LOCALIZATION OF THYROGLOBULIN ANTIGENICITY IN RAT THYROID SECTIONS USING ANTIBODIES LABELED WITH PEROXIDASE OR ^{125}I-RADIOIODINE

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ABSTRACT

In the hope of localizing thyroglobulin within the follicular cells of the thyroid gland, antibodies raised against rat thyroglobulin were labeled with the enzyme horseradish peroxidase or with ^{125}I-radioiodine. Sections of rat thyroids fixed in glutaraldehyde and embedded in glycol methacrylate or Araldite were placed in contact with the labeled antibodies. The sites of antibody binding were detected by diaminobenzidine staining in the case of peroxidase labeling, and radioautography in the case of ^{125}I labeling.

Peroxidase labeling revealed that the antibodies were bound by the luminal colloid of thyroid follicles and, within follicular cells, by colloid droplets, condensing vacuoles, and apical vesicles. ^{125}I labeling confirmed these findings, and revealed some binding of antibodies within Golgi saccules and rough endoplasmic reticulum. This method provided a visually less distinct distribution than peroxidase labeling, but it allowed ready quantitation of the reactions by counts of silver grains in the radioautographs. The counts revealed that the concentration of label was similar in the luminal colloid of different follicles, but that it varied within the compartments of follicular cells. A moderate concentration was detected in rough endoplasmic reticulum and Golgi saccules, whereas a high concentration was found in condensing vacuoles, apical vesicles, and in the luminal colloid. Varying amounts of label were also observed over the different types of colloid droplets, and this was attributed to various degrees of lysosomal degradation of thyroglobulin.

It is concluded that the concentration of thyroglobulin antigenicity increases during transport from the ribosomal site of synthesis to the follicular colloid, and then decreases during the digestion of colloid droplets which leads to the release of the thyroid hormone.

KEY WORDS thyroid · thyroglobulin · immunocytochemistry · immunoradioautography · immunohistological quantitation

Thyroglobulin is found only in the thyroid gland, where it constitutes more than half of the proteins present. It is a glycoprotein with a 19S sedimentation coefficient, and it is readily dissociated into 12S subunits, and, by vigorous means, into even smaller components (5, 26, 56, 60, 62). Although the biochemical information on thyroglobulin is considerable, little is known of its localization

within the follicles composing the thyroid gland.

Using a spectrophotometric method, Gersh and Caspersson (20), observed in 1940 that the follicular colloid and the colloid droplets present in follicular cells contained a substance which had absorption properties similar to those of iodinated amino acids, and was presumed to be thyroglobulin. In 1948, Leblond and Gross (37) observed that radioiodine accumulated in the colloid within material precipitated by half-saturated ammonium sulfate, and was considered to be thyroglobulin. More recently, the presence of thyroglobulin in the colloid was confirmed by immunofluorescence (17) and micropuncture analysis (28, 61).

However, the early investigations only suggested the presence of thyroglobulin in follicular cells. Indirect evidence was provided by electron microscope radioautography with labeled amino acids (6, 48) and labeled sugars (25, 69). Thyroglobulin precursors were said to first appear in the rough endoplasmic reticulum where they were presumably synthesized, to migrate to the Golgi apparatus where the carbohydrate side chains were completed, and to be transported by the apical vesicles to the colloid as fully formed thyroglobulin. According to these conclusions, many of the compartments in follicular cells should contain thyroglobulin or its precursors, but direct evidence is lacking. Therefore, immunological methods were used in the hope of locating thyroglobulin antigenicity[1] within the organelles of follicular cells.

Antibodies prepared against thyroglobulin were labeled with horseradish peroxidase or radioiodine. When peroxidase was used, the thyroid sections were exposed to the labeled antibodies, which were then stained and localized under the electron microscope. A good ultrastructural localization was obtained, but weakly stained structures were not readily distinguishable from the background, and the results did not lend themselves well to quantitation.

When the antithyroglobulin antibodies were labeled with radioiodine, the antibodies bound to the thyroid sections were detected by radioautography. This procedure allowed quantitation by counts of silver grains over the cell organelles of thyroid cells. Hence, while peroxidase labeling provided good resolution, the radioiodine labeling made it possible to measure the amount of antibodies bound by the various cell organelles.

[1] Thyroglobulin antigenicity refers to the property of the molecules which binds the antibodies produced against native thyroglobulin.

MATERIALS AND METHODS

Preparation of Labeled Antithyroglobulin Antibodies

PREPARATION AND IDENTIFICATION OF THYROGLOBULIN: Rat thyroid glands were homogenized and precipitated with 35% ammonium sulfate (30). After dissolution of the precipitate in phosphate-buffered saline (PBS, composed of 0.01 M phosphate at pH 6.8 and 0.15 M sodium chloride), and dialysis against the same buffer, the protein was layered on Sephadex G-200 in a column (60 × 3.5 cm) equilibrated with PBS flowing at a rate of 20 ml/h at 4°C. 5-ml fractions were collected, and their absorption was read in the spectrophotometer at 280 nm. Peak fractions were pooled and subjected to sucrose gradient centrifugation (30). After this step, 0.1-ml fractions were collected and read at 280 nm. The peak fractions were again pooled and considered to contain thyroglobulin. The protein content measured by the method of Lowry et al. (39) was 3–4 mg/ml.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed according to the method of Maizel (40). The gels contained 0.1% SDS and were discontinuous, with a 3.6% acrylamide spacer (pH 6.7) and a 5.0% acrylamide resolving gel (pH 8.3). The electrode buffer consisted of Tris HCl (0.6%) and glycine (2.88%) at pH 8.3, to which SDS (0.1%) was added. For electrophoresis, 20 μl of thyroglobulin preparation was added to 10 μl of 0.5 M Tris HCl (pH 6.7), 10 μl of β-mercaptoethanol for reduction of S-S groups, 10 μl of Bromophenol Blue, 10 μl of 7% SDS, and 10 μl of glycerol; the mixture was boiled for 2 min and applied to the gel. After completion, the gels were stained for protein with Coomassie brilliant blue (Merck and Co., Inc., Rahway, N. J.) (40), or for glycoproteins by the periodic acid-Schiff technique (73). The rat thyroglobulin preparation as well as the one received from M. J. Izumi, Harvard University, was run on the gels together with a purified guinea pig thyroglobulin, obtained from Dr. E. A. Burkhard, National Institutes of Health (Fig. 1). Two major molecular species were consistently seen in rat preparations, and they migrated at a slightly faster rate than the slowest migrating band of the guinea pig preparation. The bands were shown to contain radioactivity after the animals were injected with radioiodine (unpublished observations), and they also stained for glycoprotein. Minor components were also distinguishable above and below the two major species. When reduction with mercaptoethanol was omitted, there was a single major band migrating at a rate similar to that of the two major species in Fig. 1; other distinct components were also observed between this band and the top of the gel. However, with or without reduction,

