A Non-Autophagic Pathway for Diversion of ER Secretory Proteins to Lysosomes

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Abstract. Intracisternal granules (ICG) develop in the rough ER of hyperstimulated thyrotrophs or thyroid hormone-secreting cells of the anterior pituitary gland. To determine the fate of these granules, we carried out morphological and immunocytochemical studies on pituitaries of thyroxine-treated, thyroidectomized rats. Under these conditions the ER of thyrotrophs is dramatically dilated and contains abundant ICG; the latter contain β subunits of thyrotrophic hormone (TSH-β). Based on purely morphologic criteria, intermediates were identified that appeared to represent stages in the transformation of a part rough/part smooth ER cisterna into a lysosome. Using immunocytochemical and cytochemical markers, two major types of intermediates were distinguished: type 1 lacked ribosomes but were labeled with antibodies against both ER markers (PDI, KDEL, ER membrane

proteins) and a lysosomal membrane marker, lgp120. They also were reactive for the lysosomal enzyme, acid phosphatase, by enzyme cytochemistry. Type 2 intermediates were weakly reactive for ER markers and contained both lgp120 and lysosomal enzymes (cathepsin D, acid phosphatase).

Taken together these results suggest that in hyperstimulated thyrotrophs part rough/part smooth ER elements containing ICG lose their ribosomes, their membrane is modified, and they sequentially acquire a lysosome-type membrane and lysosomal enzymes. The findings are compatible with the conclusion that a pathway exists by which under certain conditions, secretory proteins present in the ER as well as ER membrane and content proteins can be degraded by direct conversion of ER cisternae into lysosomes.

CURRENTLY there is great interest in the mechanisms of protein degradation in the ER. It has become apparent that newly synthesized proteins, primarily unassembled subunits of multimeric proteins or those that are in some way defective (misfolded, aggregated, etc.) and therefore transport incompetent, are degraded in the ER (1, 13, 14). Relatively little is known about the pathways by which either defective proteins in transit or resident ER membrane and luminal proteins are disposed of, but both lysosomal (27, 32) and nonlysosomal (1, 14, 15, 23) mechanisms have been proposed. The best documented lysosomal disposal route is via autophagy which involves sequestration of ER elements by an enveloping smooth membrane probably derived from the ER (2, 6, 7, 12). Tooze et al. (32) have demonstrated that intracisternal granules (ICG)[1] which develop within the ER of CoCl$_2$-treated exocrine pancreatic cells are eliminated by autophagy. However, some time ago we obtained morphological and cytochemical evidence suggesting that ICG which develop within the ER of thyrotrophs of the rat anterior pituitary are disposed of by a seemingly

different lysosomal mechanism that does not involve autophagy (8, 9). ICG form in thyrotrophs when secretion of thyrotrophic hormone (TSH) is first hyperstimulated by thyroidectomy, and then abruptly stopped by treatment with thyroxine (T$_4$). These granules cannot traverse the exocytic pathway and are disposed of.

To obtain further information on the nature of this lysosomal mechanism for disposal of ICG we have studied the distribution of ER resident proteins, a lysosomal membrane protein, and lysosomal enzymes in thyrotrophs containing ICG by immunofluorescence and by immunogold labeling. Our results provide evidence suggesting that regions of the RER shed their ribosomes and are directly converted into lysosomes by acquiring a lysosome-like membrane followed by accumulation of lysosomal enzymes.

Materials and Methods

Materials

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1. *Abbreviations used in this paper:* AcPase, acid phosphatase; ICG, intracisternal granules; PDI, protein disulfide isomerase; PLP, periodate-lysine-paraformaldehyde; MPR, mannose-6-phosphate receptor; T$_4$, Thyroxine; TSH, thyroid stimulating hormone.

anti-rabbit IgG coupled to 5 nm colloidal gold was obtained either from Janssen Life Science Products (Piscataway, NJ), Amersham Corp. (Arlington Heights, IL) or BioCell Research Laboratories (Cardiff, UK). Protein A was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), gold chloride from Fisher Scientific (Fair Lawn, NJ), and tannic acid from Mallinkrodt (St. Louis, MO). Gold particles were prepared according to the method of Frens (15 nm) (11) or Slot and Geuze (5-10 nm) (24) and conjugated to protein A (25).

Animals

Thyroidectomized Sprague-Dawley rats (100-150 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). 40-43 d after the operation, T_4 (10 μg/100 g body weight) was injected subcutaneously daily for 1-4 d. Animals were sacrificed on the second to fifth day after initiating the T_4 injection.

Antibodies

Rabbit antiserum against the bovine TSH-β subunit (Midas) was obtained from Dr. I. A. Kourides (Memorial Sloan-Kettering Cancer Center, NY). Rabbit antiserum against ovine LH-α subunits (which also recognizes TSH-α) was provided by Dr. James A. Magner (Michael Reese Hospital and Medical Center, Chicago, IL) (19). A rabbit antiserum that recognizes four ER membrane proteins (29, 58, 66, and 91 kD) was from Dr. Daniel Louvard (Pasteur Institute, Paris, France) (16). Rabbit antiserum against bovine protein disulfide isomerase (PDI), and mouse monoclonal IgG (ID3) against a KDEL-containing peptide (KDDDKAVKDEL) were from Dr. Stephen Fuller, European Molecular Biology Laboratory (Heidelberg, Germany) (31). Affinity purified rabbit antibody raised against cathepsin D and acid phosphatase (AcPase) from rat liver were provided by Dr. Keitaro Kato (Kyushu University, Fukuoka, Japan) (34), and rabbit antiserum against rat liver lgp120 was from Dr. William A. Dunn, Jr. (University of Florida, Gainesville, FL) (6). Rabbit IgG raised against the cation-independent mannose-6-phosphate/insulin-like growth factor II (MPR) receptor was characterized previously (4). A second rabbit antibody against MPR receptor was kindly provided by Dr. Michael Czech (University of Massachusetts, Boston, MA).

Preparation of Tissue for Morphology and Immunocytochemistry

Rats were perfused under anesthesia first with physiological saline for 3-5 min and then with either (a) periodate-lysine-paraformaldehyde (PLP) fixative (20); (b) 3% paraformaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4; or (c) 3% paraformaldehyde, 0.05% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for ~5 min. The pituitary was removed, the anterior lobe trimmed into 1-mm^3 blocks, and the tissue blocks were immersed in the same fixatives for 6, 2, or 1 h, respectively.

For morphology alone, tissue blocks were postfixed in 1% OsO$_4$ in acetate-veronal buffer, stained in block in 0.5% uranyl acetate (pH 6.0) followed by dehydration in ethanol and embedding in Epox. A few blocks were postfixed in 1.5% potassium ferrocyanide containing 1% OsO$_4$ for 1-2 h, followed by staining in block in 0.5% aqueous uranyl acetate prior to dehydration and embedding.

For cryosectioning, tissue blocks were infiltrated with 50% polyvinyl-

pyrrolidone in 2.3 M sucrose-PBS for 2 h at room temperature or overnight at 4°C. They were then mounted on aluminum nails, frozen in Freon cooled by liquid nitrogen, and then in liquid nitrogen. Semithin (0.5 μm) or ultrathin cryosections were cut from frozen tissue blocks with an Ultracut E ultramicrotome equipped with a cryo-attachment (model FC4E; Reichert Jung S. A., Vienna) using the methods of Tokuyasu (29).

For enzyme cytochemical demonstration of AcPase, pituitary glands of rats were perfused with 1.5% glutaraldehyde in 0.067 M cacodylate buffer, pH 7.4, containing 1% sucrose; the pituitary was removed and immersed in fresh fixative for an additional 2 h. Non-frozen sections were cut at 10-40 μm with a Smith-Farquhar TC-II Tissue Chopper (Ivan Sorvall, Inc., Newton, CT) (26). Sections were collected in 0.1 M cacodylate buffer, pH 7.4, containing 7% sucrose.

Immunofluorescence

Semithin cryosections were placed on gelatin-coated glass slides, quenched with 50 mM NH$_4$Cl for 10 min, and incubated in primary antibody in 10% FCS in PBS for 2 h at room temperature, followed by TRITC- or FITC-conjugated goat anti-rabbit F(ab')$_2$ for 1 h at room temperature. Sections were mounted in 90% glycerol in PBS containing 0.1% p-phenylenediamine and examined by epifluorescence with a Photomicroscope III (Carl Zeiss, Oberkochen, Germany).

Immunogold Labeling

Ultrathin cryosections were collected on formvar and carbon-coated, glow-discharged nickel grids (29) and incubated on droplets of primary antibodies diluted with 10% FCS in PBS. The incubation times were 1 h for anti-AcPase, anti-cathepsin D, anti-LH-α subunit and anti-TSH-β subunit antibodies, and 5 h to overnight for anti-lgp120, anti-ER, anti-PDI, anti-KDEL, and anti-MPR. After several rinses, the sections were incubated with 5 or 10 nm goat anti-rabbit IgG or 6-8 nm protein A-gold conjugate diluted in 10% FCS in PBS for 0.5-1.0 h.

