

Isolation of Insoluble Secretory Product from Bovine Thyroid: Extracellular Storage of Thyroglobulin in Covalently Cross-linked Form

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Abstract. Extracellular storage of thyroglobulin (TG) is an important prerequisite for maintaining constant levels of thyroid hormones in vertebrates. Storage of large amounts is made possible by compactation of TG in the follicle lumen with concentrations of at least 100–400 mg/ml. We recently observed that the luminal content from bovine thyroids can be isolated in an intact state and be separated from soluble TG. For this purpose, bovine thyroid tissue was homogenized and subjected to various steps of purification. This procedure resulted in a pellet of single globules measuring 20–120 μm in diameter. Scanning electron microscopy revealed a unique cobblestone-like surface pattern of isolated globules, showing in detail the impressions of the apical plasma membranes of thyrocytes which had formerly surrounded the luminal content before tissue homogenization. Isolated thyroid globules were rapidly digested by trypsin but extremely resistant to various

protein solubilization procedures. Homogenization of isolated globules resulted in the release of $\sim 3\%$ of total protein, showing that only a minor proportion of TG was loosely incorporated in thyroid globules whereas $\sim 22\%$ appeared to be interconnected with the globule matrix by disulfide bridges. Analysis by SDS-gel electrophoresis and immunoblotting confirmed that the protein released by this procedure consisted of TG. The vast majority ($\sim 75\%$) of the globule matrix protein was found to be covalently cross-linked by non-disulfide bonds. TG in isolated globules was highly iodinated (~ 55 iodine atoms per 12-S TG subunit) suggesting that the covalent nondisulfide cross-linking occurs in part during the iodination of TG and that this process involves the formation of intermolecular dityrosine bridges. Mechanisms must exist which solubilize or disperse the insoluble luminal content prior to endocytosis of TG.

CELLS with a regulated pathway of secretion are characterized by the ability to store the secretion product in granules and to release the granule content following specific signals for exocytosis. The secretory pathway in thyroid follicle cells is also regulated in that thyroglobulin (TG)¹ is temporarily stored within apically located export vesicles (11, 53, 54). However, the transport pathway of TG is unique in that additionally to the apical vesicles a large extracellular storage site exists within the lumina of thyroid follicles. Luminal TG provides a physiologically important reservoir which is regulated in size by the exocytotic addition of newly synthesized TG and by endocytic removal of TG from the lumen. Endocytically removed TG is conveyed primarily to lysosomes for the proteolytic liberation of thyroid hormones. It can be concluded from numerous experiments

that the extracellular storage of TG in the follicle lumen is a prerequisite for maintaining constant levels of thyroid hormones in the vertebrate organism (41). Storage of large amounts of TG is made possible by compactation, i.e., the tight packaging of TG molecules in the follicle lumen. TG from the lumina of single follicles can be collected by micropuncture, and protein concentrations of 100–400 mg/ml have been reported (21, 47). Higher luminal concentrations are assumed to exist but have not been determined due to the viscosity of luminal TG at higher concentrations (53).

TG in the follicle lumen has been shown to have a variable but generally low diffusion rate (36) which is regulated in part by thyroid-stimulating hormones (TSH) (18). In resting glands, it may take months until newly synthesized TG exported into the follicle lumen reaches equilibrium distribution within the luminal cavity (35) and in pathological conditions, e.g., animal or human goiter, equilibration time may become infinite (48). The low rate of diffusion has been explained by the viscosity of TG in the follicle lumen (18, 30). It is unknown, however, as to whether the viscous luminal content consists of condensed but nevertheless single TG molecules or whether this TG is structurally organized by

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1. *Abbreviations used in this paper:* TG, thyroglobulin; SEM, scanning EM.

specific intermolecular interactions. Moreover, structurally organized luminal content could provide the basis for the sorting of newly exported TG from its storage form in the follicle lumen.

In search for evidence which could show the existence of structurally organized TG we have screened thyroid tissue fractions for the presence of stable polymeric TG. We report here on the isolation and characterization of globules which represent the intact luminal content of thyroid follicles. Unexpectedly, isolated thyroid globules are almost insoluble although TG, the principal constituent of thyroid secretion, is a soluble protein.

Materials and Methods

Thyroid Tissue

The laryngeal region of the trachea containing the thyroid was removed immediately after killing of cattle (ox) in the local slaughterhouse. Pig thyroid glands were removed about 10 min after exsanguination. The tissue was transported on ice within 10 min to the laboratory. Thyroid glands were dissected and stored on ice until further preparation of tissue.

Material

Percoll was obtained from Pharmacia Biosystems GmbH (Freiburg, Germany). Trypsin and goat anti-rabbit antibody (alkaline phosphatase labeled) were from Miles GmbH (Frankfurt, Germany) or from Sigma Chemie (München, Germany). FITC (Isomer I) and dialysis tubing were purchased from Serva Feinbiochemica GmbH and Co. (Heidelberg, Germany). All other chemicals and biochemicals were of highest purity grade available. All glassware was freshly siliconized to reduce adhesion of luminal globules to the glass surface.

Separation of Insoluble from Soluble TG

Freshly removed bovine thyroid glands, stored on ice, were carefully freed of fat and connective tissue by the use of acetone-cleaned razor blades and cut into 2-mm tissue fragments. Aliquots of 5-ml tissue fragments were transferred into 50-ml Falcon tubes and diluted with 15 ml distilled water containing protease inhibitors (37 mg Na-p-tosyl-L-argininmethyl ester, 0.1 mg antipain, 0.1 mg pepstatin, 75 μ l trasylol, 0.25 mM phenylmethylsulfonyl fluoride in 100 ml Tris-HCl buffer, pH 7.2).

Homogenization of thyroid fragments was performed at 4°C by the use of a Polytron-Homogenizer (15 s, position 4.5; Kinematika, Kriens, Luzern, Switzerland). The homogenate was diluted 1:10 with distilled water and vigorously shaken (30 s) to separate insoluble TG (thyroid globules) from cellular remnants and soluble TG (Fig. 1). After centrifugation (60 s, 100 g) the pellet was subjected to the steps required for purification of thyroid globules whereas the supernatant was used for the separation of soluble TG. The pellet was resuspended in water and filtered through 150- μ m Thermapor nylon gauze (Reichert Chemietechnik GmbH, Heidelberg, Germany) which had been extensively cleaned with acetone and distilled water before use. The filtrate was transferred into 15-ml Polyallomer tubes and centrifuged 60 s at 100 g and the resulting pellet was repeatedly washed. The composition of the resulting pellet was controlled by light microscopy and usually found to be highly enriched in thyroid globules but still contaminated by collagen fibers. This preparation was further purified by Percoll gradient centrifugation. A step gradient was prepared with 10 ml undiluted Percoll, 10 ml Percoll diluted 1:2 and 15 ml Percoll diluted 1:10. The sample pellet was diluted in distilled water and applied on top of the gradient. Centrifugation was performed in Polyallomer tubes for 2.5 h at 15,000 g and 4°C using a 55.2 Ti rotor in an L7-65 ultracentrifuge (Beckman Instruments Inc., Düsseldorf, Germany). Thyroid globules collecting at the interface between 10 and 50% Percoll (see Fig. 2) were diluted in water, filled into Polyallomer tubes, and centrifuged using an SW50.1 rotor (Beckman Instruments) for 1 h at 20,000 g at 4°C. The pellets containing the highly purified thyroid globules were stored at -80°C. TG solutions and globule pellets were frozen at -20°C. The same protocol for isolation of thyroid globules was applied to separate insoluble TG from porcine thyroid glands.