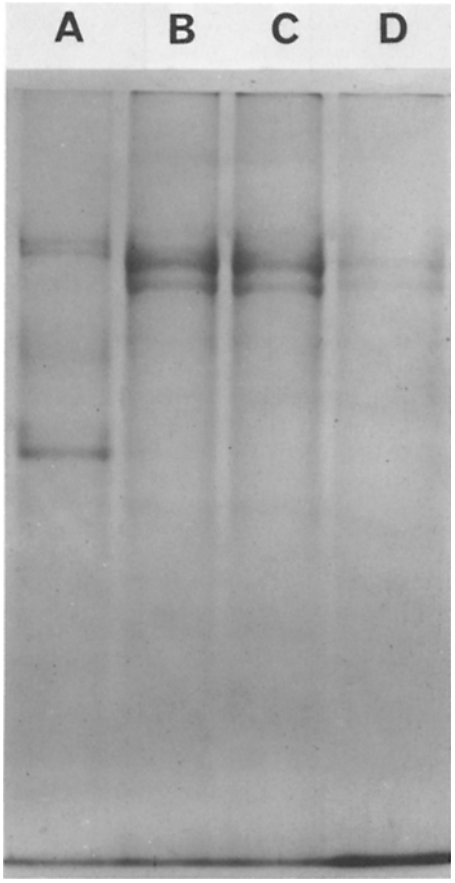


FIGURE 1 Polyacrylamide-SDS slab gel electrophoresis of rat and guinea pig thyroglobulin preparations. Slab *A* shows a guinea pig thyroglobulin from Dr. A. E. Burkhard. Slabs *B* and *C* show a rat preparation obtained as outlined in Materials and Methods. Slab *D* shows a rat thyroglobulin preparation from Dr. Izumi. The proteins were mixed with 1% SDS in Tris-HCl buffer pH 6.7, reduced with 2-mercaptoethanol, and then denatured by boiling before electrophoresis. They were used in concentrations of 1–2 mg/ml (20 μl/slab). Acrylamide concentration was 5%. The dark line at the base of the pictures indicates the front. Coomassie Blue staining.

the electrophoretic mobility was similar to that of previously characterized rat thyroglobulin preparations (56, 19, 26).

PREPARATION, PURIFICATION, AND TEST OF THE ANTIBODIES: Two New Zealand 3-mo-old rabbits were bled by ear vein puncture, and the serum was treated by half-saturated ammonium sulfate (32) to obtain "normal rabbit immunoglobulin."

The same rabbits received an intramuscular injection of 3 mg of a thyroglobulin preparation in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Thereafter, they received weekly injections of the same dose, but without adjuvant. After 4 wk they were bled and, after another 2 wk they were given two booster shots at weekly intervals, and again bled. The "antithyroglobulin sera" thus obtained were subjected to affinity chromatography (11), using thyroglobulin bound to Sepharose (1.5 mg/ml of wet gel; Sepharose 4B, Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) to obtain purified "antithyroglobulin antibodies."

For control, another rabbit was injected with 3 mg of rat serum proteins with adjuvant, and then treated according to the same injection schedule as the animals given thyroglobulin. Bleeding yielded an "anti-rat protein serum."

Immunodiffusion tests (51), in which a thyroglobulin preparation was run against the antithyroglobulin serum, yielded a single reaction band, but none against the anti-rat protein serum (Fig. 2, left). Therefore, the thyroglobulin preparation did not contain a detectable amount of serum protein contaminants. When the thyroglobulin preparation and a crude supernate of rat thyroid homog-

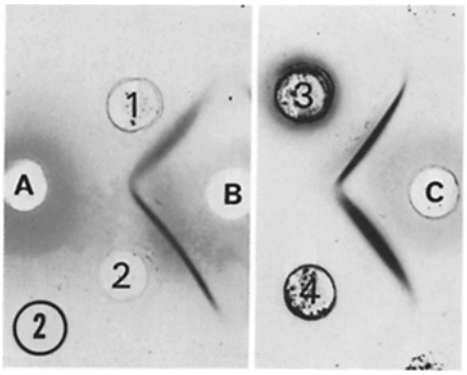


FIGURE 2 Precipitin reactions observed by immunodiffusion in agar gel (using 10-μl aliquots). At left, antiserum against rat serum proteins was placed in well *A* and antithyroglobulin serum in well *B*. A rat thyroglobulin preparation was placed in wells *1* (3 mg/ml) and *2* (5 mg/ml). Precipitin bands showing identity occurred only between the antithyroglobulin antiserum and the thyroglobulin preparations. Amido black B stained (31). On the right, antithyroglobulin serum was placed in well *C* and allowed to diffuse against a thyroglobulin preparation in well *4* (3.3 mg/ml), and the supernate of centrifuged rat thyroid homogenate in well *3* (8 mg/ml). A single precipitin band was obtained between the antithyroglobulin serum and either the thyroglobulin preparation or the supernate of the thyroid homogenate. These bands show identity. The slide was stained with 0.25% Coomassie brilliant blue in 25% isopropanol and 10% acetic acid for 15 min; it was then washed in the same solvent without Coomassie Blue, and subsequently dried.

enate were run against the antithyroglobulin serum, single precipitin bands were obtained, and showed identity (Fig. 2, right). Hence, the thyroglobulin preparation did not contain detectable amounts of thyroid proteins other than thyroglobulin.