For double labeling, the sections were incubated with the primary antibody followed by incubation with 5 or 8 nm protein A-gold. The sections were then washed and incubated with free protein A (0.5 mg/ml) diluted in PBS containing 10% FCS for 30 min after which they were incubated with the second antibody, followed by 8-15 nm protein A-gold. Sections were fixed with 2% glutaraldehyde in PBS (10 min), postfixed in 1-2% aqueous OsO$_4$ (10 min), and stained in 2% aqueous uranyl acetate (15-20 min). Embedding and adsorption staining was done in 3% polyvinyl alcohol containing 0.001-0.002% lead citrate according to Tokuyasu (30). Sections were examined at 80 kV in either a Philips 410 (Philips Electronic Instruments, Mahwah, NJ) or JEOL 1200-EXII (JEOL USA, Peabody, MA) electron microscope.

AcPase Cytochemistry

After an overnight wash at 4°C, non-frozen sections were incubated for 20-90 min in Gomori medium, pH 5.0, as modified by Barka and Anderson (3) using β-glycerophosphate as substrate and lead nitrate as capture agent (26). Sections were postfixed at 4°C for 45 min in 1% OsO$_4$ in 0.05 M acetate-Veronal buffer, pH 7.4, stained for 30-45 min at room temperature with 0.5% uranyl acetate in acetate-Veronal buffer, pH 6.0, before dehydration and embedding in Epon. Thin sections were stained with lead citrate.

Figure 1. Thyrotrophs from the pituitaries of thyroidectomized, T$_4$ (4 d)-treated rats as seen in semithin cryosections (A and B) and Epon-embedded tissue (C and D). (A) Phase contrast micrograph showing thyrotrophs (T) with enlarged vacuoles that correspond to dilated ER cisternae (er). (B) Immunofluorescence showing the same field labeled for PDI, an ER content marker, demonstrating the presence of PDI in the enlarged ER of the thyrotrophs (arrows). (C and D) A number of vacuolated ER cisternae (er) containing dense ICG (arrowheads) as well as lysosomes (ly) containing barely recognizable remnants of ICG are seen. Also present are structures which appear to be intermediate in their characteristics between ER cisternae and lysosomes. Early intermediates (Type 1) contain dense, tightly packed ICG surrounded by a narrow, electron-lucent space filled with finely granular material. Late intermediates (Type 2) differ in that their contained ICG are less compact and their remaining contents more condensed. In C it can be seen that ER cisternae containing ICG typically have a part rough/part smooth membrane with clusters of ribosomes (arrows) separated by smooth regions lacking ribosomes. Note that ICG are in general much larger (~200-500 vs. ~100-200 nm) than secretion granules (sg). (A and B) Tissue was fixed in 3% formaldehyde, 0.5% glutaraldehyde, and prepared for cryomicrotomy. (C) Tissue was fixed in 2% formaldehyde, 3% glutaraldehyde and postfixed in OsO$_4$ before Epon embedding. (D) Tissue fixed as in A and B followed by reduced OsO$_4$ and embedding in Epon. G, Golgi apparatus. Bars: (A and B) 5 μm; (C and D) 0.1 μm.
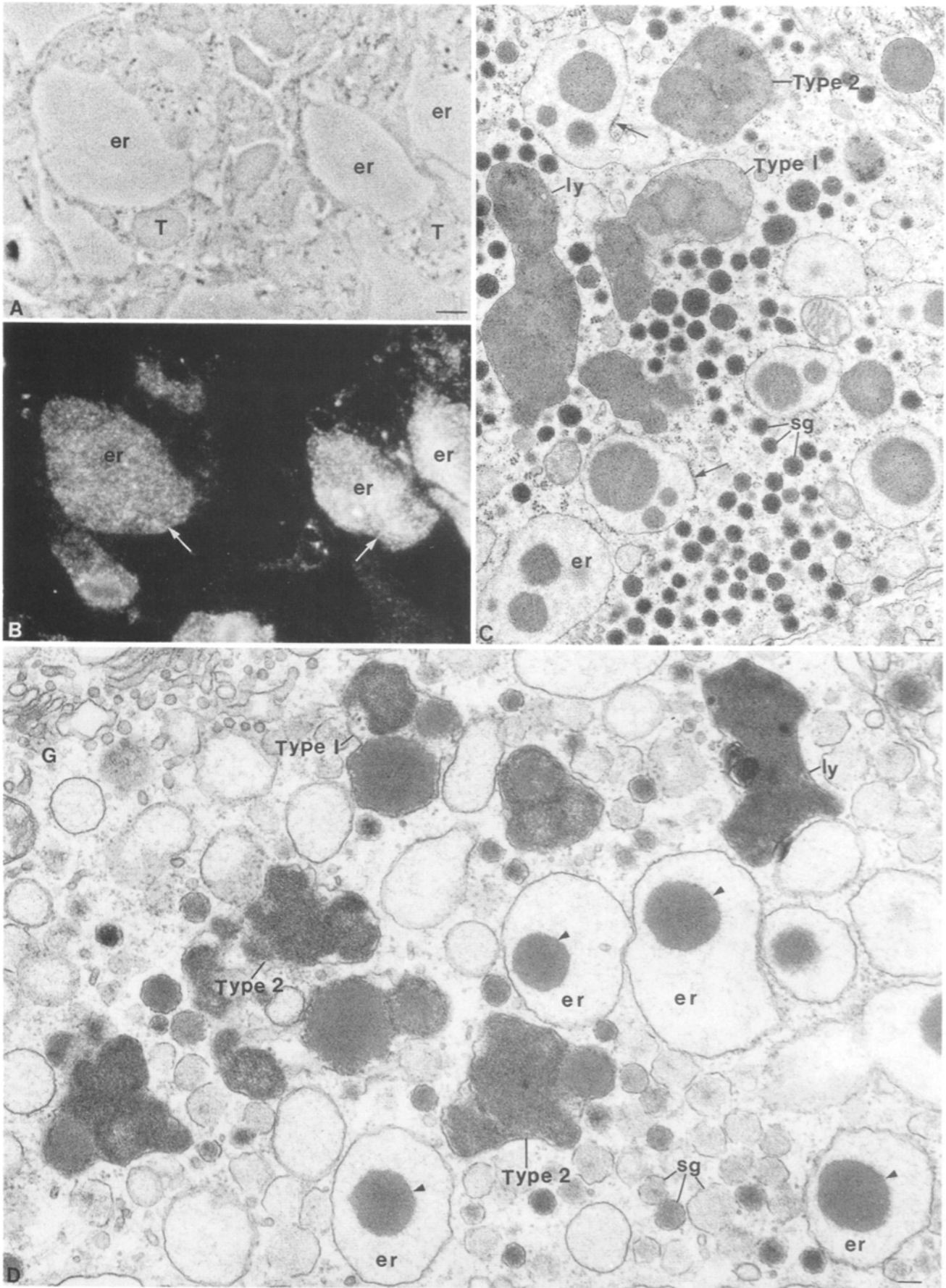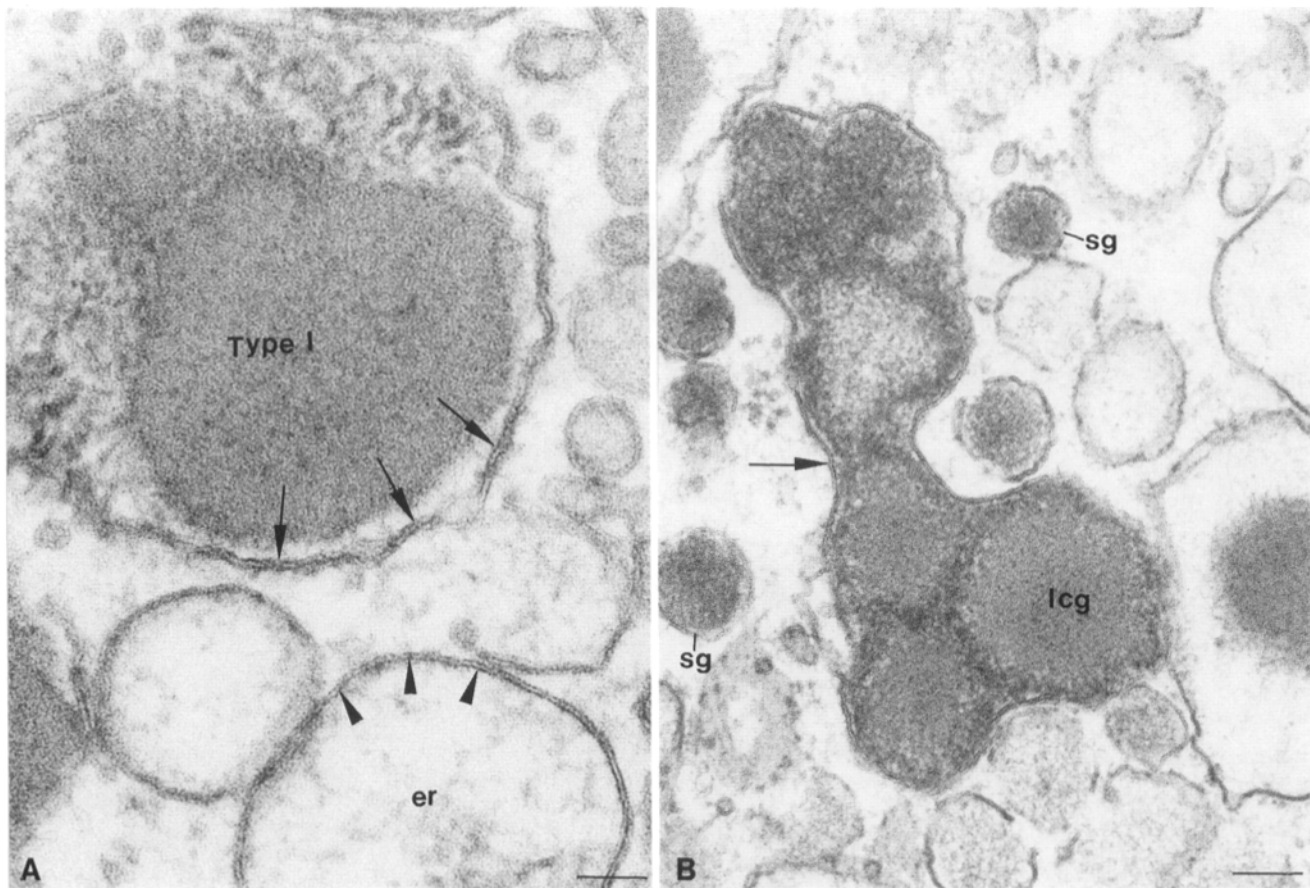
Figure 2. (*A*) Enlargement of membranes of the ER (*er*) and an early (*Type 1*) intermediate in Epon-embedded tissues. The limiting membrane of the latter (*arrows*) is thicker than that of the ER membrane (*arrowheads*) and is comparable to that of lysosomes. (*B*) Structure which appears to have arisen by fusion of a type-1 intermediate containing compact ICG (*below arrow*) and a type 2 intermediate containing more dispersed ICG (*above arrow*). *sg*, secretion granule. Tissue fixed in formaldehyde-glutaraldehyde followed by reduced OsO_4 to enhance membrane staining. Ribosomes are not well visualized after this procedure. Bars: (*A*) 0.2 μm; (*B*) 0.1 μm.