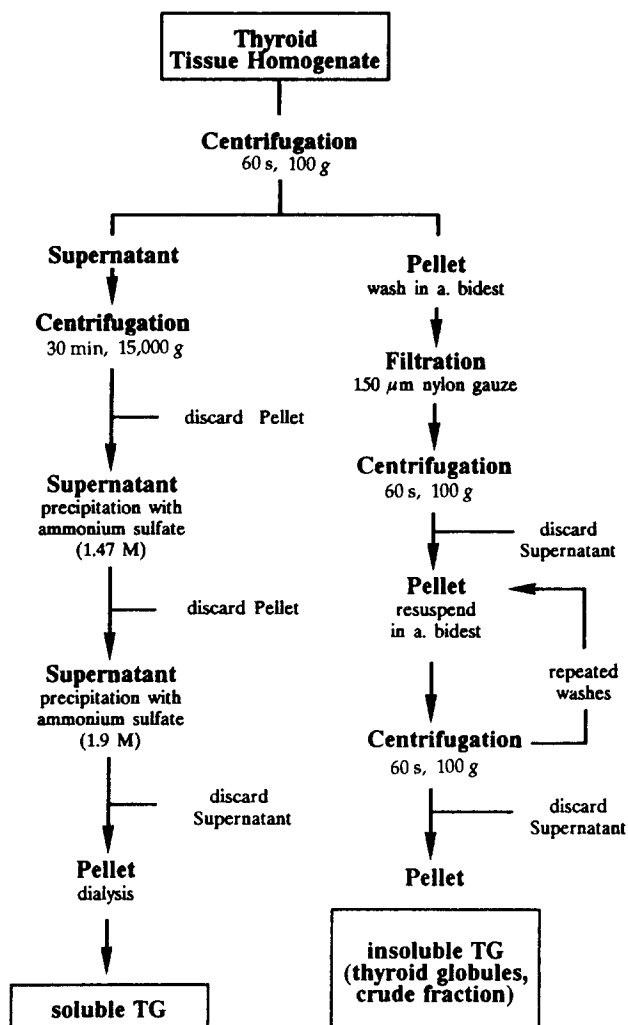


Figure 1. Flow chart of procedure to isolate insoluble TG (thyroid globules; right) from soluble TG (left). The crude globule fractions may be further purified by centrifugation on a Percoll gradient (see Fig. 2).

FITC-labeling of Thyroid Globules

For better visualization of thyroid globules and contaminants during Percoll gradient centrifugation, the crude globule fraction (Fig. 1) was labeled with FITC. 5 mg FITC were dissolved in 1 ml DMSO and added to the thyroid globule preparation suspended in 10 ml 50 mM borate buffer, pH 9.0. After 4 h the globule suspension was dialyzed for 24 h against distilled water, always at room temperature and in the dark. After dialysis the fluorescent sample was applied to Percoll gradient centrifugation as above.

Exposure of Thyroid Globules to Solutions of Distinct Osmolality

Isolated thyroid globules were placed into a hemocytometer and viewed by light microscopy. The volume of single globules was determined by the area of the globule and the depth of the chamber in the hemocytometer. The volume was monitored during the application of distilled water and of various dilutions of PBS.

Stability of Isolated Thyroid Globules

It was observed that suspension and prolonged storage in water or in PBS failed to dissolve isolated TG globules. The globules were also insensitive to freezing and thawing. Dispersion of insoluble TG globules was attempted

according to the following procedures: (a) Treatment with EDTA; Ca^{2+} is known to participate in the formation of TG aggregates (23). Hence, isolated TG globules were suspended in 10 mM EDTA in PBS and stored at room temperature or at 37°C at sterile conditions for prolonged periods of time (up to 4 wk). (b) Treatment with SDS and DTT; isolated thyroid globules were heated to 100°C for 5–60 min in 100 μl of 10% glycerol containing 10 mM DTT, 1% SDS in 0.12 M Tris-HCl buffer, pH 6.8. (c) Treatment with urea; isolated TG globules were suspended in 6 M urea and heated for 60 min to 90°C. (d) Trypsin digestion; globules were suspended in bovine pancreas trypsin (0.1 mg/ml) in phosphate buffered saline, pH 7.4, at 37°C. The digestion was monitored microscopically and ended after 1 h. (e) Acid hydrolysis; globules were suspended in 6 M HCl and heated to 110°C for 20 h.

Mechanical Disruption and Fractionated Chemical Dispersion of Thyroid Globules

Isolated globules collected in Eppendorf microvessels formed small translucent $\sim 10\text{-}\mu\text{l}$ pellets which were resuspended in 25 μl Tris-HCl buffer, pH 7.2, containing protease inhibitors as described above. Homogenization was performed at 4°C using a Teflon Micropistille Eppendorf tightly fitting into the bottom of the microvessel. The homogenate was centrifuged for 30 s in a microfuge (Beckman Instruments Inc.) and the supernatant I (containing "loosely incorporated TG") used for gel electrophoretic analysis. The pellet was boiled for 5 min in 100 μl of 10 mM DTT, 1% SDS, 0.12 M Tris-HCl buffer, pH 6.8, in 10% glycerol and centrifuged for 30 s in a microfuge. The supernatant II contained "TG bound by disulfide bridges." Protein in supernatants I and II was determined (see below) and both supernatants were used for gel electrophoretic analysis and immunoblotting (see below). The final pellet represented the insoluble residue (containing "TG interconnected by covalent bonds other than disulfide bridges"). To show the presence of TG in insoluble residues, the pellet was extensively washed in PBS and TG was visualized by immunofluorescence and immunogold procedures (see below). Since the usual protein assays were not applicable for the insoluble residue was hydrolyzed under nitrogen in 6 N HCl at 110°C for 20 h. Soluble bovine TG, isolated as described above, was used as a standard and treated in the same way. The hydrolysates were neutralized and total free amino groups were determined using the colorimetric ninhydrin assay (38).

Amino Acid Analysis

300 μg of highly purified soluble TG and 100 μg of isolated thyroid globules were subjected to acid hydrolysis (see above). Amino acid analysis was performed by Drs. G. Multhaup and K. Beyreuther, Laboratory for Molecular Neuropathology, University of Heidelberg (Germany) using an ABI-analyzer (Model 420A; Applied Biosystems, Inc., Foster City, CA) and applying the PITC technique.

Protein Assays

Protein was determined either according to Bradford (4) with the modification of Sedmark and Grossberg (44) or according to the amidoblack microassay of Heil and Zillig (22). Soluble TG isolated according to the flow chart in Fig. 1 was used as a standard.

Statistical Analysis

The mean and the 95% normal range were computed for the percent values of protein in the thyroid globule subfractions and for the number of iodine atoms per TG subunit. The 95% normal range defines the range where one expects to find 95 values out of 100 independent experiments (34, 39). Due to the logarithmic transformation used for computation (1, 34) the 95% normal range is asymmetrically distributed around medium values.

Preparation of Anti-TG Antisera

Soluble bovine or porcine TG was isolated by ammonium sulfate precipitation as outlined in Fig. 1 and further purified chromatographically using an FPLC-system (Pharmacia GmbH, Freiburg, Germany). The highly purified TG was used to raise rabbit polyclonal antibodies following standard procedures. The initial injection of 200 μg TG was administered in complete Freund's adjuvans followed by two injections of 100 μg TG in incomplete Freund's adjuvans given in 10-d intervals. Animals were bled 2 wk after the third injection, and serum samples were screened for the presence of anti-TG antibodies by the use of immunoblots after SDS-PAGE (see below) of bovine thyroid homogenates.

SDS-PAGE and Immunoblotting

Samples of dissolved thyroid globules were run on a gradient of 5–18% according to the method of Laemmli (32) in a horizontal gel electrophoretic apparatus. Standard molecular mass markers for Coomassie-stained gels were 200 (myosin), 116 (β -galactosidase), 97 (phosphorylase B), 66 (BSA), 45 (ovalbumin), and 31 (carbonic anhydrase) kD. In addition, we used bovine TG (preparation from our laboratory) as a marker (330 kD) for the TG subunit. Staining of gels was performed with Coomassie Blue or by the silver technique (27). Western blotting for the detection of TG was performed according to the method of Towbin et al. (50) using rabbit antiserum against bovine TG and goat anti-rabbit antibody labeled with alkaline phosphatase.

Immunolabeling for Light and Electron Microscopy

Thyroid globules isolated as described above were centrifuged in Eppendorf microtubes (Microfuge E, Beckman Instruments Inc.) 3 min at room temperature. The pellet was fixed in 3% formaldehyde in PBS for 1 h at room temperature, washed in distilled water, resuspended in 500 μl 10% gelatin (45°C) and centrifuged as described above. The gelatin containing the pelleted thyroid globules was applied to immunofluorescence visualization of TG.

0.5- μm -thick frozen sections were prepared with a cryomicrotome (UltraCut E, C. Reichert AG, Wien, Austria) with glass knives and mounted on poly-L-lysine-coated glass slides. The mounted sections were incubated with 0.1% BSA in PBS (10 min at room temperature), washed and incubated with rabbit antiserum raised against bovine TG (1 h at 37°C). For the visualization of possible cellular remnants (e.g., microvilli or nuclei), anti-actin antibodies and nuclear staining with bis benzimide H 33258 (28) were used. After washing in PBS sections were incubated with FITC-labeled goat anti-rabbit IgG (1 h at 37°C), washed in PBS and prepared for light microscopy by the use of phenylenediamine. Sections were examined in an Axiophot light microscope (Carl Zeiss, Inc., Oberkochen, Germany).