In immunoelectrophoresis tests (51), the antithyroglobulin serum and affinity-purified antithyroglobulin antibodies were electrophoresed and run against a thyroglobulin preparation, or a crude supernate of thyroid homogenate (Fig. 3, first experiment). Conversely, the crude supernate of a rat thyroid homogenate was electrophoresed and then run against antithyroglobulin antiserum (Fig. 3, second experiment). The results showed single precipitin bands in all cases, thus confirming the immunodiffusion tests. Furthermore, the affinity purification of the antibodies did not alter their immunological reactivity. The position and shape of the bands identified the antibodies as IgG molecules.

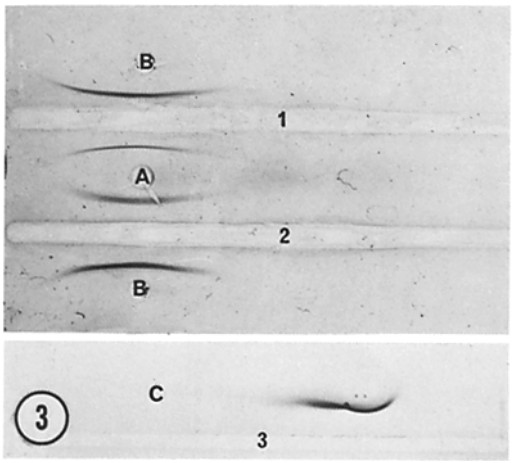


FIGURE 3 Precipitin reactions in agar gel after electrophoresis and diffusion. Two experiments are represented. In the first one, antithyroglobulin serum was placed in the middle well (*A*) and affinity-purified antithyroglobulin antibodies (4.4 mg/ml) were placed in the outer two wells (*B*). They were electrophoresed in Immuno Agaro slides Tm (Millipore Corp., Bedford, Mass.) at 10 mA for 35 min, and then allowed to diffuse overnight against the supernate of a rat thyroid homogenate (8 mg/ml) in well *1*, and a thyroglobulin preparation (3.3 mg/ml) in well *2*. Hence, whether the antithyroglobulin serum or the purified antibodies were allowed to react against the supernate of a thyroid homogenate or against the purified thyroglobulin preparation, similar single precipitin bands were obtained. In the second experiment, the supernate of a rat thyroid homogenate (7.5 mg/ml) was placed in well *C*, electrophoresed as above, and allowed to diffuse overnight against an antithyroglobulin antiserum in well *3*. Again a single precipitin band was observed. It is concluded that the antibodies were monospecific for thyroglobulin.

COUPLING OF ANTIBODIES TO PEROXIDASE: For detection of thyroglobulin in tissue sections, the purified antithyroglobulin antibodies were coupled to horseradish peroxidase (type VI, Sigma Chemical Co., St. Louis, Mo.) by the two-step procedure of Avrameas and Ternynck (1), employing glutaraldehyde as a bifunctional reagent. The same procedure was used to label normal rabbit immunoglobulin for treatment of sections used as controls.

Immunodiffusion tests revealed that the peroxidase-antithyroglobulin antibody conjugate, run against thyroglobulin, yielded a single band showing identity with that produced by unconjugated antithyroglobulin antibodies (not shown).

IODINATION OF ANTIBODIES: The chloramine-T procedure of Greenwood et al. (24), as modified by McConahey and Dixon (41), was employed to label antithyroglobulin antibodies with ^{125}I-iodine (Charles E. Frosst and Co., Montreal, Canada) to a specific activity of 10 μCi/μg. The reaction mixture was chromatographed on a Sephadex G-200 column equilibrated with 0.05 M phosphate buffer at pH 7. 1-ml fractions were collected in 0.2 ml of 2.5% bovine serum albumin, and their radioactivity was monitored in a Packard liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Clearly separated peaks of radioactivity were observed for the unreacted ^{125}I-iodine and the radioiodinated antibodies. For control work, normal rabbit immunoglobulins and bovine serum albumin were radioiodinated by the same method.

Inasmuch as iodination may damage proteins (4), it was necessary to check whether the immunological reactivity of the radiolabeled antithyroglobulin antibodies was retained. Because their amount was small (60 μg per experiment), they were first mixed with carrier-unlabeled antithyroglobulin antiserum; they were then tested by immunodiffusion against a thyroglobulin preparation, and single precipitin bands were observed (Fig. 4, top). To check for the presence of radioactivity by radioautography, the slide carrying the immunodiffusion profiles was placed in contact with a slide coated with Kodak NTB2 emulsion. Radioactivity was then observed in precipitin bands obtained between thyroglobulin and the labeled antithyroglobulin antibodies (Fig. 4, bottom). Therefore, the labeling of the antibodies did not interfere with their antithyroglobulin reactivity.