Results

Secretory Processes in Thyrotrophs of the Thyroidectomized Rat Pituitary

In pituitary glands of normal rats the thyrotrophs, or TSH producing cells, are a minority population, but after thyroidectomy they increase in number, enlarge dramatically (up to 10× normal), and predominate (8, 9, 10) due to hyperstimulation as a consequence of the removal of the negative feedback control on TSH secretion. Most of the enlargement of these cells results from ballooning of the ER due to a partial block in ER to Golgi transport of secretory proteins (21). The dilated ER appears as giant vacuoles by light microscopy (Fig. 1 *A*). Normally TSH-α is synthesized in excess and when TSH-β is synthesized it dimerizes with TSH-α, rapidly exits the ER (18), and is transported to the Golgi where the protein is packaged into granules (21). However, in thyroidectomized animals TSH-β synthesis increases, and this subunit accumulates in the ER (22). With increasing time after thyroidectomy the ER cisternae also contain increasing numbers of ICG (Fig. 1, *C* and *D*) that are in general larger (200–500 nm) than secretion granules (100–200 nm).

When thyroidectomized rats are treated with T_4 the size of the ER cisternae gradually diminishes over time due to

transport of secretory proteins out of the ER and their packaging into granules (8, 9). This leaves behind the ICG which must be disposed of. Over the course of the first 2–4 d of treatment, many of the ICG appear to be taken up and disposed of in lysosomes (8, 9). There is no evidence this occurs by autophagy—that is, there is no morphologic evidence of wrapping of the ER cisternae containing ICG with smooth-surfaced cisternae as described in the liver (2, 6, 12) or in pancreatic exocrine cells (32).

ER Cisternae Initially Lose Ribosomes and Acquire a Thicker Membrane

To obtain information on the mechanism of ICG disposal we studied thyrotrophs from thyroidectomized animals treated (2–4 d) with T_4 in routine Epon sections. In such sections multiple ER cisternae could be seen that contained one or more ICG (Fig. 1, *C* and *D*). However, most ER cisternae containing ICG had some of the properties ascribed to transitional elements in that they were part rough/part smooth. The ribosomes were typically present in patches or clusters separated by smooth areas rather than uniformly distributed over the entire membrane (*arrows*, Fig. 1 *C*). In addition, structures which appeared to represent intermediates between ribosome-studded, ER cisternae containing ICG and
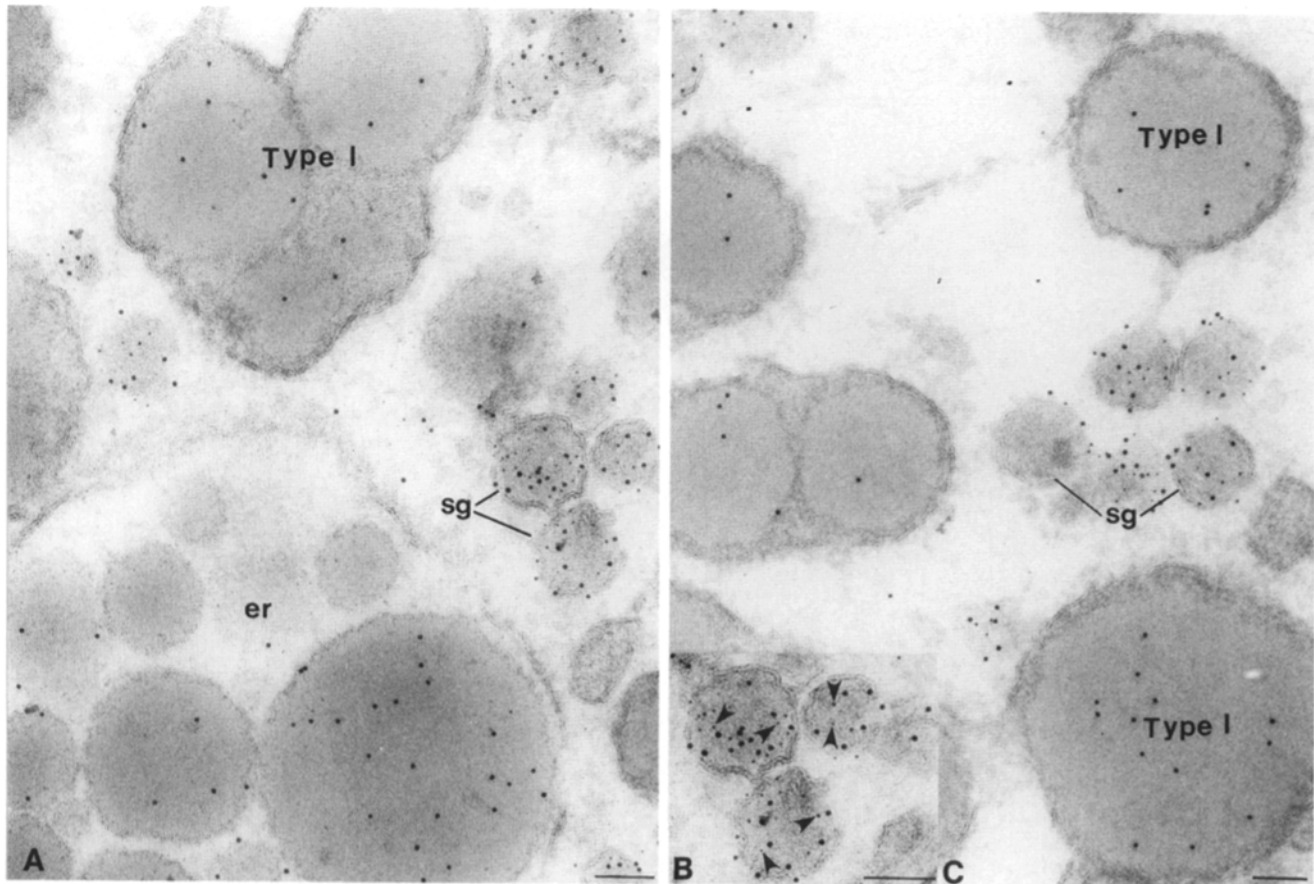
Figure 3. (A) Double immunogold labeling for TSH-α (5 nm gold) and TSH-β (8 nm gold). ICG in the ER *(er)* or in early *(Type 1)* intermediates are labeled only with TSH-β (large gold), whereas both TSH-α (small gold) and TSH-β subunits are detected in secretion granules *(sg).* Enlargement of several secretion granules demonstrating the presence of both TSH-β (large gold) and TSH-α (small gold, *arrowheads).* Bars: *(A, B,* and *C),* 0.1 μm.

fully condensed lysosomes containing barely recognizable ICG were frequently seen (Fig. 1, *C* and *D*). Based on morphologic criteria a series of intermediates could be identified in what is apparently a continuous process. For convenience these intermediates were divided into two main types, type 1 and type 2.

The early or type 1 intermediates (Figs. 1, *C* and *D*, and 2–4) resembled ER cisternae in that they contained one or multiple ICGs with the same density as those in the rough ER cisternae—i.e., they were quite compact with fine granular or fibrous material at their periphery. The main morphologic differences between these early intermediates and bona fide ER cisternae were that they lacked ribosomes, their content was typically denser, their shape more polymorphic, and their membrane thicker (8–9 nm) than the ER membrane (6–8 nm) (Fig. 2 *A*). No ribosome-free, ICG-containing cisternae could be found that possessed a thin, ER-like membrane. It appears, therefore, that ribosomes detach from the rough ER, and the latter acquires a thicker membrane.