For electron microscope observations fractions of thyroid globules fixed in 3% formaldehyde and embedded in gelatin were dehydrated in graded ethanol at progressively lower temperatures and embedded in Lowicryl K4M (6). Thin sections (80–100 nm) were prepared with diamond knives, mounted on nickel grids coated with parlodion and carbon, pretreated with 1% ovalbumin in PBS (overnight at 4°C) and incubated with rabbit antiserum raised against bovine TG (90 min at room temperature). After washing with 0.1% ovalbumin in PBS the sections were incubated (2 h at room temperature) with goat anti-rabbit IgG complexed to 17-nm gold particles prepared according to the procedure of Frens (17) or Slot and Geuze (46) and containing 1% ovalbumin in PBS. Before use the IgG-gold complexes were freed of aggregates by centrifugation (10 s in a microfuge; Beckman Instruments Inc.). After washing, the labeled sections were stained with 1% uranylacetate and examined in a Philips 301 electron microscope (Philips GmbH, Unternehmensbereich Electronic, Kassel, Germany).

Scanning EM

Freshly isolated thyroid globules were spread on coverslips coated with poly-L-lysine. The globules became immediately adherent and were fixed in 2% glutaraldehyde at room temperature in 0.1 M cacodylate buffer, pH 7.6, and postfixed in 1% unbuffered osmium tetroxide (2 h at 4°C). After dehydration in graded series of acetone, the globules were critical point dried (Critical Point dryer CPD 010; Balzers Union Ltd., Lichtenstein) using acetone and oil-free carbon dioxide as described by Porter et al. (40), sputter coated with gold (Eiko sputter coater IB3, Mito, Japan) and viewed in a scanning electron microscope, using the Cambridge Stereoscan S200 (Leica GmbH, Bensheim, Germany) or the Philips XL20 (Philips Industrial and Electro-Acoustic Systems Division, Eindhoven, Holland).

For scanning EM (SEM) of tissue and cell surface characteristics, 1-mm fragments of thyroid tissue were rinsed extensively in MEM for 1 h at 4°C, to remove the luminal content from thyroid follicles opened during the preparation of tissue fragments. The fragments were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, 6 h at room temperature, postfixed in 1% unbuffered osmium tetroxide (1 h at 4°C), dehydrated in graded series of acetone and critical point dried as described above. The dried tissue fragments were mounted on SEM studs and sputter coated as above.

Soluble TG was precipitated by ammonium sulfate (Fig. 1), pelleted, fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, 6 h at room temperature and dialyzed against water. The pellet was then prepared for SEM as described for tissue samples.

Discontinuous Percoll Gradient Centrifugation

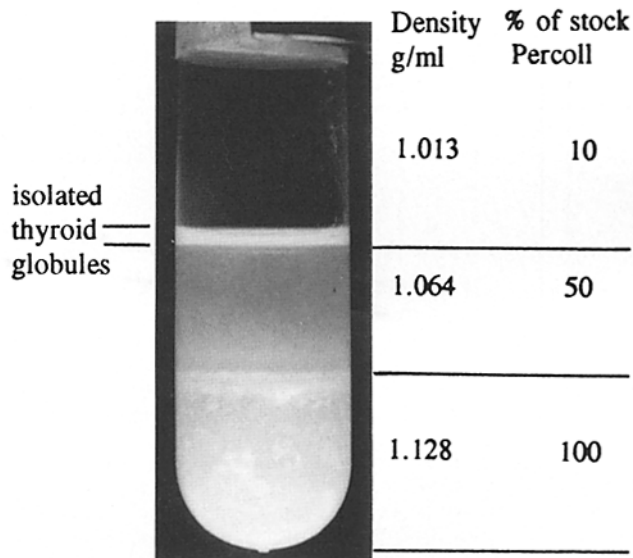


Figure 2. Purification of crude globule fractions by discontinuous Percoll gradient centrifugation. The crude fraction (see Fig. 1) was laid upon a step gradient consisting of undiluted, 50 and 10% Percoll in 0.15 M NaCl. After centrifugation (2 h at 15,000 g) the globules were found at the interface between 10 and 50% Percoll. Collagen and cell fragments migrated to higher gradient densities.

X-ray Microanalysis

Soluble TG and isolated thyroid globules were studied by x-ray microanalysis. For this purpose, bovine tissue and isolated thyroid globules were prepared for SEM as described above, except that the coating of specimen was performed exclusively with carbon. The preparations were analyzed at 20 kV and at a tilt of zero in a Philips XL-30 scanning electron microscope (Philips Industrial and Electro-acoustic Systems Division) supplied with an EDAX x-ray microanalysis system. According to the bulk character of our preparations the correction factors C_Z , C_A , and C_F were taken into account during calculation. The sulfur content of bovine TG (148 sulfur atoms per TG subunit) is known from the primary structure of bovine TG and its cysteine and methionine content (37) and the posttranslational sulfation of TG (25, 26). This provided the basis for calculating the iodine content per TG molecule. For this purpose, the $K\alpha$ -line of sulfur (2.307 keV) and the $L\alpha$ -line of iodine (3.938 keV) (2, 7) from the energy dispersive spectrum were used for calculating the percent mass relation of iodine to sulfur and the number of sulfur atoms per TG subunit.

Results

This report describes the isolation and characterization of thyroid globules from bovine thyroid tissue and their separation from soluble TG (Figs. 1 and 2). The morphological and analytical studies indicate that the isolated globules represented a previously unknown form of TG in the follicle lumen. We, therefore, use the term "isolated thyroid globules" throughout this report. Compositionally, this term corresponds primarily to "insoluble TG."

It should be pointed out that insoluble TG in the form of TG globules is distinct from "psammoma bodies" which are also insoluble but which represent the endstages of extrafollicular calcification processes (29).

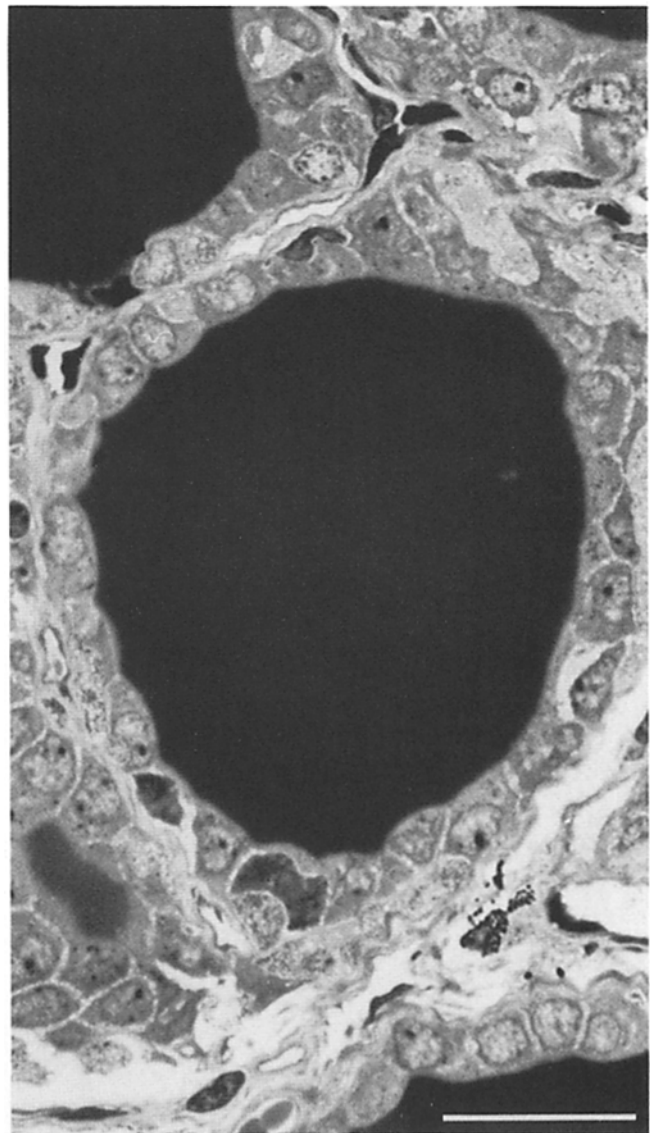


Figure 3. Light micrograph of bovine thyroid follicles. PAS-staining. The follicle lumina were enclosed by a tight monolayer of thyrocytes and completely filled with homogeneously stained TG. Convex or concave apical cell surfaces shaped in part the surface of the follicle content. This report shows that a considerable proportion of the luminal content was covalently crosslinked and almost insoluble. The impressions of the apical cell surfaces remained visible after isolation of thyroid globules (i.e., luminal content). Bar, 25 μ m.