PREPARATION OF MICROSCOPE MATERIAL: 100-g Sherman rats, anesthetized with ether, were perfused intracardially with 2.5% glutaraldehyde (Taab Laboratories, Emmer Green, Reading, England) in 0.05 M Sorensen's buffer (Sorensen Co., Manchester, N. H.) (pH 7.2) containing 0.1% sucrose and 0.5% dextrose (400 mosmol). The fixative solution was administered at room temperature through the left ventricle by means of a cooled polyethylene catheter (Venocath-16) attached to a ministatic pump (Manostat Corp., New York) set at a reading of 0.3. The perfusion continued for a 15-min period, after which time the thyroid gland

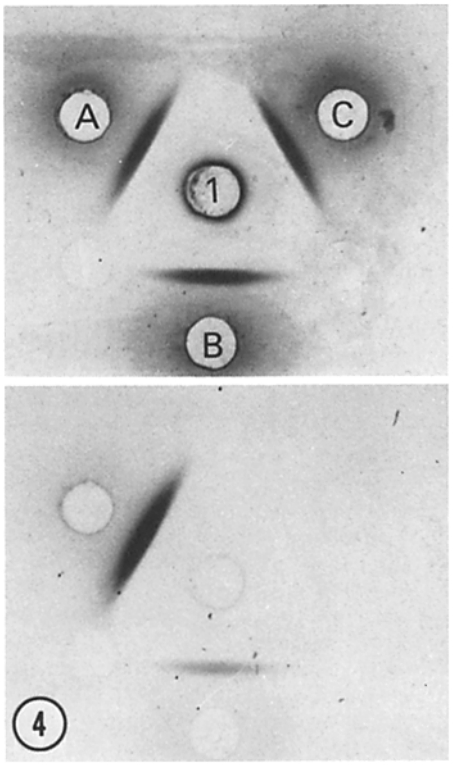


FIGURE 4 The top panel shows precipitin reactions in agar gel. A thyroglobulin preparation (5 mg/ml) placed in well *1* was allowed to diffuse against radiolabeled antithyroglobulin antibodies, using a 20-μg/ml solution in *A* and a 3-μg/ml solution in *B*, both mixed 1:1 with unlabeled antithyroglobulin antiserum to serve as carrier. Well *C* received unlabeled antithyroglobulin antibodies mixed with free radioactive iodide containing a radioactivity equal to that in well *A* (Coomassie Blue stain as in Fig. 2, right). Single precipitin bands can be seen between well *1* and the other three. The bottom panel shows a radioautograph of the agar gel shown above. By comparison with the top figure, it is seen that radioactivity is present in the regions corresponding to the precipitin bands occurring between the radiolabeled antibodies and the thyroglobulin preparation. The band related to well *A* gives a stronger radioautograph than that related to well *B*, as expected from the corresponding differences in amounts of radiolabeled antibodies. No radioactivity is observed over the precipitin band formed by the unlabeled antibodies mixed with free radioiodide.

was removed and washed in phosphate buffer for 2 h before embedding.

To examine whether glutaraldehyde fixation affected the antigenicity of thyroglobulin, immunoprecipitation tests were run by the method of Heidelberger and Kendall (29). Aliquots of antithyroglobulin serum were made to react with increasing amounts of thyroglobulin or thyroglobulin treated with 2.5% glutaraldehyde. The precipitates were centrifuged, washed twice with PBS, and then dissolved in 1 ml of 0.25 M acetic acid (21). Their relative protein content was estimated by absorption at 277 nm. The results (Fig. 5) showed that the addition of glutaraldehyde to thyroglobulin produced only a slight change in the precipitin curve.[2] Therefore, glutaraldehyde used under conditions similar to those of fixation had little adverse effect on the antigenic activity of thyroglobulin.

IMMUNOLABELING WITH PEROXIDASE-LABELED ANTITHYROGLOBULIN ANTIBODIES: Thyroid lobes were fixed and washed as described above, and then embedded in glycol methacrylate, using the method of Leduc and Bernhard (38). The semithin thyroid sections (0.5 μm) used for light microscopy were treated after mounting on glass slides, and the thin sections for electron microscopy (gold interference color) were treated, after mounting on Formvar-coated grids. To minimize nonspecific antibody binding (36, 64), the sections were exposed successively at room temperature to PBS for 1 h, 2.5% bovine serum albumin in PBS for 1 h, and normal rabbit serum (diluted 1:30 with PBS), first for 30 min at room temperature, and then 30 min at 4°C. Using a modification of the method of Moriarty et al. (44), the sections were placed at 4°C for 12–48 h in contact with dilutions of peroxidase-conjugated antibodies varying in protein concentration from 5 to 500 μg/ml. Optimal results were obtained by using concentrations of 50 μg/ml with overnight incubation. The sections were washed with PBS and stained with 3,3′-diaminobenzidine (DAB) by the method of Graham and Karnovsky (23), as modified by Moriarty and Halmi (45). Then they were quickly rinsed in distilled water and subsequently osmicated in 2% OsO_4 for 5 min. They were again rinsed in distilled water and dried. Slides for light microscopy were mounted under cover slips. Dried grids were examined without further staining in a Siemens Elmiskop I electron microscope set at 60 kV.

IMMUNOLABELING WITH RADIOLABELED ANTITHYROGLOBULIN ANTIBODIES: Thyroid lobes were fixed and washed as described above, and then rinsed in Veronal acetate buffer at pH 5 for 1 h, and treated for 2 h at room temperature with the same buffer containing 0.5% uranyl acetate (16). Both methacrylate (38) and Araldite embedding (22) were used successfully.

For light microscopy, semithin sections were cut at 0.5 μm. For electron microscopy, sections showing gold

[2] The thyroglobulin concentration (1 mg/ml) and reaction conditions used with glutaraldehyde produced no visible aggregates of thyroglobulin. Therefore, it is not likely that cross-links were induced between the thyroglobulin molecules. It may be concluded that thyroglobulin molecules are still allowed free interaction in subsequent antibody precipitation tests.