The late or type 2 intermediates had many of the same features as the type 1, but the outlines of the ICG were less sharp, and their content was usually somewhat denser and more polymorphic (Fig. 1, *C* and *D*). There was sometimes evidence of beginning dispersion of the ICG at their periphery. Also, when multiple ICG were seen within a single cisterna they were often aggregated into clusters (Fig. 1, *C*

and *D*). Occasionally images were encountered suggesting that fusion takes place between type 1 and type 2 intermediates (Fig. 2 *B*). In no case was there any evidence of a double membrane or enveloping cisterna surrounding these structures comparable to that surrounding forming autophagic vacuoles. All of the intermediates had a single thick membrane.

These observations suggested that ER cisternae containing ICG are gradually modified by a progressive process during which they lose their ER characteristics (attached ribosomes; thin membrane; content of low density) and gradually acquire lysosomal characteristics (smooth, thick membrane; heterogeneous, dense content). They raised a number of questions concerning the nature of the membranes and content of the ER cisternae and intermediates containing ICG. To answer these questions we turned to immunocytochemistry.

Intracisternal Granules Contain TSH-β

We first set out to determine the nature of the ICG. Toward this end we labeled sections of T$_4$-treated (4 d) thyroidectomy cells with antibodies that recognize TSH-α and TSH-β. TSH is a heterodimer with the β subunit being specific for TSH and the α subunit shared in common with several other glycoprotein hormones (follicle stimulating and luteinizing hormones and chorionic gonadotropin). By immunofluores-

Table I. Results of Immunogold and Enzyme Cytochemical Labeling

	§RER	Type 1	Type 2	Lysosome	Secretory granule
TSH-β	+	+	+	±	+
TSH-α	−	−	−	−	+
PDI	+	+	−	−	−
KDEL	+	+	−	−	−
ER membrane proteins	+	+	+	±	−
Lgp120	−	+	+	+	−
Cathepsin D	−	−	+	+	−
AcPase-E*	−	+	+	+	−
AcPase-I‡	−	−	+	+	−

* AcPase-E: AcPase activity detected by enzyme cytochemistry.
‡ AcPase-I: AcPase immunoreactivity detected by immunogold labeling.
§ RER, type 1 and type 2 intermediates, lysosomes, and secretory granules were identified by morphologic criteria described in the Results. With the exception of AcPase-E, presence or absence of labeling is based on the presence or absence of gold particles over a given organelle. Labeling for AcPase-E is based on the presence or absence of lead phosphate reaction product. "+" indicates organelle designated is regularly labeled for a given marker. "−" indicates organelle is not labeled above background. "±" indicates occasional labeling is seen.

cence, the dilated ER cisternae of thyrotrophs were weakly labeled for TSH-β, and the surrounding cytoplasm was strongly stained (not shown). By immunogold procedures, heavy gold labeling was found over the ICG and secretion granules (Fig. 3, A, B, and C). The remaining luminal contents of the ER and Golgi elements were lightly labeled. It was apparent that the strong cytoplasmic immunofluorescence was due to heavy labeling of secretion granules which showed the heaviest concentration of gold. Gold particles were also found over ICG within type 1 and type 2 intermediates (Fig. 3 A and see Fig. 7 B), but few if any were found over lysosomes (summarized in Table I).

By contrast TSH-α was detected only in secretion granules: the cytoplasm surrounding the dilated ER cisternae was stained by immunofluorescence, and only the secretion granules were significantly labeled by immunogold procedures (Fig. 3, A, B, and C). Since these cells are known to synthesize and secrete TSH-α, one would expect to detect TSH-α in the ER. The lack of reactivity could be due to: (a) its low ER concentration as a result of rapid transit of TSH-α to the Golgi; (b) low sensitivity of the TSH-α antisera; or (c) failure of the antiserum to recognize the ER form of this subunit.

We conclude that ICG consist of aggregates containing TSH-β, and therefore TSH-β provides a convenient marker for ICG.

### ER Content and Membrane Markers Are Found in type 1 and 2 Intermediates

We next wanted to know if type 1 and type 2 intermediates contained ER markers. To obtain information on this point we carried out labeling for ER membrane and content proteins. By immunofluorescence two ER content markers, anti-PDI (Fig. 1 B) and anti-KDEL (not shown), were concentrated in the dilated ER of thyrotrophs, with the intensity of labeling varying from one cell to another. By immunogold, the contents of the dilated ER cisternae were immunoreactive, but the ICG were not labeled (Fig. 4 C). These two ER content markers were also detected in the contents of type 1 intermediate structures (Fig. 4 C).

The findings by immunofluorescence were very similar with an anti-ER serum that recognizes four ER membrane proteins in NRK cells (16): staining was concentrated in the contents of the dilated ER cisternae (Fig. 4, A and B). It was surprising to find that the contents of the dilated ER cisternae of thyrotrophs were reactive with an antiserum that recognizes ER membrane proteins. When this was checked at the EM level by immunogold labeling, gold particles were seen over both the membranes and contents of ER and type 1 and type 2 intermediates in thyrotrophs (Figs. 4 E and 5, A-D). However, in other anterior pituitary cells (Fig. 4 D) it was predominantly on the ER membranes (rather than their contents). The findings establish that intermediate structures with ICG contain ER membrane and content markers. The fact that labeling of the ER lumen is seen in thyrotrophs but not in other cell types, suggests that in hyperstimulated thyrotrophs one or more of the ER membrane proteins recognized by the antibody undergoes cleavage releasing its ectodomain into the ER lumen.

Co-Localization of ER Markers and a Lysosomal Membrane Protein in Intermediates

We next wish to determine whether both ER and lysosomal proteins could be detected in the same intermediate structures. We found that an antibody against the lysosomal membrane protein, lgp120, labeled not only the membranes of lysosomes (Fig. 5 C), but also the membranes of type 1 and 2 intermediates with clearly recognizable ICG (Figs. 5, A, B, and D and 6, A and B). In fact, the membrane of any structure containing ICG that lacked ribosomes was immunoreac-