Morphological Characteristics of Luminal TG In Situ

The lumina of bovine thyroid follicles contained densely packed TG which stained homogeneously with the PAS-technique for visualization of carbohydrates (Fig. 3). The luminal content was completely surrounded by a tight monolayer of thyroid follicle cells. Their apical cell surface exhibited convex or concave curvatures thereby shaping the circumference of the luminal content.

Yield of Thyroid Globule Preparations and Purification from Crude Fractions

Homogenization of 10 g bovine thyroid tissue and separation of TG according to the flow chart in Fig. 1 yielded ~50-70

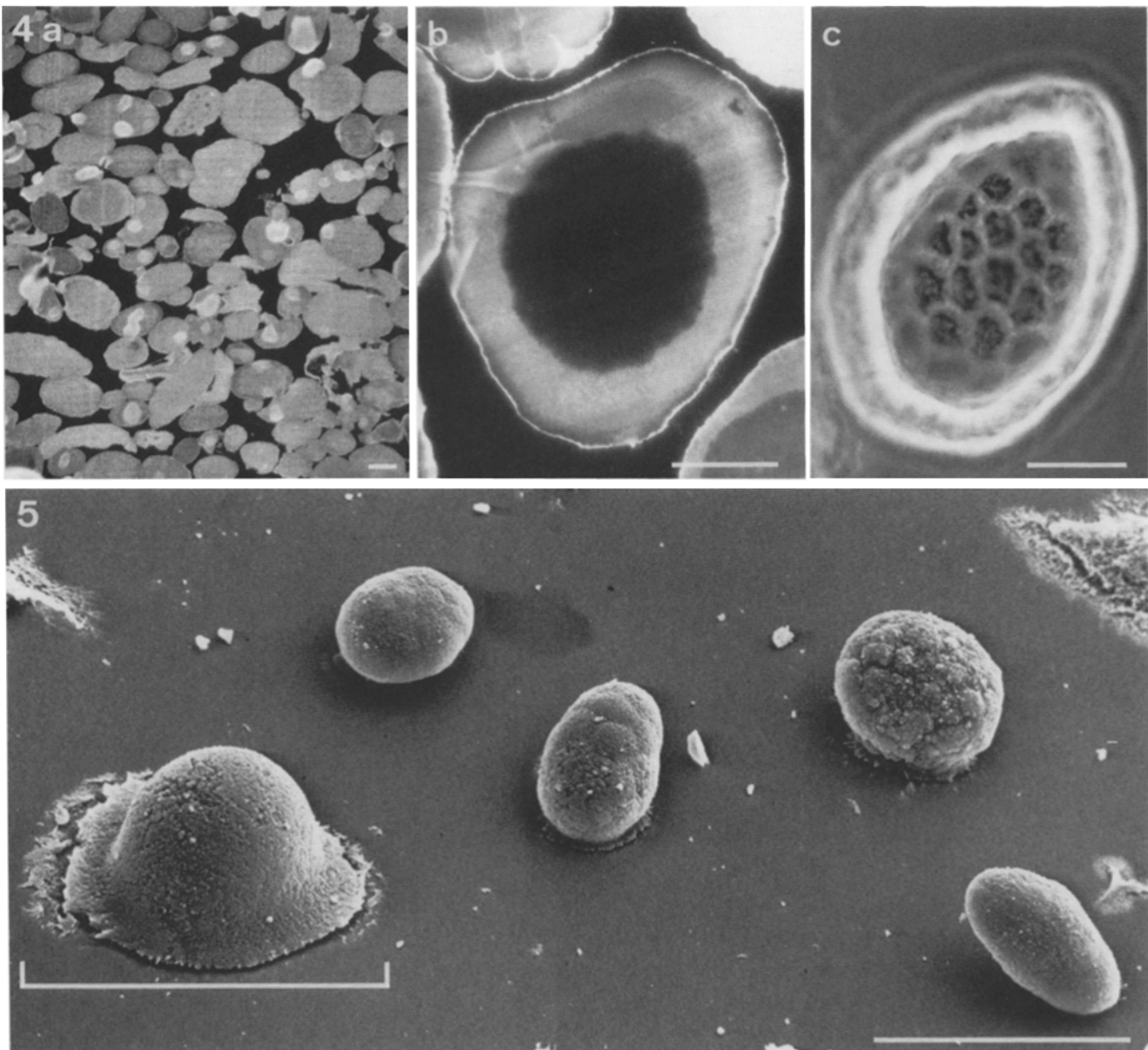


Figure 4 and 5. Fractions of isolated thyroid globules. (Fig. 4) Immunofluorescence microscope detection of TG in 1- μ m-thick frozen sections from globule pellets. Note the heterogeneity in intensity of staining and in shape of isolated globules (a). Occasionally the globule matrix was composed of concentric layers of distinct immunoreactivity (b). By phase contrast most isolated globules showed a characteristic surface structure (c). Bar, 20 μ m. (Fig. 5) Scanning electron micrographs revealed that grooves subdivide the globule surface into hexagonal or pentagonal fields. Occasionally, the globules were less rigid as they appeared to 'melt' on the glass support (bracket). Bar, 50 μ m.

mg soluble TG and 5–10 mg of isolated thyroid globules (crude fraction). These results did not, however, reflect the real ratio between soluble TG and isolated globules, because an unknown but certainly large proportion of globules was lost during the isolation procedure due to adherence of globules to centrifuge tubes and to pipettes. Use of suitable centrifuge tubes and siliconization of all glassware (see Materials and Methods) did reduce but not avoid this loss of globules during isolation. The yield of thyroid globules from porcine thyroid glands was much lower and estimated to be <10% of the yield from bovine tissue.

Another source for the loss of isolated globules was observed after the first step of centrifugation (60 sec at 100 g) which resulted in the sedimentation of many but not all globules. Hence, an unknown proportion of isolated globules was

still present in the supernatant used for the purification of soluble TG. Here they sedimented after the first step of precipitation with ammonium sulfate. We conclude from these observations that the structurally organized TG which remained intact during isolation was more common in the bovine thyroid tissue than the proportion of isolated globules in the crude preparation might suggest.

Crude fractions prepared according to the flow chart in Fig. 1 contained primarily globules which were, however, contaminated by collagen fibers and cellular remnants. Further purification was achieved by the use of Percoll gradient centrifugation. The globules concentrated at the interface between 10 and 50% Percoll whereas collagen and cellular remnants migrated to various depths of the gradient. FITC labeling of crude globule preparations allowed to locate