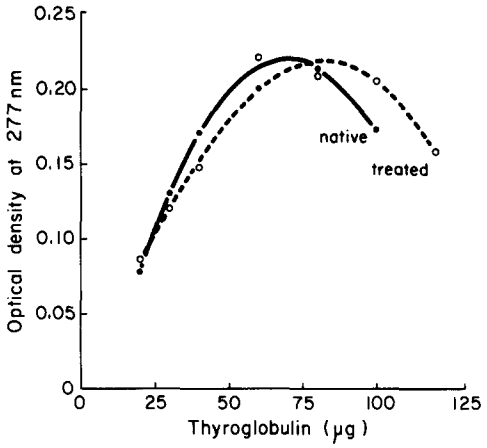


FIGURE 5 Quantitative precipitin reaction of antithyroglobulin serum against either native thyroglobulin (solid line) or thyroglobulin treated with 2.5% glutaraldehyde for 30 min (dashed line). Increasing amounts of native or treated thyroglobulin were added to 0.10-ml aliquots of antithyroglobulin serum; the precipitates were washed, dissolved in 1 ml of 0.25 M acetic acid, and the protein content was estimated by measurement of the absorbance at 277 nm. Native and glutaraldehyde-treated thyroglobulin produced similar precipitin curves, with only a slight shift of the treated curve towards the region of high antigen concentration.

interference color were used. To minimize the background, sections were handled without mounting on glass slides or grids; they were floated on the different solutions and transferred from one to the other by small plastic O rings in which they were held by surface tension. The sections were first etched with 5% hydrogen peroxide for 3 min, a treatment later shown to be unnecessary and then passed through PBS for 60 min. They were treated with bovine serum albumin and normal rabbit serum as done before labeling with peroxidase-conjugated antibodies. Sections were then floated on the radiolabeled antibody solution, using concentrations of 1–30 μg/ml for 12–48 h in a humidity chamber at 4°C. After incubation, sections were floated over five successive PBS baths containing 1% normal rabbit serum, and, in the hope of insuring the binding of the attached antibodies to the sections, they were exposed to 2.5% glutaraldehyde in PBS for 15 min and rinsed in distilled water. It is assumed that the binding of the antibodies takes place on the section surface in contact with the solutions.

For light microscope radioautography, the sections were mounted on glass slides and coated with Kodak NTB2 emulsion, exposed for 1–21 days, and developed in Kodak D-170. They were then stained through the emulsion with 1% toluidine blue in a saturated borax solution.

For electron microscope radioautography, the sections were mounted on celloidin covered glass slides (34) and coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) on the surface opposite to that binding the antibodies. The sections were exposed for 5–60 days. The radioautographs were then treated by gold latensification before "solution physical" development in the Agfa-Gevaert developer (Peerless Photo Products, Inc., Teterboro, N. J. [35]). Contrast was enhanced by staining with saturated, aqueous, uranyl acetate plus lead citrate (68).

Radioautographs of semithin methacrylate and Araldite sections, exposed to labeled antithyroglobulin antibodies and processed identically, were compared to assess the effect of the embedding medium. Relative density was measured over 25 follicular colloids in both types of sections, using a Welsh Solid State Densichron (Norton Co., Safety Products Div., Cranston, R. I.). The density of the colloid reaction was greater with methacrylate than Araldite sections by a factor of ~40. However, the distribution of the reactions was the same in both cases. Since the ultrastructural quality was far superior with Araldite than with methacrylate, the distribution of the reactions was mostly investigated in Araldite-embedded tissues.

QUANTITATION OF RADIOAUTOGRAPHIC RESULTS: After treatment of sections from the same thyroid block with radioiodinated antibodies against thyroglobulin (experimental sections), or radioiodinated normal rabbit immunoglobulin (control sections), radioautographs were prepared and silver grains were counted over the organelles in thyroid cells. Using preparations exposed for 10 days, and developed as indicated above, most silver grains consisted of a single, compact, round silver deposit; in a small proportion of the cases, two to four small silver deposits packed close to one another were considered as constituting a single grain (35). Micrographs were taken at × 10,000 in a linear series along successive follicular cells, and printed at × 30,000.

First, direct counts were used, that is, the silver grains were assigned to the organelle located below their center, except that any grain straddling the membrane of an organelle was assigned to that organelle. 1,028 grains were recorded in 47 micrographs of experimental sections, and 529 grains in 95 micrographs of control sections. An index of the volume of the structures was obtained by the "point hit" technique (8). This was done by having a transparent plastic sheet, on which evenly spaced dots had been drawn, placed over the micrographs, and recording the frequency with which the dots occurred over the intracellular structures. Two results were obtained: (*a*) the surface area of the investigated cells was estimated from the total number of point hits over the cells in the micrographs; thus, the 6,265 hits recorded in control sections corresponded to a cellular area of 1.05 mm^2, and the 3,056 hits recorded in experimental sections corresponded to a 0.51 mm^2 area; (*b*) an index of the percent cell volume occupied by that organelle was obtained from the number of point hits over each organelle. The counts of silver grains over each

organelle and expressed per 0.168 mm^2 of cellular area were then divided by the percent point hits to give the concentrations of radioactivity in experimental and control sections. Finally, the control concentrations were deducted from the experimental ones to yield the specific concentrations of radioactivity (Table IV).