Figure 4. Distribution of ER markers in a T₄-treated, thyroidectomized rat pituitary. (*A* and *B*) Phase contrast and immunofluorescence micrographs demonstrating labeling of thyrotrophs (16). Semithin cryosections were reacted with an anti-ER antiserum that recognizes four ER membrane proteins. Surprisingly, the contents of the dilated ER cisternae (*er*) of many of the thyrotrophs are strongly labeled (*). Diffuse cytoplasmic staining is also seen in several other cell types, and one thyrotroph (*T*) shows prominent labeling of the ER membrane (*arrowhead*), but the strongest labeling is seen within the dilated ER of thyrotrophs. The presence of immunoreactivity in the ER lumen suggests that ER membrane proteins are cleaved and released into the lumen of the modified ER. (*C*) Immunogold labeling demonstrating localization of PDI within the contents of two ER cisternae (*er*) and an early (*Type 1*) intermediate. The ICG within these compartments are not labeled. The type 1 intermediates can be distinguished from ER cisternae by the greater density of their contents (*). (*D* and *E*) Immunogold labeling with anti-ER antiserum. In a mammosomatotroph (shown in *D*) most of the gold particles are closely associated with membranes of the rough ER which is in keeping with the fact that the four proteins recognized by this antiserum are membrane proteins. In the thyrotroph, however (shown in *E*), these ER membrane antigens are detected in the ER contents and in the contents (*arrowheads*) of early (*Type 1*) intermediates as well as associated with ER membranes (*arrows*). Little labeling of the lysosome (*ly*) is seen above background. Bars: (*A* and *B*) 10 μm; (*C*) 0.1 μm; (*D*) 0.2 μm; (*E*) 0.1 μm.
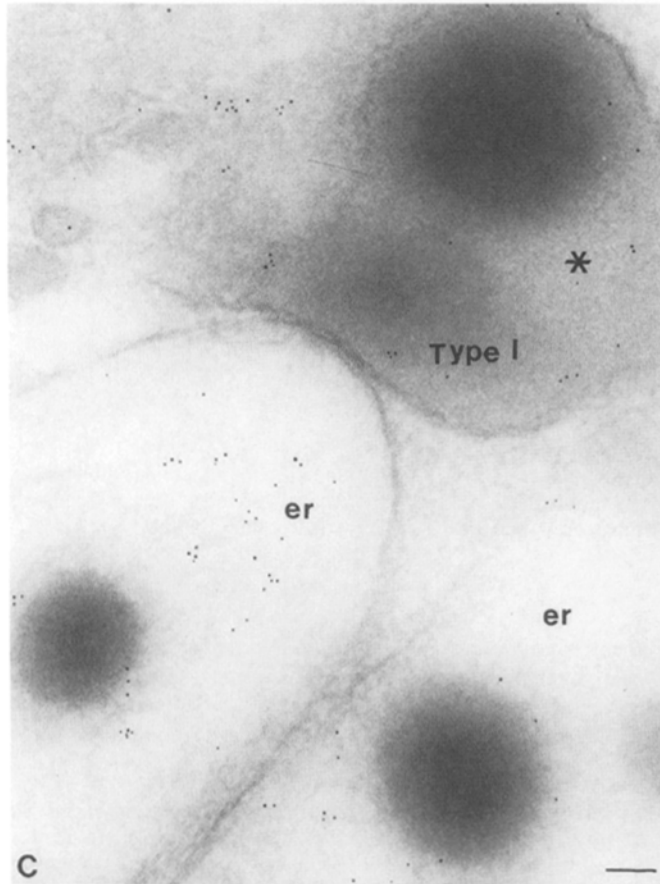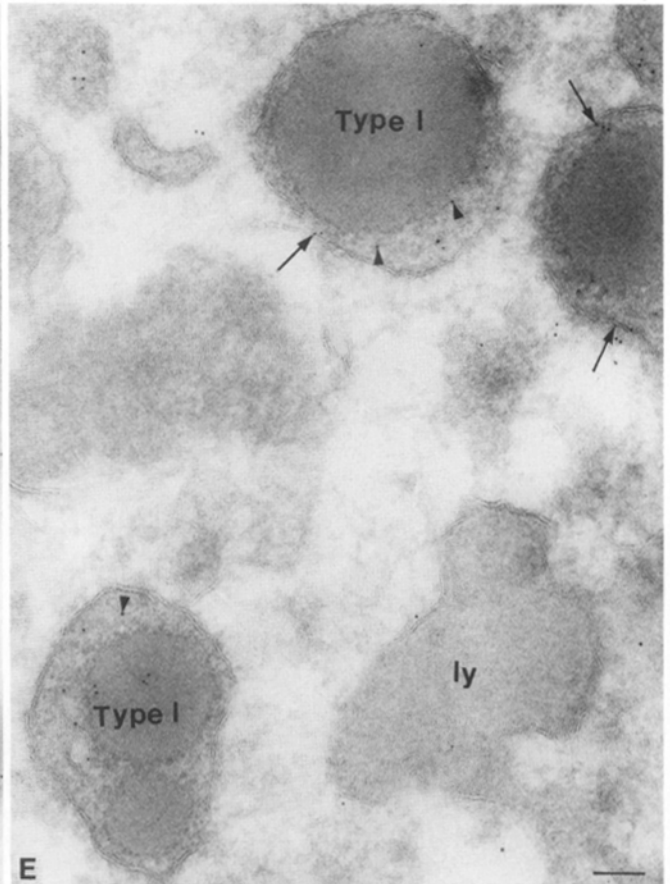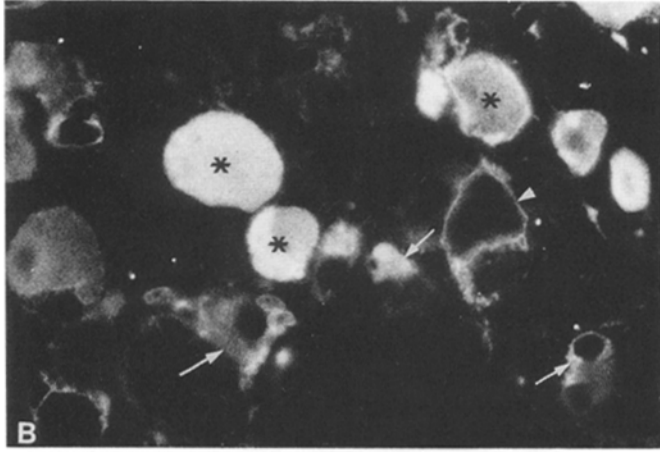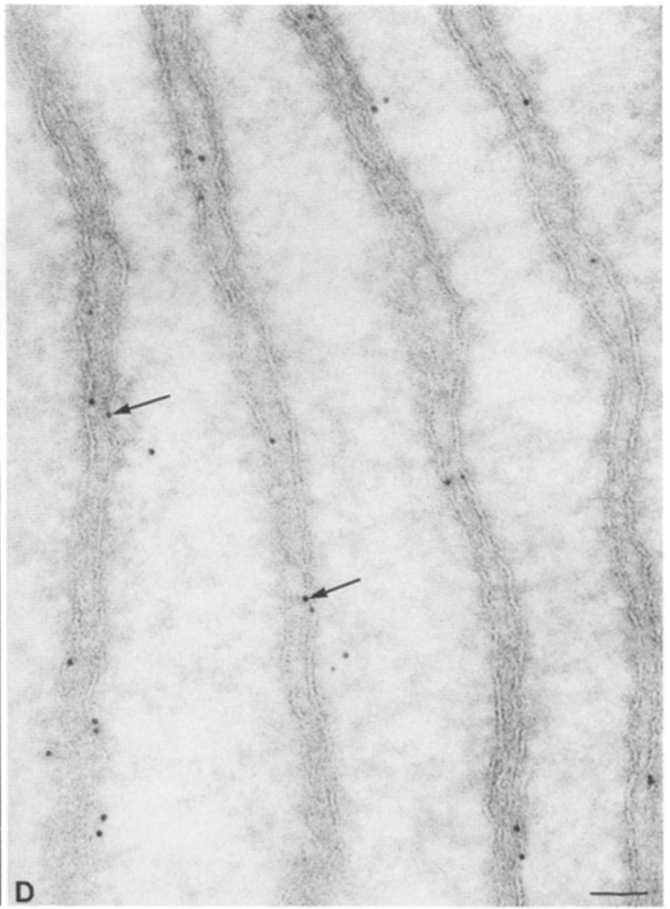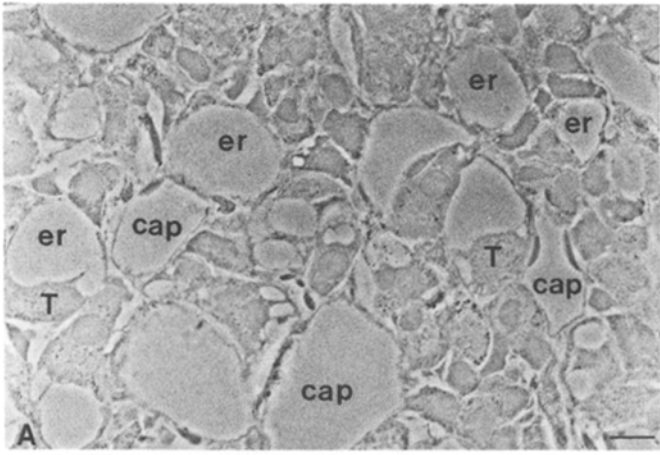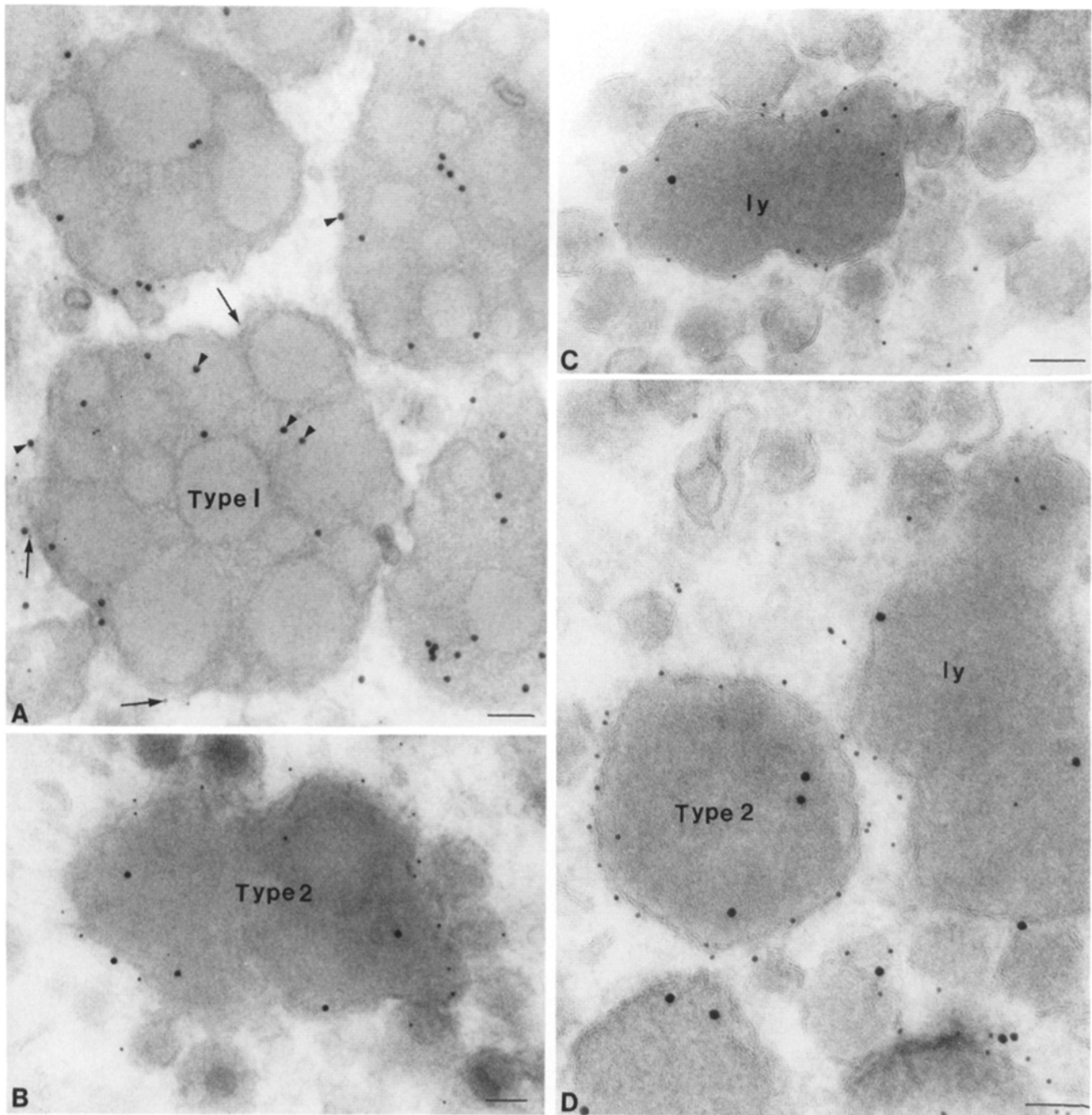
Figure 5. Double immunogold labeling for the lysosomal marker, lgp120 (5 nm gold), and ER markers (15 nm gold). (*A*) A type 1 intermediate whose membrane and contents (*arrowheads*) are labeled with an antibody raised against ER membrane proteins and whose membranes are weakly reactive for lgp120 (*arrows*, small gold). (*B, C,* and *D*) Type 2 intermediates and lysosomes (*ly*) whose membrane and contents are labeled with the anti-ER antiserum (large gold) and whose membranes are labeled for lgp 120 (small gold). Bars, 0.1 μm.