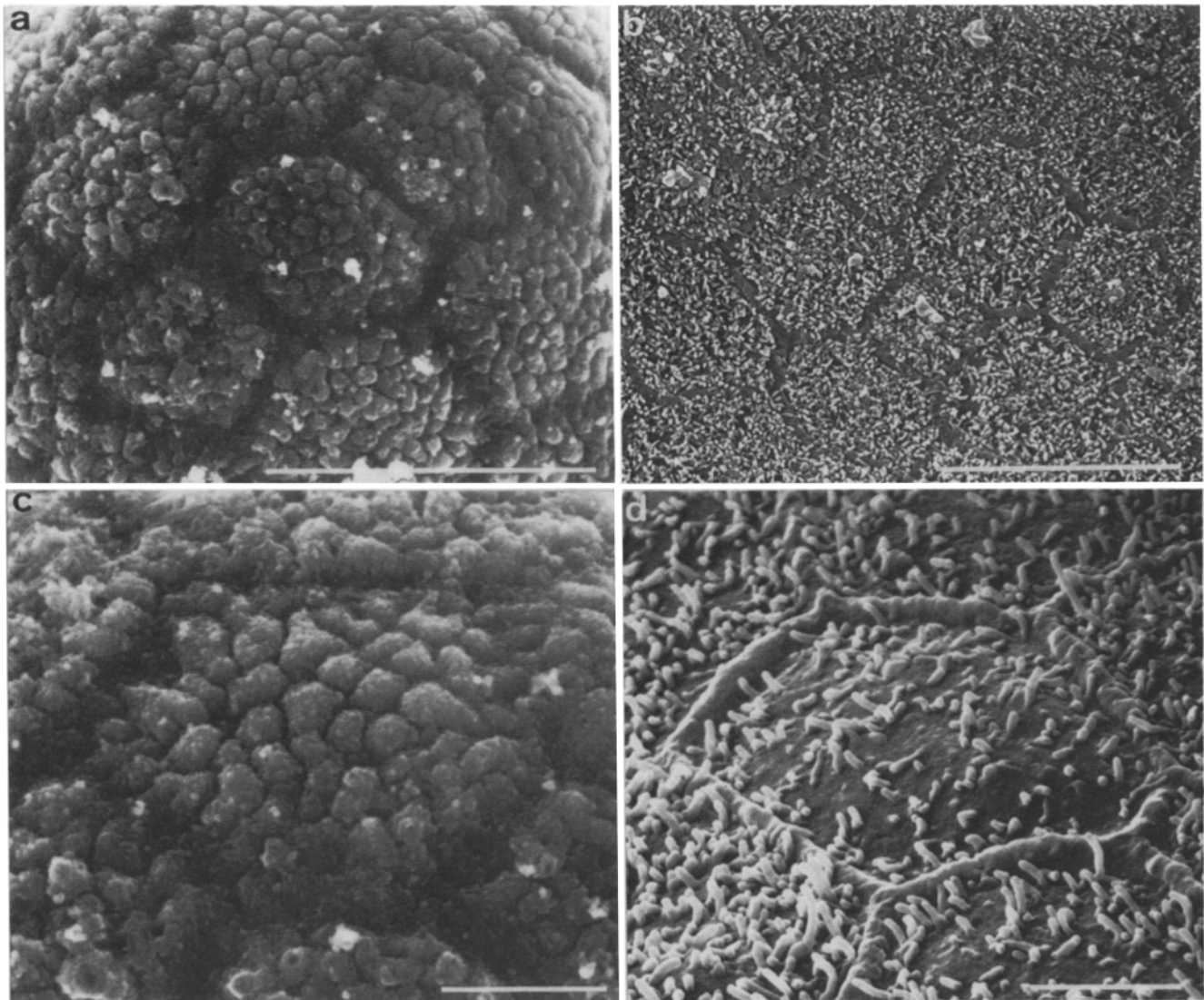


Figure 6. Scanning electron micrographs of isolated globules (*a* and *c*) and of luminal surface characteristics of thyroid follicle cells (*b* and *d*). The hexagonally or pentagonally shaped areas (*a* and *c*) appeared to correspond to the structure of the luminal cell surfaces (*b* and *d*). Ridges at the border of the apical plasma membranes (*d*) appear to have formed the grooves on the globule surfaces (*c*). The surfaces also showed a characteristic substructure of ~ 250 -nm particles. This surface pattern remained unchanged even after 30 min boiling of isolated thyroid globules in SDS and DTT (not shown). Bars: (*a* and *b*) $10\ \mu\text{m}$; (*c* and *d*) $2.5\ \mu\text{m}$.

the globules after centrifugation by the use of a UV lamp (Fig. 2).

Morphological Characteristics of Isolated Thyroid Globules

Fractions of thyroid globules isolated according to the flow chart in Fig. 1 and further purified by Percoll gradient centrifugation (Fig. 2) were pelleted and analyzed by fluorescence microscopy after immunocytochemical visualization of TG. The result (Fig. 4 *a*) shows that the fractions consisted of heteromorphous but mainly spherical or ovoid shaped globular bodies. All globules in a given fraction showed positive staining for TG and impurities were not detectable. Most isolated globules stained homogeneously. A few globules in each fraction showed concentric layers of different immunocytochemical staining intensity (Fig. 4 *b*). By phase contrast, a peculiar pattern on the surface of most globules

was detectable (Fig. 4 *c*). This pattern might suggest the presence of remnants of epithelial cells. Fluorescence microscope visualization of nuclear DNA or of actin and electron microscope cross sections showed, however, that cells or cellular remnants were absent from fractions of isolated globules and that the peculiar surface structure was an inherent characteristic of the globules.

Fractions of isolated thyroid globules adsorbed on coverslips coated with poly-L-lysine were subjected to scanning electron microscope analysis. Fig. 5 confirms that most globules were spherical or oval in shape. Some globules appeared to be less rigid in structure and to "melt" on the coverslip surface (*bracket* in Fig. 5). The surface of such globules was smooth as compared with the surfaces of rigid globules which always showed the peculiar surface architecture observed already by light microscopy. Hexagonally or pentagonally shaped areas (of $\sim 5\ \mu\text{m}$ in diameter) were surrounded by grooves. This characteristic surface pattern was

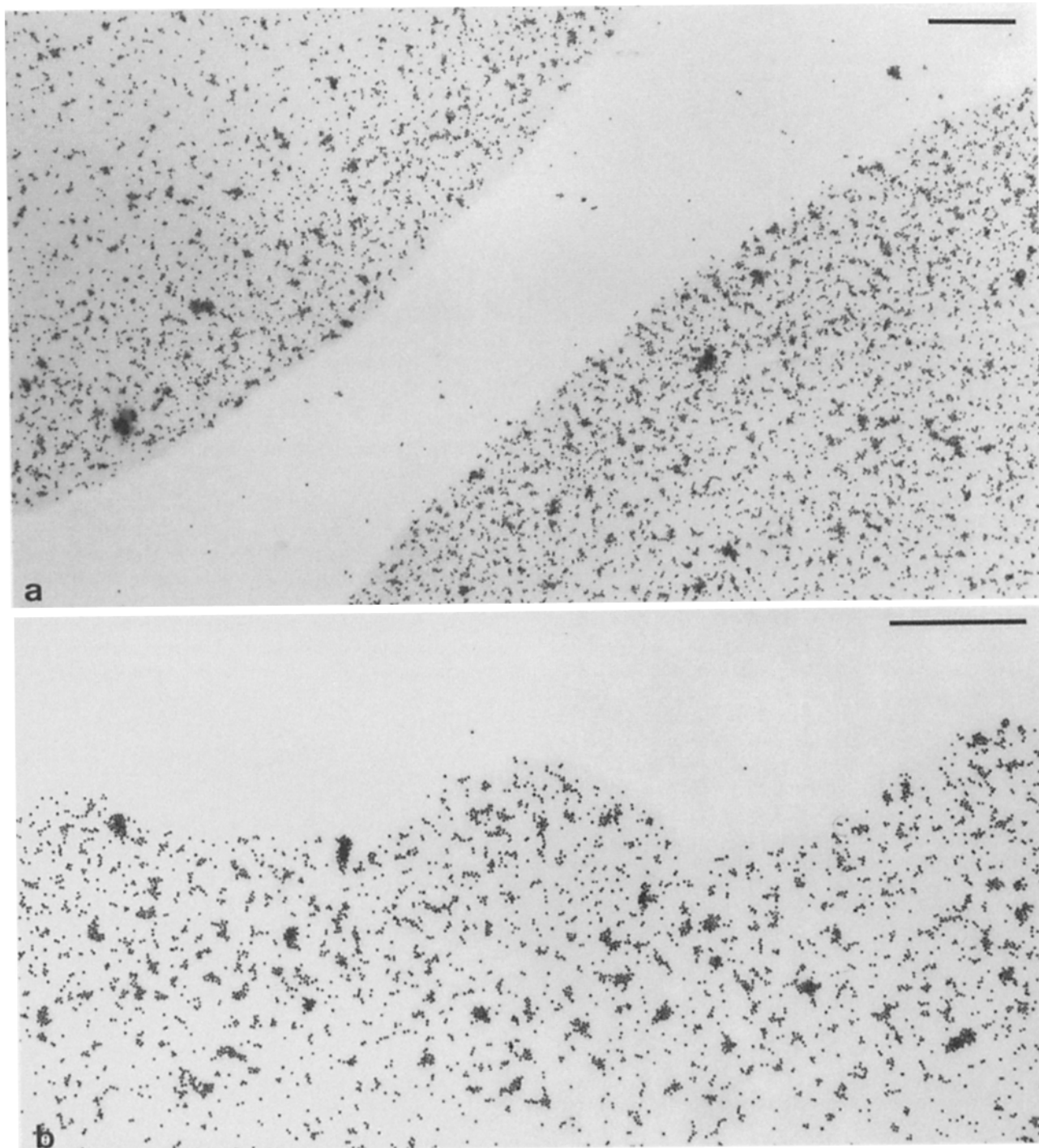


Figure 7. Portions of isolated thyroid globules embedded in Lowicryl. Immunogold detection of TG. Part of the immunogold staining occurred in clusters possibly located over the 250-nm particles (see Fig. 6). A small proportion of globules was smooth surfaced (*a*) and might correspond to the less rigid globules (see Fig. 5). In most cases, however, the globule surfaces were shaped by concave (*b*) or convex (see Fig. 5) indentations. Note the absence of cells or cellular remnants on the globule surfaces. Bars, 1 μm .

resistant against various attempts to dissolve isolated thyroid globules, including the boiling in SDS and DTT for up to 30 min (not shown). A comparison of higher magnifications of the globule surface with the apical surfaces of thyrocytes showed that the grooves (Fig. 6, *a* and *c*) corresponded to ridges in the periphery of apical cell surfaces located close to the tight junctions (Fig. 6, *b* and *d*). In addition, the glob-

ule matrix was apparently composed of aggregates measuring ~ 250 nm in diameter (Figs. 6 and 7).

