fractions; hatched columns, radioactivity immunoprecipitated by anti-thyroglobulin antibodies. Arrows on (*c*) and (*d*) indicate the position of the lysosome subpopulations: L1 (filled arrow), L2 (open arrow).

1.06 g/ml (fractions 4–8 of Fig. 4*a*) should contain thyroid endocytotic vesicles as defined by Yoshinari *et al.* (1985). However, these thyroglobulin-containing vesicles are probably mixed with a large number of thyroglobulin-containing vesicles from endoplasmic reticulum, identified by their content of NADH–cytochrome *c* reductase (Alquier *et al.*, 1985). Indeed, we have extracted thyroglobulin from the vesicles with a density ranging from 1.04 to 1.06 g/ml (gradient fractions 4–8 of Fig. 4*a*) and showed that its iodine content was close to zero. In contrast, the intralysosomal thyroglobulin appears to be iodinated (results not shown). Thyroglobulin present in lysosomes (secondary lysosomes) is therefore different from the large fraction of thyroglobulin associated with low-density vesicles.

The presence of thyroglobulin in lysosomes does not seem to be the result of artefactual entry into lysosomes during the purification procedure. We have verified that ^{125}I-thyroglobulin (labelled by chemical oxidation) added to the cell homogenate was not recovered in purified lysosomes. The possibility of non-physiological fusion between thyroglobulin-containing vesicles and lysosomes in crude vesicle fractions appears very unlikely since all the purification–separation steps have been conducted at 0–4 °C, a condition known to be unfavourable for membrane fusion. Data from osmotic-pressure-dependent lysis of lysosomes showing a differential release between a soluble lysosomal component (acid phophatase) and thyroglobulin suggest that intralysosomal thyroglobulin is not in a soluble state. Preliminary investigations indicate that thyroglobulin could be reversibly bound to the luminal face of lysosomes in a manner dependant upon the concentration of calcium. We postulate that complexes between thyroglobulin and lysosomal membrane components would dissociate upon decreasing calcium concentration; this hypothesis is in agreement with the results of Figs. 3 and 7. Indeed, the treatment of lysosomes in hypo-osmotic medium, which leads to an entry of water, would cause a decrease in the calcium concentration and the release of thyroglobulin.

The very similar polypeptide composition of L1 and L2 lysosome subpopulations indicates that the two types of vesicles originate from the same cell compartment. The L2 lysosome subpopulation exhibited the expected properties for secondary lysosomes. Indeed, secondary

lysosomes, as the product of the fusion between light ligand-containing vesicles and dense lysosomes (Dean, 1984), were expected to have a density lower than primary lysosomes, but higher than endocytotic vesicles. The proteolysis of thyroglobulin inside secondary lysosomes should result in the generation of low molecular mass material, mainly aminoacids, which leave the lysosome together with water molecules (Lloyd, 1984); this outflow probably induces an increase of the density of the vesicles and the transition from secondary to primary lysosomes. Thus, it could be imagined that the existence of a continuous spectrum of lysosomes in which thyroglobulin hydrolysis is at different stages impedes the complete separation of lysosome subpopulations corresponding to the theoretical definition of primary and secondary lysosomes. Functional studies on cultured thyroid cells strongly suggest that the transition from secondary to primary lysosomes is related to the degradation of thyroglobulin. Indeed, when lysosomal proteolytic activites were blocked by chloroquine plus leupeptin treatment of the cells ^{125}I-thyroglobulin was demonstrable in secondary lysosomes. In contrast, under the same experimental conditions, but in the absence of the lysosomotropic inhibitors, the lysosomal radioactivity, composed to large extent of thyroglobulin degradation products, was found at the density of primary lysosomes.

The combination of two methodological approaches: subcellular fractionation and cell labelling and the use of immunological techniques has allowed us to obtain data supporting the concept of thyroid phagolysosomes, intermediate vesicles between the vesicles carrying thyroglobulin and lysosomes.

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