tive for lgp120. When double labeling was done with anti-PDI or anti-ER antisera and anti-lgp120, type 1 and type 2 intermediates (Figs. 5, *A–D* and 6 *B*) were regularly labeled with both organelle markers. Counts of gold particles over intermediates in the same field indicated that labeling for ER markers declined and labeling for lgp120 increased as the ICG contents became less distinct, which presumably corresponds to the conversion of a type 1 to a type 2 intermediate. Particularly significant was the co-localization of both ER membrane proteins and lgp120 in the membrane of the same early intermediate (Fig. 5 *A*) with compact ICG. These data indicate that ER cisternae acquire lysosomal membrane proteins very early, before loss of ER membrane and content proteins.

Lysosomal Enzymes Are Detected in Intermediates As Well As Lysosomes

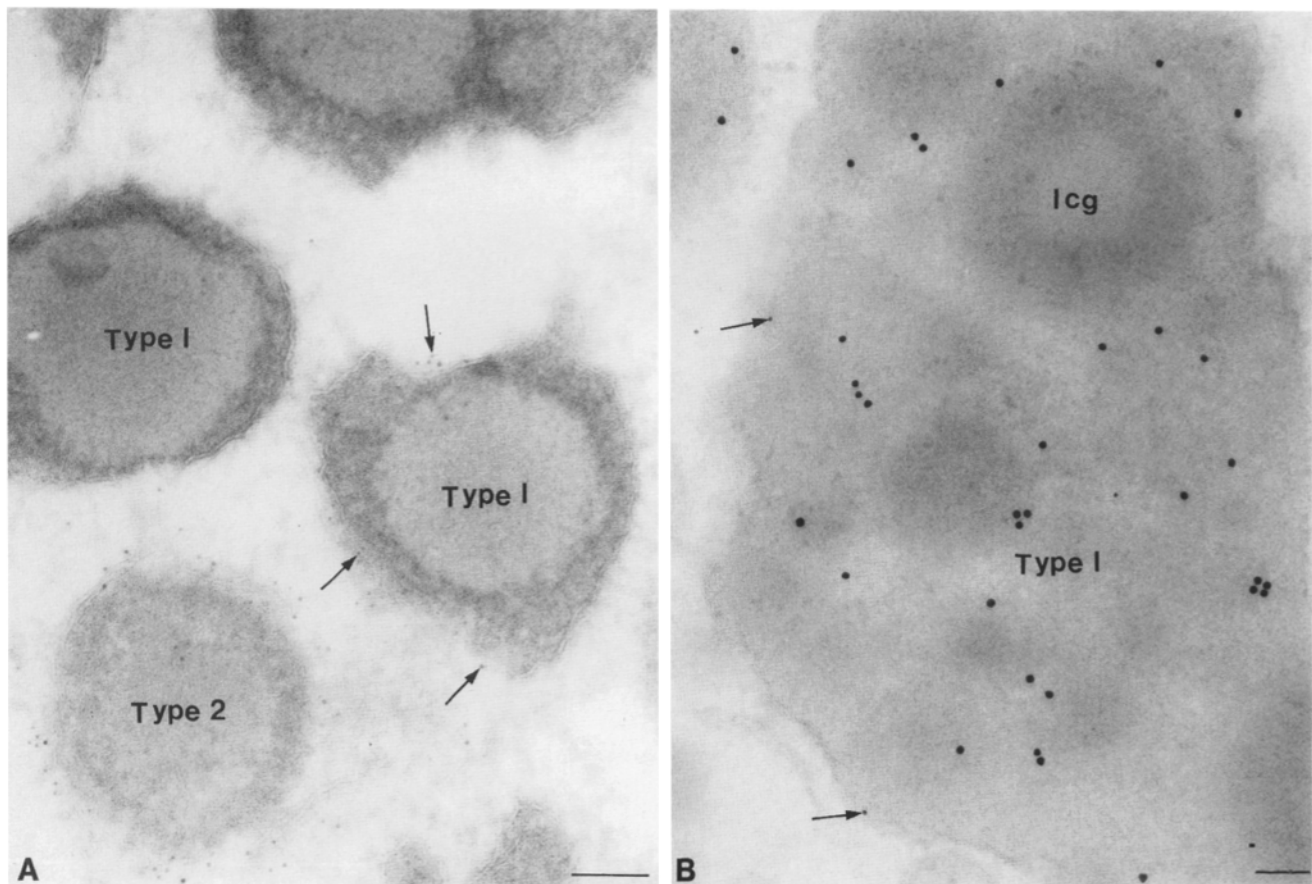To determine the distribution of lysosomal enzymes, AcPase

Figure 6. Immunogold labeling for the lysosomal membrane protein, lgp120. (*A*) Two early (*Type 1*) intermediate structures are seen: one shows little labeling and the other moderate labeling (*arrows*) for lgp120 along its limiting membrane. A more advanced (*Type 2*) intermediate is heavily labeled. (*B*) Double labeling for lgp120 (5 nm gold) and PDI (15 nm gold). An early (*Type 1*) intermediate with several ICG (*Icg*) contains both the ER content marker (large gold) and the lysosomal membrane marker (*arrows*, small gold). Bars, 0.1 μm.

and cathepsin D were localized by immunocytochemistry. The distribution of both enzymes was similar as both were found in lysosomes and in type 2 intermediates, but they were not seen in type 1 (Fig. 7, *A* and *B*). When double-labeling was carried out with anti-lgp120 and anti-AcPase or anti-cathepsin D, many type 1 intermediates were identified which showed membrane labeling for lgp120, but lacked labeling for lysosomal enzymes (not shown).

When AcPase activity was localized using an enzyme cytochemical reaction (Fig. 8, *A* and *B*), reaction product was detected in the same compartments as after immunocytochemical localization—i.e., in late or type 2 intermediates and in lysosomes. However, by enzyme cytochemistry the transmost Golgi cisterna and some type 1 intermediates were also AcPase positive (Fig. 8 *A*).

When double immunogold labeling was carried out for TSH-β and either AcPase or cathepsin D using two sizes of colloidal gold, both TSH-β and the lysosomal enzymes could be detected in intermediate structures, with TSH-β being localized to ICG and lysosome enzymes to their remaining contents (Fig. 7 *B*).

From these results (summarized in Table I) we conclude that intermediates containing ICG acquire the lysosomal membrane protein lgp120 before the acquisition of lysosomal

enzymes. A similar sequence occurs during formation of autophagic vacuoles (32).

MPR Are Not Involved in Delivery of Lysosomal Enzymes to ER Cisternae

When we carried out labeling for MPR there was no significant immunoreactivity detected in the Golgi complex, ER, or endosomes of thyrotrophs cells with two different antibodies to the receptor. Labeling was seen however, in endosomes in other cell types in the same field. This suggests: (*a*) that the overall level of expression of MPR in hyperstimulated thyrotrophs is rather low; and (*b*) that this receptor may not be involved in the delivery of lysosomal enzymes to the ER.