Thin sections from pellets of isolated globules embedded in Lowicryl were stained for the visualization of TG using the immunogold procedure (Fig. 7, *a* and *b*). Intense and specific labeling throughout the globule matrix was observed over all globules. In addition, all globules showed also

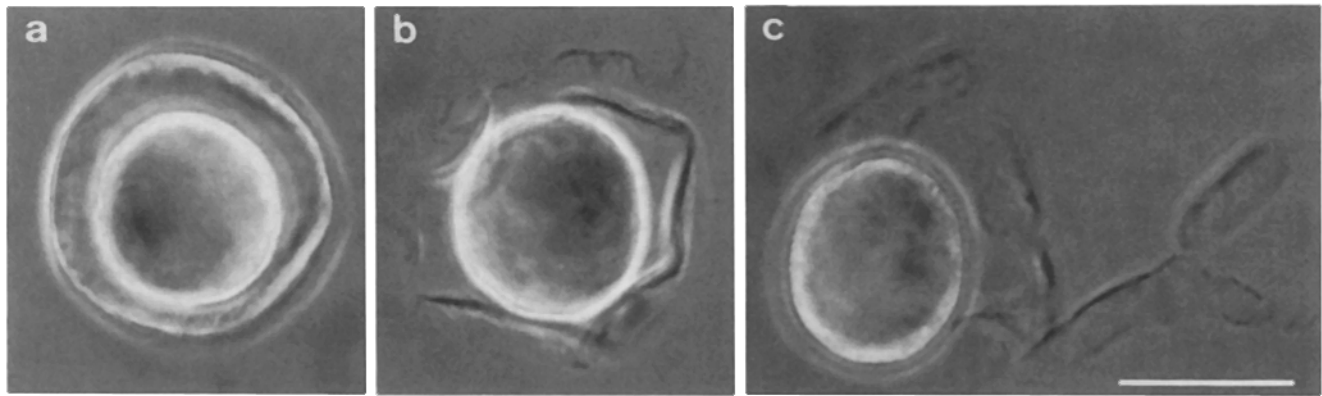


Figure 8. Sequential removal of concentric layers by trypsin digestion of isolated thyroid globules. An isolated thyroid globule (a) was exposed to 0.1 mg/ml trypsin. After 4 min the outer layer ruptured (b), and after 9 min of digestion detached from the central core (c). After 30 min, no macromolecular remnants of the globule were detectable. Bar, 50 μ m.

clusters of 10–20 gold particles. Gold particle clusters were observed despite the removal of gold aggregates before immunocytochemical detection of TG. Over the gelatin surrounding the globules no or only very few gold particles were found. The surface architecture with grooves observed by SEM was also recognized in thin sections (Fig. 7 b).

Protein Nature of Isolated Globules

Most attempts to disperse or dissolve isolated globules failed. Isolated globules were resistant to freezing and thawing, to prolonged storage in water or in 10 mM EDTA, and to the treatment with SDS and DTT (5–60 min at 100°C). Although small amounts of protein were released upon treatment with SDS and DTT, the globules and their peculiar surface architecture remained intact (not shown). Suspension in water or in PBS did not cause swelling or shrinking, indicating that the globules were osmotically inert. Isolated globules were completely dissolved by digestion with trypsin (1 h, 37°C) or by hydrolysis with 6 M HCl (20 h, 110°C), thus showing their proteinaceous composition. The first steps of trypsin digestion were characterized by the sequential removal of concentric layers (Fig. 8). Their array showed similarities to the concentric layers of distinct immunofluorescence intensity as visualized by the reaction with anti-TG antibodies (Fig. 4 b).

Table I. Proportion of Covalently Interconnected TG in Isolated Thyroid Globules

States of incorporation	Percent of total globule protein	
	Medium values	95% normal range
Loosely incorporated TG (supernatant I)	3	0.5–19
TG bound by disulfide bridges (supernatant II)	22	16–28
TG interconnected by covalent bonds other than disulfide bridges (insoluble residue)	75	65–86

Medium values and 95% normal range limits of protein in supernatant I, supernatant II, and in the insoluble residue from four independent experiments. The amount of total globule protein ranged between 0.234 and 1.887 mg.

Proportion of Covalently Cross-linked TG in Isolated Globules

The immunocytochemical observations had shown before that isolated globules contain high concentrations of TG (Figs. 4 and 7). Analyses by PAGE and by immunoblot were, however, impeded by the insolubility of globules and their resistance against the treatment with urea or with SDS and DTT. We, therefore, used a procedure of mechanical disruption and fractionated dispersion of thyroid globules. Upon homogenization of globules a soluble proportion of TG was

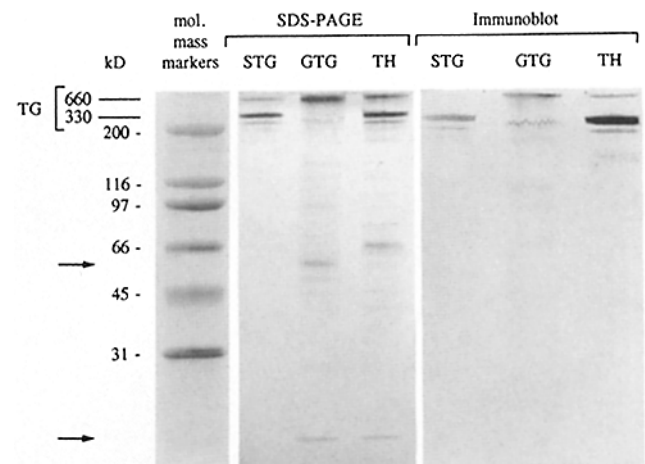


Figure 9. PAGE (left) and immunoblot (right) analyses of thyroid tissue homogenates (TH), globule TG interconnected by disulfide bridges (GTG), and of loosely incorporated (soluble) TG (STG). STG and GTG derived from the supernatants I and II, respectively (see Table I). STG from globules showed the same electrophoretic mobility as the soluble TG isolated according to the flow chart in Fig. 1. Molecular mass standards are indicated on the left. Note that STG was dissociable into 330-kD subunits whereas GTG was primarily detectable as the 660-kD band indicating the presence of covalent nondisulfide bridges. Tissue homogenates (TH) contained both, the 660-kD TG and the 330-kD subunit. Two protein bands (at 60 and 12 kD) from the GTG fraction did not show immunoreactivity with anti-TG antibodies (arrows). The 60-kD band appeared immunoreactive with antitransglutaminase antibodies (not shown), whereas the 12-kD band which was also observed in tissue homogenates (TH), remained unidentified.

Table II. Amino Acid Composition of Soluble and Insoluble TG

Amino acid	Expected values	Soluble TG	Insoluble TG
	mol %	mol %	mol %
Asp/Asn	6.97	8.30	9.79
Glu/Gln	12.38	12.15	13.51
Ser	9.53	9.56	8.06
Gly	7.71	8.30	8.76
His	1.13	1.20	1.44
Arg	6.58	6.64	6.21
Thr	4.66	5.54	5.41
Ala	8.66	8.75	8.51
Pro	6.98	7.75	6.53
Tyr	2.62	2.19	2.04
Val	6.04	5.92	6.46
Met	0.87	0.89	0.54
Cys	4.44	2.20	0.74
Ile	2.62	2.43	2.97
Leu	9.49	10.32	10.02
Phe	5.13	5.40	5.05
Lys	2.69	2.48	3.96

Amino acid analysis from acid hydrolysates of insoluble TG and of soluble TG. The data are medium values from three different determinations and given in mol %. Expected values are derived from SWISS-PROT (Vers. 22.0, European Molecular Biology Laboratory, October 1989). Methodologically caused differences in the amino acid composition are observed in the values for cysteine, methionine, and lysine (see Results). Due to deamination, the data for glutamine or asparagine are summarized in the values for Gln/Glu or Asp/Asn, respectively.

released which appeared to be loosely incorporated into the globule matrix. The pellet of the homogenized and washed globules was then heated with SDS and DTT to remove TG interconnected by disulfide bridges. The remaining insoluble pellet represented the proportion of TG interconnected by nondisulfide bridges. The protein content of this globule portion was determined, after hydrolysis, using the ninhydrin assay. The results are summarized in Table I which shows that most of the globule TG was covalently cross-linked and only a small proportion (~3%) was present as soluble TG.