Because silver grains do not always overlie the source of radioactivity which produces them, several methods were developed to minimize this difficulty. The methods of Williams (70) and Nadler (46) were used and corrected counts were obtained. A first approach was based on the finding of Fertuck and Salpeter (18) that, in an Ilford L4 emulsion developed after gold latensification in Elon ascorbic acid (Photographic Developing Agent, Eastman Kodak Co., Rochester, N. Y.), a ^{125}I source producing a silver grain had a 50% probability of being located within 80 nm of the center of the grain. Hence, a circle with an 80-nm radius entered over the grain would include the radioactive structure responsible for the grain with a 50% probability. Since our ^{125}I-radioautographs were prepared by a similar procedure, it was decided to record the organelles contained within 80-nm circles centered over each grain. Such circles (which at × 30,000 had a 2.4-mm radius) were drawn on a plastic sheet. When a circle was centered over a grain, only one structure was included and the grain was said to be "exclusive." If two or more structures were within the circle, the grain was said to be "shared." This procedure yielded the distribution of exclusive and shared grains in the micrographs of the experimental sections. In the next step, 80-nm circles were placed at random over the micrographs, and the included organelles were recorded as "circle hits," in the hope of defining what the distribution of randomly scattered grains would be (70). Using the chi-square method, the distribution of circle hits was then compared to the actual distribution of silver grains obtained with the 80-nm circles, as proposed by Williams (70). The next step was to use Nadler's method (46) by applying the formula of this author to the data, and thus obtain corrected counts for the experimental sections. The counts recorded per unit area were divided by the percent point hits to obtain the concentrations of radioactivity (Table V). The whole procedure was then repeated, using the micrographs of the control sections, and again, concentrations were calculated. Subtracting control from experimental concentrations yielded the corrected specific concentrations (Table V).

In a second approach, it was decided to use larger circles, so as to obtain a greater probability of including the radioactive source of a silver grain. Geometric analysis of the effect of radiation from the surface of the section on the silver bromide crystals of the emulsion, indicated that a circle with a 160-nm radius centered over a grain would include its source with a 90% probability (Nadler, unpublished observations). Such circles were used to obtain exclusive and shared grains, as well as circle hits. The corrected counts were then obtained in the experimental and control sections, and the corrected specific concentrations were calculated (Table V).

Immunocytological Controls

In both the immunoperoxidase and radioautographic studies, tests were used to control the validity of the method, as well as the specificity and sensitivity of the reactions.

METHOD CONTROLS: First, sections were subjected to the same sequence of treatments as the experimental ones, except that PBS replaced the antithyroglobulin antibodies labeled with peroxidase or radioiodine. Second, the possibility of nonspecific interaction between thyroid sections and labels was tested by treatment of the sections with unconjugated peroxidase (1–2 mg/ml), or free radioiodide (of the same radioactivity as the radiolabeled antithyroglobulin antibody), or radiolabeled bovine serum albumin (15 μg/ml; sp act, 10 μCi/μg).

SPECIFICITY CONTROLS: Three controls were used as recommended by Coons and Kaplan (10). (*a*) To test whether labeled antithyroglobulin antibodies could be nonspecifically adsorbed by thyroid sections, they were replaced by normal rabbit immunoglobulin labeled with peroxidase (250 μg of the complex/ml), or with radioiodine (15 μg/ml; sp act, 10 μCi/μg). (*b*) The second control tested whether or not a thyroid section would react with a solution of labeled antithyroglobulin antibodies after the latter had been adsorbed onto an excess of thyroglobulin. For the immunoperoxidase study, a peroxidase-labeled antithyroglobulin preparation (2.5 mg/ml) was adsorbed with excess thyroglobulin (20 mg/ml). For the radioautographic study, radiolabeled antithyroglobulin antibodies (0.5 ml of a 20-μg/ml solution; sp act, 10 μCi/μg) were mixed with carrier antithyroglobulin antiserum (0.25 ml), and reacted against thyroid soluble proteins (7 mg/ml in 0.25 ml PBS). In both cases, the solutions were then centrifuged, and the supernates were incubated overnight with thyroid sections at 4°C. (*c*) The third control tested whether prior exposure of the sections to unlabeled antithyroglobulin antibodies prevented or reduced the binding of labeled ones. For the immunoperoxidase studies, sections were exposed to 3 mg/ml of unlabeled antithyroglobulin antibodies, before 0.05 mg/ml of peroxidase-labeled ones. For radioautography, 15 μg/ml of unlabeled antithyroglobulin antibodies was used, before the same amount of radioiodine-labeled ones. In either case, incubation times were approximately 16 h.

SENSITIVITY TESTS: The dependency of the reaction on the concentration of antibodies was examined by treating thyroid sections with solutions containing concentrations of peroxidase-labeled antithyroglobulin antibodies varying from 5 to 250 μg/ml of solution, and of ^{125}I-labeled antithyroglobulin antibodies of 2, 8, 16, and 20 μg/ml.

RESULTS

Immunolabeling with Peroxidase-Conjugated Antithyroglobulin Antibodies

Light microscope examination of semithin methacrylate sections of rat thyroid gland, exposed to peroxidase-conjugated antibodies against rat thyroglobulin, showed a dark brown stain extending throughout the follicular colloid, but restricted in the cells to colloid droplets and tiny granules preferentially located in the apex (Fig. 6). The colloid droplets, with a 1-4-μm range, exhibited varying staining intensities, being as dense as the follicular colloid in most instances, but almost unstained in others. The size of the stained tiny granules approximated the limit of resolution of the light microscope (0.1–0.2 μm); they were often observed immediately beneath the cell-colloid interface, and lined up against it. In a particular follicle, one or two cells might show many of these granules, while the others had few or none. The cells at the periphery of the follicles, identified as calcitonin cells, showed no stained component.

Electron microscope examination confirmed the strong staining of the follicular colloid (Figs. 7, 8). Within follicular cells, the staining of the colloid droplets was usually as intense as that of the follicular colloid (Fig. 9). However, some of the droplets stained less intensely, or did not stain at all.