Discussion

We have used immunocytochemical markers for ER, lysosomal, and secretory proteins to investigate the mechanism of disposal of ICG in thyrotrophs of the thyroidectomized, T₄-treated rat. By morphology alone, we were able to distinguish intermediates in what appeared to be a continuous process involving direct conversion of ER cisternae into
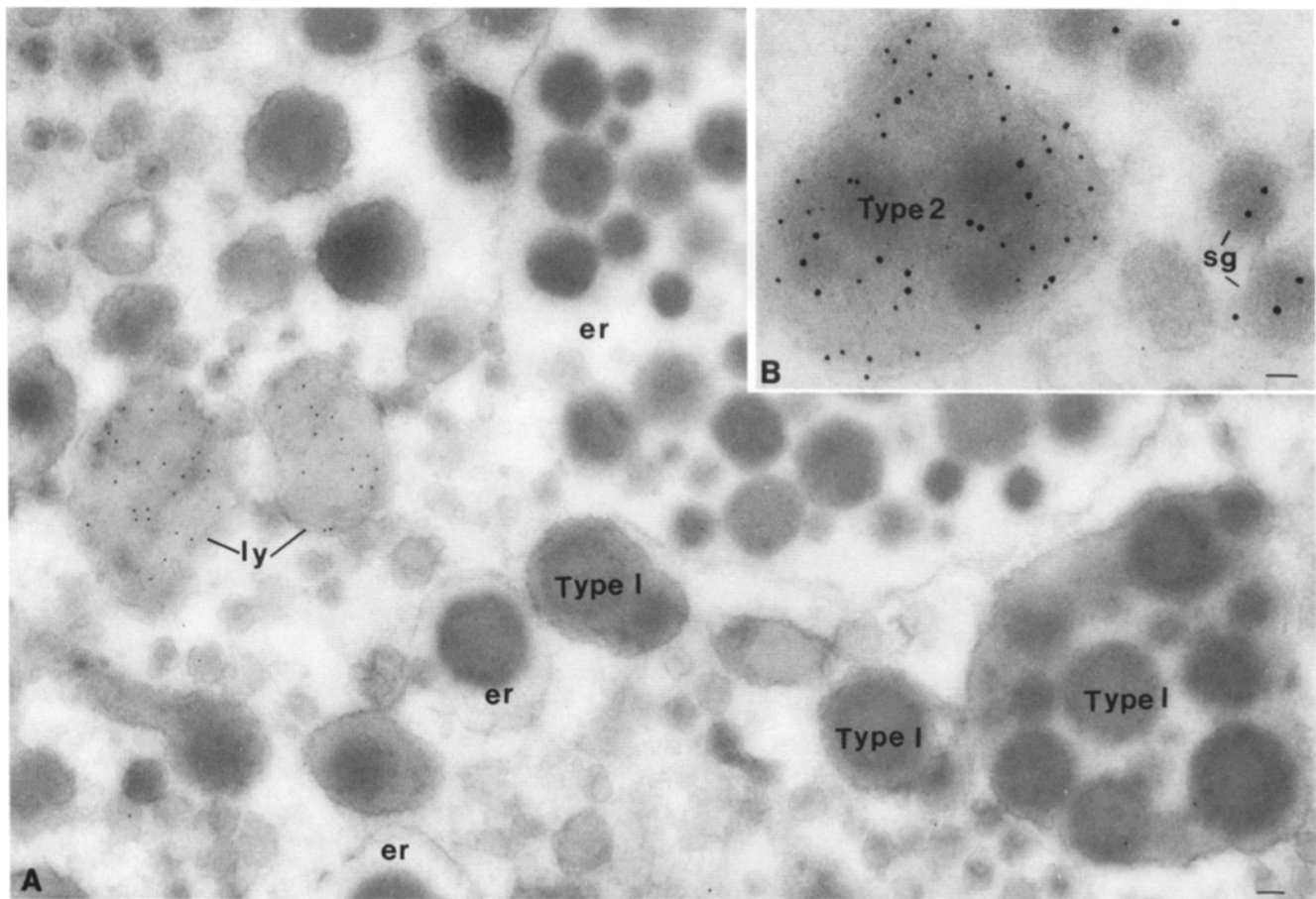
Figure 7. Immunogold localization of cathepsin D (8 nm gold). (*A*) Two lysosomes (*ly*) with contents of low density are strongly labeled, whereas the ER cisternae (*er*) and early (*Type 1*) intermediates are devoid of label. (*B*) Double labeling for cathepsin D (8 nm gold) and TSH-β (15 nm gold) demonstrating colocalization of a lysosomal enzyme (*arrowheads*) and TSH-β in a type 2 intermediate. Secretion granules (*sg*) are labeled only with TSH-β (large gold). Bars, 0.5 μm.

lysosomes. Based on our immunocytochemical localizations of ER and lysosomal markers we distinguished two main types of intermediates—type 1 and type 2 (Table I). From our combined morphologic and immunocytochemical results we can reconstruct the process for disposal of ICG as follows (Fig. 9): An ER cisterna with some of the properties of transitional elements (part rough/part smooth) containing one or more ICG begins to lose its ribosomes, its contents become condensed, and its shape more irregular. At the same time, it appears that some ER membrane proteins undergo cleavage and are released into the ER lumen. Concomitantly the ER cisternae acquire a thicker, lysosome-type membrane. These early or type 1 intermediates are identified by the presence of clearly recognizable ICG, ER content markers (PDI and other KDEL-containing proteins), ER membrane proteins, and the lysosomal membrane protein, lgp120, but they lack lysosomal enzymes. Type 1 intermediates gradually become more condensed, the profiles of the ICG become less sharp, and they acquire lysosomal enzymes as demonstrated by immunogold labeling for AcPase and cathepsin D. That AcPase is enzymatically active was demonstrated by enzyme cytochemistry. It is the presence of lysosomal enzymes that distinguishes type 2 or late intermediates from type 1 (see Table I). The ICG within type 2 intermediates begin to disperse at their periphery and eventually can no longer be recognized within the dense contents. In the strict sense, a structure becomes a lysosome when it acquires lysosomal enzymes, but, as already indicated, the conversion of an ER cisterna to a lysosome is a continuous process and the delineation of type 1 and type 2 structures and lysosomes is somewhat arbitrary. We distinguish late intermediates from lysosomes by the presence of still recognizable ICG. Fully mature lysosomes contain barely recognizable ICG or have a homogeneous content in which specific digestive residues cannot be recognized. Membranous whorls or myelin figures, commonly seen in autophagic vacuoles, are rarely seen in lysosomes of thyrotrophs.

The process we have described for the direct conversion of an ER cisterna to a lysosome is novel. The only previous study dealing with ICG disposal was in the exocrine pancreas of guinea pigs treated with $CoCl_2$ which causes massive induction of ICG that are eventually disposed of by autophagy (32). Is it possible that in our case disposal also occurs by autophagy, but we have not been able to detect the earliest stage of autophagic vacuole formation? We do not consider this likely, as multiple rat pituitaries were investigated over a period of 1–4 d after thyroidectomy and T_4 treatment, and images suggesting the presence of an enveloping cisternae were rare, whereas they are not difficult to detect in other situations where autophagy occurs. Moreover, autophagic
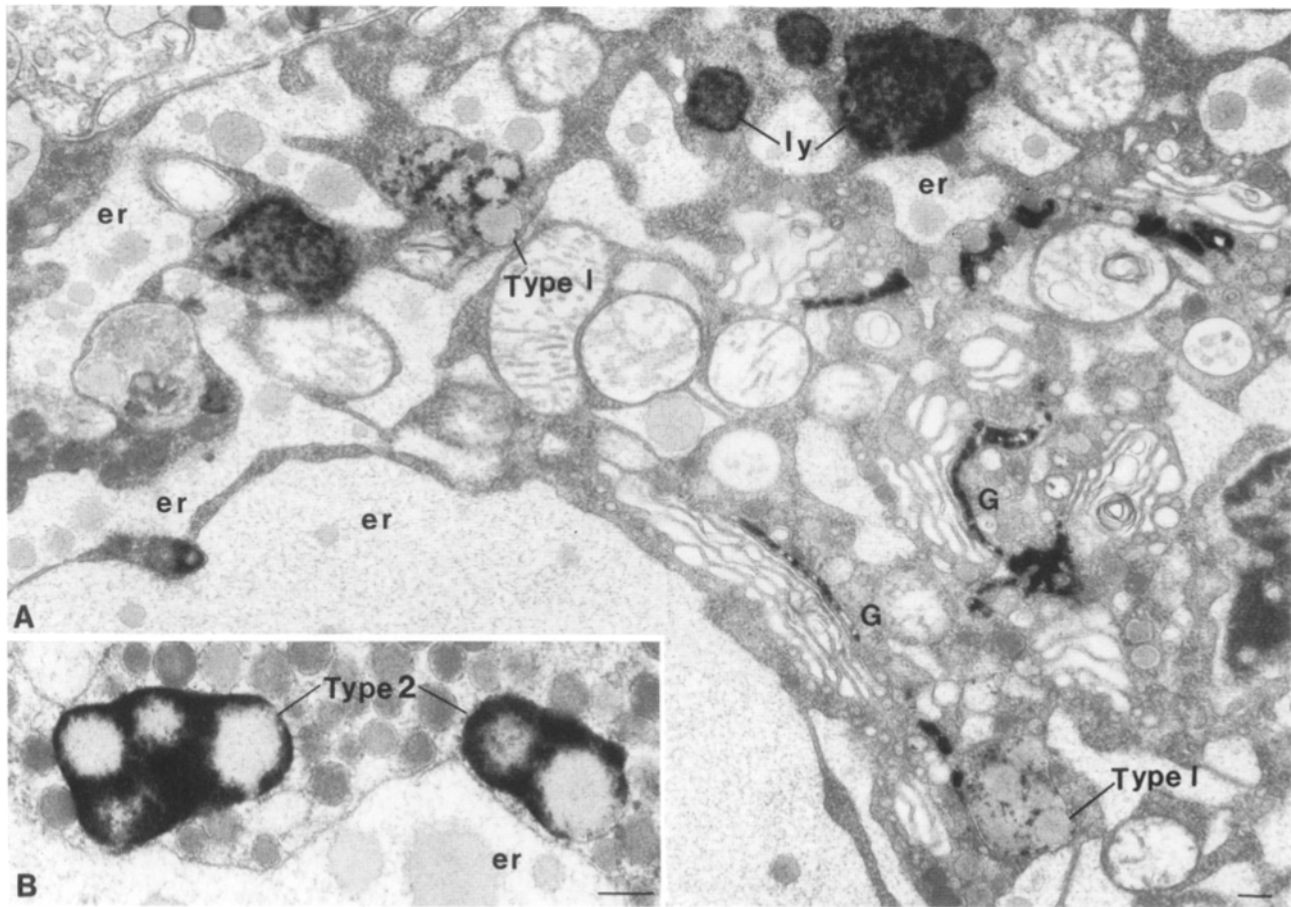
Figure 8. Enzyme cytochemical localization of AcPase. Heavy deposits of acid phosphatase reaction product are seen in lysosomes (*ly*), in the transmost cisterna of the Golgi complex (*G*), and around the ICG in type 2 intermediates (*inset, B*). A weaker reaction is present in early (*Type 1*) intermediates. The ER cisternae (*er*) are not reactive. Bars, 0.2 μm.