The identification of TG in the three globule subfractions was achieved by SDS-PAGE and immunoblotting (*supernatants I and II* in Table I) or by immunocytochemistry (*insoluble residue* in Table I). Electrophoretic analysis showed that the TG from supernatants I was detectable primarily as the 330-kD TG subunit whereas the TG from supernatant II was not completely dissociable and, hence, detected mainly as 19-S TG (660 kD) (Fig. 9), indicating that TG from supernatant II was cross-linked by nondisulfide bridges. Such cross-links between the subunits have been described before as being characteristic for highly iodinated TG species (9). In contrast, soluble TG isolated from thyroid tissue according to the flow chart in Fig. 1 was dissociable into 12-S (330 kD) subunits and found to resemble the TG band from supernatant I. Both species, the 330-kD subunit and the complete 660 kD molecule, were detectable in the thyroid tissue homogenates (Fig. 9) indicating the coexistence of both TG species in bovine thyroid glands.

In addition to the 330- and the 660-kD TG, two protein bands at 60 and 12 kD became detectable in thyroid globule extracts (Fig. 9). Both proteins were not immunolabeled using a polyclonal anti-TG antiserum and may represent specific constituents of the luminal content not related to TG.

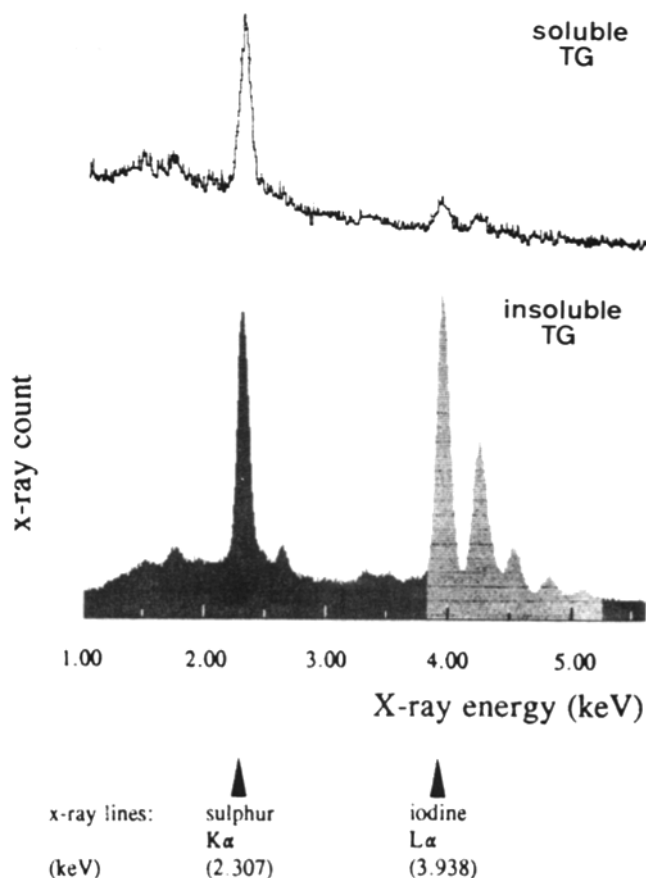


Figure 10. X-ray microanalysis of isolated thyroid globules (*insoluble TG*) and of soluble TG. Note the prominent iodine peaks (*light area*) of insoluble TG. The $K\alpha$ line of sulfur and the $L\alpha$ line of iodine were used to calculate the mass relation of both elements and to determine the iodine content of insoluble TG (thyroid globules) and of soluble TG (summarized in Table II).

It is unknown, whether the two proteins were involved in the cross-linking of TG in insoluble thyroid globules.

Owing to the stability of covalent cross-links, the insoluble residue of globule homogenates was not applicable for SDS-PAGE and immunoblotting because of the stable covalent cross-links. Hence, immunofluorescence and immunogold procedures were applied to show the presence of TG in the insoluble residue. After extensive washing to remove SDS and DTT the insoluble residue was resuspended and applied to the immuno light- and electronmicroscope detection of TG (see above). All fragments of the insoluble residue from globule homogenates showed intensive immunofluorescent staining for TG (results not shown). Amino acid analyses showed a close compositional similarity between isolated thyroid globules and soluble TG (Table II). Hence, we conclude that the globules consisted primarily of TG.

Iodine Content of Isolated Thyroid Globules

TG isolated from supernatant II was not dissociable into 12-S subunits (Fig. 9), suggesting that the TG in globules contained a particularly high iodine content. We, therefore, analyzed the iodine content in globules using x-ray microanalysis. Fig. 10 shows the characteristic spectrum of soluble TG and of an isolated TG globule (*insoluble TG*). Deter-

Table III. Iodine Content of Bovine Thyroglobulin

	Mass in percent of total mol wt	Atoms iodine per TG subunit (330) kD)		
		Mean	SD	95% normal range
Soluble TG	0.5 ± 0.05 (SD)	12	2.8	7-18
Insoluble TG	2 ± 0.7 (SD)	53	17.4	19-84

The data are derived from x-ray microanalyses (see Fig. 10) of soluble TG (50 determinations) and of insoluble TG (isolated thyroid globules; 22 determinations). Iodine concentrations between 0.2 and 1.0% are considered normal (31). The results show that the number of iodine atoms per TG subunit in isolated globules exceeds normal values.

minations in 22 globule (*insoluble TG*) preparations showed that the mean iodine content was about fourfold higher than the iodine content of soluble TG (Table III) and about 2-10-fold higher than the mean iodine content of soluble TG considered to be normal (31).

Discussion

In this report we show that TG can be isolated from bovine thyroid glands in at least two distinct forms of aggregation: (a) soluble TG which occurs as TG subunits, as monomeric, dimeric, or trimeric molecules or as small dissociable aggregates; and (b) solid TG in almost insoluble form which occurs as large globules resistant against the usual protein dissociation procedures. We discuss here the structural and compositional characteristics of isolated thyroid globules and their biological implications with respect to central functions of the thyroid such as storage, sorting, and endocytosis of TG.

Morphology of Isolated Thyroid Globules

Isolated thyroid globules are highly variable in size and shape. Fractions of isolated thyroid globules contain spherical elongated structures with diameters ranging from 20-120 μm . Larger globules are observed but usually lost during the isolation procedure. Thyroid globules are not a curiosity restricted to bovine thyroid glands because they can also be isolated from the tissue of other mammalian species such as porcine thyroids. All globules share basic characteristics in structure and composition.

The surfaces of most thyroid globules exhibit a unique architecture with hexagonally or pentagonally arranged grooves enclosing fields which correspond in size and shape to the apical cell surface areas of thyrocytes. We, therefore, believe that the cobblestone-like surface pattern of isolated globules results from the preserved impressions of the luminal surfaces of thyrocytes which surrounded the luminal content before tissue homogenization. Apparently, the apical plasma membranes of thyrocytes shape the surface of globules. Indeed, the close proximity between the globule and the apical surfaces of thyrocytes is one of the prominent histological characteristics of thyroid tissue (see Figs. 3 and 4 c). However, microvilli and other constituents of the apical cell surface of thyrocytes such as pseudopods are not enclosed during globule formation. We conclude this from the failure to detect actin on the surface of isolated colloids by immunofluorescence microscopy or by gel electrophoresis.

Possible Nature of TG Cross-linking

Obviously, isolated thyroid globules are proteinaceous in composition as they are completely degraded by trypsin without detectable macromolecular remnants. In some globules the first steps of trypsin digestion are characterized by the sequential removal of distinct layers. Their array might correspond to the concentric layers of distinct immunofluorescence intensity as visualized by the reaction with anti-TG antibodies. This concentric heterogeneity reflects differences in composition or in structural organization of the globules and it suggests that TG export and addition to the growing globule occur in a discontinuous fashion. Isolated globules are, however, extremely resistant to protein dispersion procedures. Surprisingly, they remain unchanged in size and morphology during heating with SDS and DTT or with urea. Therefore a procedure of homogenization and fractionated chemical dispersion was developed which allows us to analyze isolated globules.