Staining was also observed in cytoplasmic vesicles with a diameter averaging 135 nm, although a few were as large as 500 nm. Like the tiny vesicles seen by light microscopy, they were often arranged in rows along the apical border (Figs. 7–9), and less often they were located along the lateral borders of the cells. By comparison with the description of Haddad et al. (25), they were identified as "apical vesicles," the secretory granules of follicular cells.

Stained vesicles were also associated with the Golgi apparatus (Fig. 10). Their range of size and staining intensity was comparable to that of the apical vesicles, and they were identified as condensing vacuoles or prosecretory granules. The Golgi saccules did not show distinct staining. Similarly, the ribosomes were stained in places (Fig. 9), but doubt was raised by the fact that these organelles are known to absorb diffusing oxidized DAB (49), and it was not clear whether lysosomes were reactive. Mitochondria however were not stained (Figs. 7 and 9).

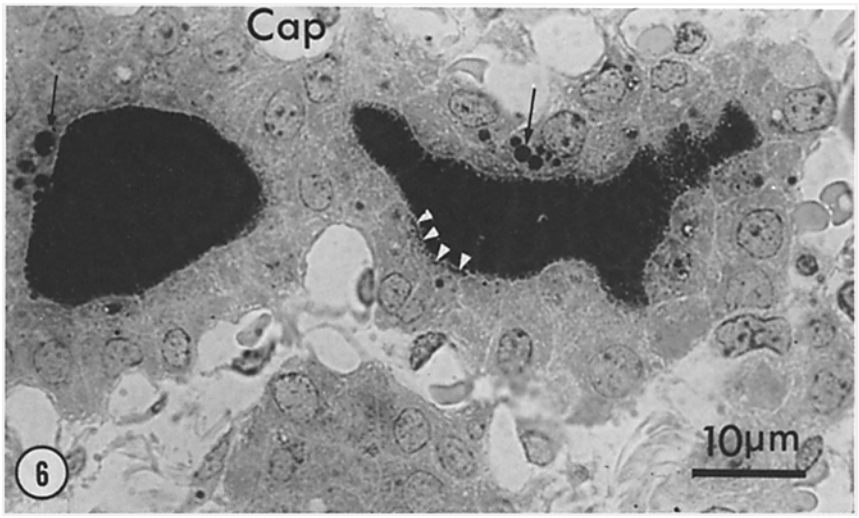
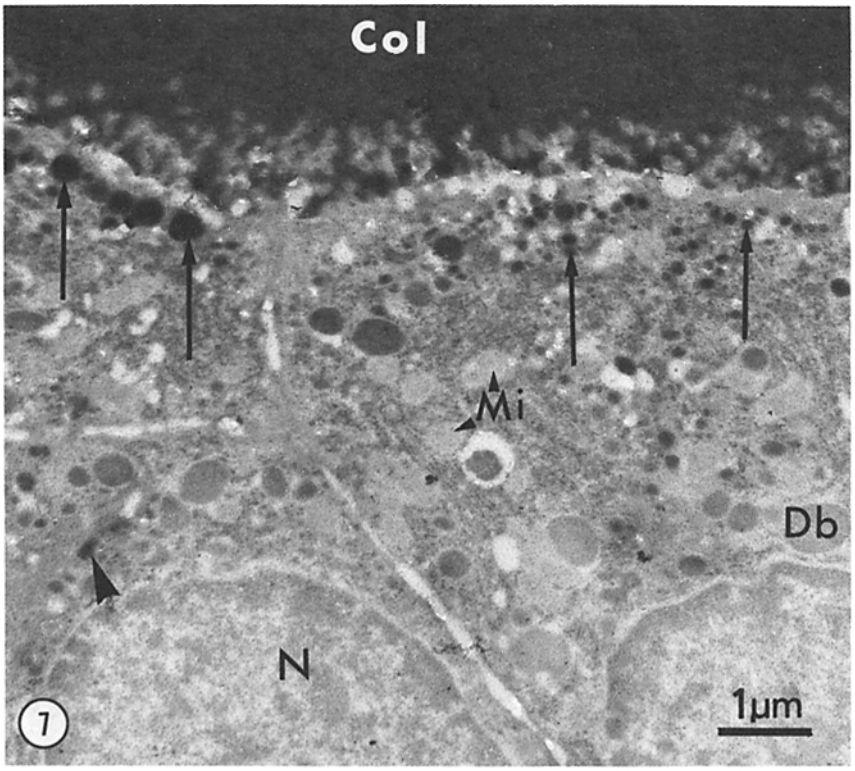


FIGURE 6 Semithin section of rat thyroid allowed to react with peroxidase-conjugated antithyroglobulin antibodies. The sites of antibody binding were identified by staining the peroxidase with DAB followed by osmium tetroxide. No counterstain was used. × 1,500. In the two thyroid follicles shown, a dark reaction product is present in the follicular colloid. Within the cells, the reaction product is in colloid droplets (arrows) and in tiny granules mainly located near the cell-colloid interface (white arrowheads). *Cap*, capillary. The stained areas are those which bind antithyroglobulin antibodies; they are interpreted as sites of thyroglobulin antigenicity. The nuclear staining is due to osmication.