vacuoles are usually readily identified by their heterogeneous content which often includes ER cisternae, mitochondria, and other membranous residues (myelin figures) (2, 6, 12, 32) that are not seen in the ICG-containing type 1 or type 2 intermediate structures or in lysosomes in thyrotrophs. Thus it appears that two different mechanisms exist for disposal of ICG in the exocrine pancreatic cell and in the pituitary thyrotroph. In the case of the thyrotroph the lysosomal disposal mechanism may serve to degrade the TSH-β that was made in excess during thyroidectomy and did not dimerize with TSH-α.

Our findings provide compelling evidence that a mechanism exists for disposal not only of ICG, but also of other ER proteins by direct conversion of an ER cisterna into a lysosome. Two questions are left unanswered by our data. (*a*) What is the mechanism by which the ER membrane is converted into a lysosomal membrane?; and (*b*) How do the ER cisternae acquire lysosomal enzymes? In regard to the first question, the membrane could come from direct fusion with preexisting lysosomes; however the membrane change occurs very early and there is no evidence of fusion of ER cisternae with lysosomes, although images suggesting fusion between type 1 and type 2 intermediates are sometimes seen. During autophagic vacuole formation in the liver (6, 7, 12) the enveloping cisterna that initiates the autophagic event is

also derived from a rough ER cisterna that loses its ribosomes, undergoes structural changes, envelopes cytoplasmic components, and rapidly acquires lysosomal membrane proteins. In neither case is the basis for the conversion of an ER membrane to a lysosome membrane known, but one can speculate that membrane replacement occurs via fusion of small vesicles carrying lysosomal membrane constituents with these special transitional elements of the ER.

The second question left unanswered by our findings is the mechanism of delivery of lysosomal enzymes to the ER cisternae. It seems unlikely that cation-independent MPR are involved since they could not be detected in these structures. Among the existing possibilities are that the enzymes are: (*a*) acquired by direct fusion with preexisting lysosomes; (*b*) ferried there via the 46 kD (cation-dependent) mannose-6-phosphate receptor; (*c*) newly synthesized enzymes from the ER itself; or (*d*) targeted by an unknown delivery mechanism. Since images suggesting fusion between type 1 and type 2 structures are sometimes seen (Fig. 2 *B*), we consider the first possibility the most likely. Experiments of the type carried out by Tooze et al. (32) with MPR fragments conjugated to gold could help to determine whether the lysosomal enzymes present in the intermediate structures were newly delivered with the mannose-6-phosphate recognition marker still intact or had already undergone processing to remove
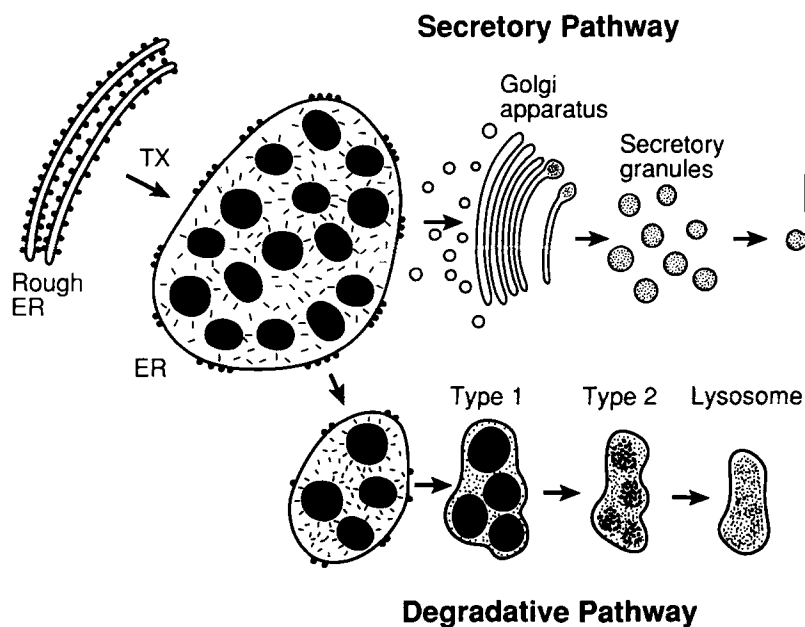
Secretory Pathway

Degradative Pathway

Figure 9. Diagram depicting the proposed degradative pathway for ICG in thyrotrophs of the rat pituitary. In the thyrotroph of normal rats, the rough ER is organized into flattened cisternae (upper left). After thyroidectomy (*TX*) which causes increased TSH secretion, the ER lumen becomes dilated and ICG form. Upon T₄ treatment which shuts off TSH secretion, some of the TSH stored in the ER lumen is transported to the Golgi complex and packaged into secretory granules, whereas the ICG which cannot be transported along the secretory pathway are degraded in lysosomes. This appears to occur by direct conversion of an ER cisterna into a lysosome in several steps as follows: A part rough/part smooth ER cisterna which is heavily labeled with ER markers (PDI, KDEL, ER membrane proteins) loses its ribosomes, its contents become more condensed, and ER membrane proteins are cleaved and released into the lumen. At the same time the membrane is converted into a thick, lysosomal-type membrane which contains lgp120, a lysosomal membrane protein, which completes its transition to a type 1 intermediate. The structure then receives lysosomal enzymes such as AcPase and cathepsin D (to become a type 2 intermediate) which is arbitrarily distinguished from a lysosome by the presence of recognizable ICG. Lysosomal enzyme delivery is believed to be accomplished by fusion between type 1 intermediates and either type 2 intermediates or lysosomes. Based on the fact that the lysosomal membrane protein, lgp120, can be co-localized with ER membrane proteins on the limiting membranes of type 1 and 2 intermediates as well as lysosomes, the transformation of the ER to a lysosome is assumed to occur by a progressive direct conversion process.

it, but they would not provide information on the route of lysosomal enzyme delivery. Evidence for involvement of (cation-independent) MPR in autophagy was obtained in the experiments of both Dunn (6, 7) and Tooze et al. (32). However, in another model of autophagy (induced by leupeptin treatment), Dunn (6, 7) found no evidence for involvement of these receptors. This indicates that MPR are not necessarily involved in lysosomal enzyme delivery in all cases of lysosomal digestion. It has already been established that some lysosomal enzymes such as AcPase reach lysosomes via the *trans*-Golgi by mechanisms that do not involve MPR (28, 33).

Protein Degradation in the ER

It has become evident that considerable turnover of newly synthesized proteins occurs in the ER. Some of this is related to normal turnover of ER resident proteins (5, 27), and some is related to the newly discovered function of the ER in disposing of unassembled subunits of multimeric proteins (1, 14, 15) and aggregated or misfolded, transport incompetent proteins (13, 14). Very little is known regarding the mechanisms by which either resident proteins or those in transit are degraded. Both lysosomal and nonlysosomal mechanisms have been claimed to exist based on the sensitivity (14, 27) or lack of sensitivity (1, 5, 15), respectively, of the process being studied to lysosomotrophic agents (primaquine, chloroquine). The only direct information on lysosomal involvement comes from the experiments of Tooze et al. (32) already summarized and experiments on two resident ER enzymes indicating that the half-life of glucosidase II (27) and HMG-CoA reductase (5) was doubled in the presence of lysosomotrophic agents. This suggests that lysosomes are involved in the turnover of these two enzymes. In the case

of glucosidase II it was suggested (27) that disposal was by autophagy based on earlier immunocytochemical observations (17).

Our findings as well as those of Dunn (6, 7) are compatible with the assumption that the distal (preGolgi) regions of the ER where transitional elements are found may represent areas of specialization which under some circumstances can participate in the diversion of secretory proteins and ER membrane and content proteins to lysosomes. The validity of this hypothesis remains to be established.

From the general standpoint it is of interest that in pituitary cells secretory proteins can be diverted to lysosomes at several intracellular sites along the exocytic pathway. In addition to the ER mechanism for disposal of ICG described here, both immature and mature secretory granules can be disposed of in lysosomes by crinophagy. This is the process by which mature secretory granules can fuse directly with lysosomes, and immature granules can fuse with multivesicular endosomes and be degraded (9, 26). Thus both pre-Golgi and post-Golgi sites exist for regulation of the secretory process through lysosomal degradation.

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