As SDS-PAGE analyses have shown in isolated thyroid globules the presence of proteins other than TG (see Fig. 9), the amino acid composition of thyroid globules is closely similar but not identical to the composition of TG. However, TG is the major constituent of isolated thyroid globules as concluded from immunofluorescence and immunogold observations and from gel electrophoretic analyses. Most TG in globules is covalently cross-linked ($\sim 97\%$). Part of the cross-links consists of disulfide bridges ($\sim 22\%$) whereas the majority is found as covalent nondisulfide bonds ($\sim 75\%$). Their presence is concluded from the resistance of isolated globules to protein dissociation procedures involving reducing agents. Only $\sim 3\%$ of TG is present without being cross-linked to the globule matrix. This soluble fraction is apparently loosely incorporated as it can be freed merely by homogenization of isolated globules.

The high degree of cross-linking in isolated globules raises questions as to the mechanisms of insoluble TG formation *in vivo*. A model which might explain this process on the basis of this report is shown in Fig. 11. According to this model the formation of insoluble TG occurs by sequential steps of aggregation and polymerization. These steps include the formation of disulfide bridges, covalent nondisulfide bonds and Ca^{2+} -mediated aggregates. In highly iodinated TG covalent nondisulfide cross-linking has been observed before between the 12-S subunits of 19-S TG (9, 10). We therefore analyzed the iodine content of globules. The data obtained by x-ray microanalysis show that with $\sim 2\%$ the iodine content in the TG in globules is extremely high inasmuch as values between 0.2 and 1.0% are considered to be normal (9). Hence, assuming that globules consist almost entirely of TG we can calculate that insoluble TG contains an average of ~ 55 iodine atoms per 12-S TG subunit as compared with ~ 12 iodine atoms measured in soluble TG.

Covalent nondisulfide cross-links are known to occur in a variety of proteins. In collagen and elastin intermolecular cross-links are formed between modified lysine side chains. For this purpose, certain lysine and hydroxylysine residues are deaminated by the action of the extracellularly located lysyl oxidase, resulting in the formation of highly reactive aldehyde groups which spontaneously react to form covalent bonds with each other or with other lysine or hydroxylysine residues (13). Covalent cross-linking may also occur by the formation of ϵ -(γ -glutamyl) lysine bonds by the action of

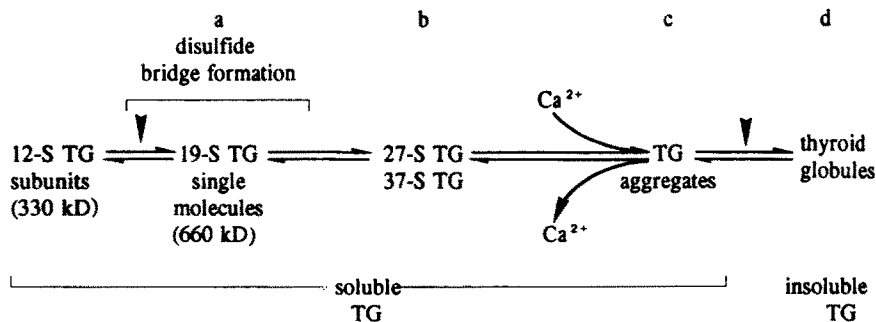


Figure 11. Model of TG association and dissociation and of insoluble TG formation. Extracellular TG may occur in soluble form (a-c) or, as shown in this report, be present as insoluble thyroid globules (d). According to this model, insoluble TG formation occurs by stepwise polymerization of TG through the formation of disulfide bridges, of Ca^{2+} -mediated aggregates and, most important, of covalent nondisulfide bonds (arrowheads). (a) 19-S TG is composed of two identical 12-S subunits (42). Dissociation of 19-S TG into 12-S TG depends on the de-

gree of iodination as noniodinated TG is completely convertible to 12-S TG by reducing agents. Highly iodinated 19-S TG is not completely dissociable into 12-S TG as it contains one or more nondisulfide cross-links the nature of which is unknown (9). (b) Under nonreducing conditions the disulfide linked dimeric (27-S) and trimeric (37-S) TG can be observed (8). Using highly cross-linked polyacrylamide gels, five TG species have been described, four of which were progressive polymers of 19-S TG (31). (c) Aggregates of TG can be dissociated by 10 mM EDTA indicating that Ca^{2+} participates in their formation (23). (d) This report shows that thyroid globules can be isolated in an intact state. TG in isolated thyroid globules showed a high degree of covalent cross-linking. The nature of the covalent cross-links is unknown. However, this report shows that the covalent cross-linking in insoluble TG coincides with a high degree of iodination. Hence, intermolecular dityrosine bridges may be formed during iodination of TG and render the globule TG resistant to most protein dissociation procedures. It is postulated that proteolytic mechanisms exist which fragment the insoluble TG to facilitate endocytosis by thyrocytes.

transglutaminases. Such cross-links have been reported to occur in hair proteins, in the epidermis, during the formation of fibrin and of the vaginal plug in rodents (for review see 16) and in apoptotic bodies, the highly cross-linked remnants of cells after programmed cell death (14). The presence of a transglutaminase in the secretory product of thyrocytes and in isolated thyroid globules is suggested by immunofluorescence microscope and immunoblot studies (our own unpublished observations).

The nature and the intermolecular localization of the covalent cross-bridges in thyroid globules are unknown. However, the high degree of TG iodination in thyroid globules suggests that it is functionally related to the covalent cross-linking in insoluble TG. Such intermolecular bridges have been described in other systems (e.g., in sea urchin oocytes where ovoperoxidase is known to harden the fertilization membrane due to the formation of phenolic dityrosine cross-links) (15, 20). This step coincides with the iodination of the fertilization membrane proteins and results in the complete resistance of the fertilization membrane to protein-solubilizing procedures. Future studies will show whether intermolecular dityrosine bridges formed during the iodination of TG are the structural basis for the insolubility of thyroid globules.

Biological Significance of Covalently Cross-linked TG

The extracellular storage of TG is a prerequisite for maintaining constant levels of thyroid hormones (41) and it is assumed that the condensation process in the follicle lumen serves to increase the storage capacity of the thyroid. Storage of high concentrations of single TG molecules without cross-linking would result in increased osmolality. In follicle lumina containing soluble TG a mean hydrostatic pressure of ~ 365 mm Hg has been described (45). We have shown, however, that TG in the follicle lumina can occur in a highly cross-linked form. TG cross-linking may render the luminal content of thyroid follicles osmotically inert and this process appears, therefore, fundamental for the storage of excessively high concentrations of TG in the follicle lumen without osmotic consequences on the volume and the structure

of thyroid follicles. Other cells with a regulated pathway of secretion are well known for their ability to store the secretion product in osmotically inert form. This, however, occurs intracellularly as compact spherical cores within secretion granules (5).

Various reports have shown that endocytosis in thyrocytes is a selective process (8, 12) which may occur by macropinocytosis mediated by pseudopods thereby preventing the immediate uptake of freshly exported TG (12). Alternatively, it has been suggested that newly exported TG molecules are internalized and hydrolyzed first whereas previously secreted TG is stored in the follicle lumen and utilized only when the organism requires large quantities of thyroid hormones. This selectivity has been summarized in the "last come, first served-concept" (43). The extracellular compaction and storage of TG described here is in support of this hypothesis and might be the structural basis for the sorting of freshly exported soluble TG which undergoes rapid endocytosis from the covalently cross-linked storage form of TG. This mechanism of extracellular sorting by compaction which helps to discriminate between freshly exported TG and older luminal content is different from the intracellular cocondensation of various zymogens which, in exocrine secretory cells, serves the sorting of proteins exported in a regulated manner from those constitutively secreted (19, 49).

The high degree of cross-linking also has consequences on our current understanding of the mechanisms of endocytosis in thyroid follicle cells. The high concentrations of TG in the follicle lumen might suggest that TG is internalized by a process of fluid phase uptake. Macropinocytosis, a thyroid specific form of phagocytosis mediated by pseudopods, has been assumed to serve this process (12, 52, 53). However, observations with thyrocytes *in vitro* indicate that TG is taken up primarily by micropinocytosis (3, 11, 24, 54) which depends on coated vesicles (3) and is possibly mediated by a low affinity binding site for TG (33). Micropinocytosis requires the availability of small globule fragments or of single TG molecules. Hence, mechanisms must exist for the regulated dissociation or fragmentation of insoluble TG. Such mechanisms are unknown but act presumably extracellularly to facilitate micropinocytosis of TG. It has been shown in

hyperstimulated glands that luminal TG is rapidly mobilized and that the luminal content can be depleted by endocytosis within 24 h (51, 55). Knowing the rapid dissociation of the luminal content in vivo the insolubility of thyroid globules in vitro was an unexpected observation in this investigation.

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