Col
Mi
Db
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1μm
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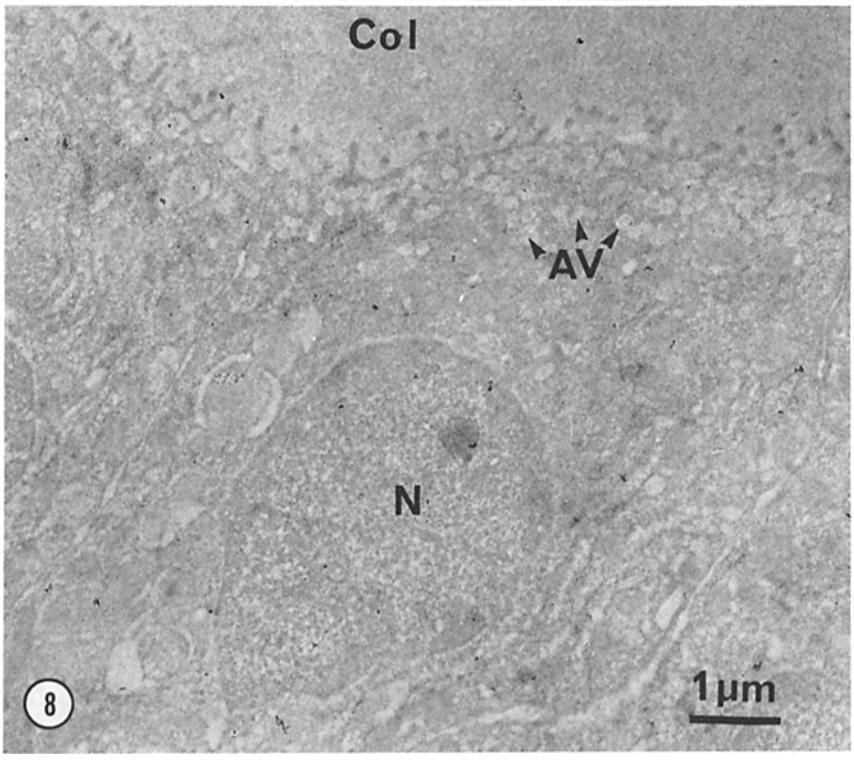
Col
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Of the immunocytological controls, two were used to assess the method. First, thyroid sections treated with buffer alone before DAB staining took up no stain, indicating that, under the conditions used, no endogenous peroxidase was active. Further, treatment of the sections with 1–2 mg of peroxidase/ml before DAB treatment also gave negative results, so that peroxidase in the doses used did not bind to the sections. In the first "specificity control," the peroxidase-conjugated antithyroglobulin antibodies were replaced by peroxidase-conjugated, normal rabbit immunoglobulin; colloid, apical vesicles, and Golgi vesicles were not stained (Fig. 8). The other specificity controls also gave negative results. Therefore, the staining reactions of thyroid sections occurred at sites that specifically bound the labeled antithyroglobulin antibodies.

The "sensitivity tests" carried out with increasing concentrations of peroxidase-conjugated antithyroglobulin antibodies showed no reaction at all with 5 μg, but reacted to a heavy background with 250 μg. However, an optimal response was achieved with 50 μg of conjugate protein/ml of solution. When the conjugate was diluted to 18–25 μg, the cellular reactions decreased and disappeared, but those of the colloid persisted, suggesting a greater concentration of specific antibody-binding sites in colloid than in cell organelles.

Immunolabeling with Radioiodinated Antithyroglobulin Antibodies

Light microscope examination of Araldite sections of rat thyroid exposed to radioiodine-labeled antithyroglobulin antibodies, and processed for radioautography, revealed that the concentration of silver grains was high over the follicular colloid, and low over follicular cells (Fig. 11).

In the electron microscope, silver grains were distributed throughout the follicular colloid (Fig. 12). Grain counts per unit area over five different colloids (Table I) showed no significant difference by analysis of variance ($P > 0.20$). Therefore, label concentration was uniform in the examined colloids. The distribution of the grains at the cell-colloid interface was examined by counting 100 grains in photographs. Of these, 4 were directly over microvilli, 32 touched on their edge, and 64 were over the intervening colloid. Therefore, it was likely that the silver grains arose from radioactivity associated with the colloid.

In follicular cells, most silver grains were located over colloid droplets, apical vesicles, Golgi apparatus, or rough endoplasmic reticulum. Occasional grains were also found over nuclei, mitochondria, and dense bodies, but similar numbers of grains were found over these organelles in control preparations.

The labeling of many of the colloid droplets was definite (Fig. 13), especially when they were as electron-dense as the follicular colloid. The colloid droplets showing a smaller degree of labeling were usually less electron-dense (Figs. 14, 15). Grain counts confirmed this conclusion (Table II). In polymorphic colloid droplets, the denser portions were more intensely labeled (Fig. 16).

In the apical region of follicular cells, many silver grains were associated with the small, electron-dense apical vesicles. Comparison of Figs. 17 and 18 indicated that the number of labeled apical vesicles increased with the length of the radioautographic exposure.

Silver grains were also found over vesicles associated with the Golgi apparatus (Fig. 19), and these were of the same size and electron density as

FIGURE 7 Apex of a thyroid follicular cell allowed to react with peroxidase-conjugated antithyroglobulin antibodies and stained with DAB and osmium tetroxide. × 12,000. A dark reaction product is seen in the follicular colloid (*Col*) and small cytoplasmic vesicles predominating at the cell apex (arrows); these are identified as apical vesicles. Reaction product is also present in a Golgi vesicle (arrowhead). Mitochondria (*Mi*) are unreactive. The light grey shade of nucleus (*N*) and dense bodies (*Db*) may not be different from that in control preparations.

FIGURE 8 Control section of the apex of a thyroid cell allowed to react with peroxidase-conjugated normal rabbit immunoglobulin and stained with DAB and osmium tetroxide. × 12,000. No reaction product is seen within colloid (*Col*), apical vesicles (*AV*), or other structures. Hence, the reaction observed in these compartments, after exposure to antithyroglobulin antibodies (Fig. 7), indicates binding of these antibodies and, presumably, sites of thyroglobulin antigenicity. In addition, the absence of a reaction here shows that the procedure did not reveal any of the endogenous peroxidase known to occur in these cells. *N*, nucleus.

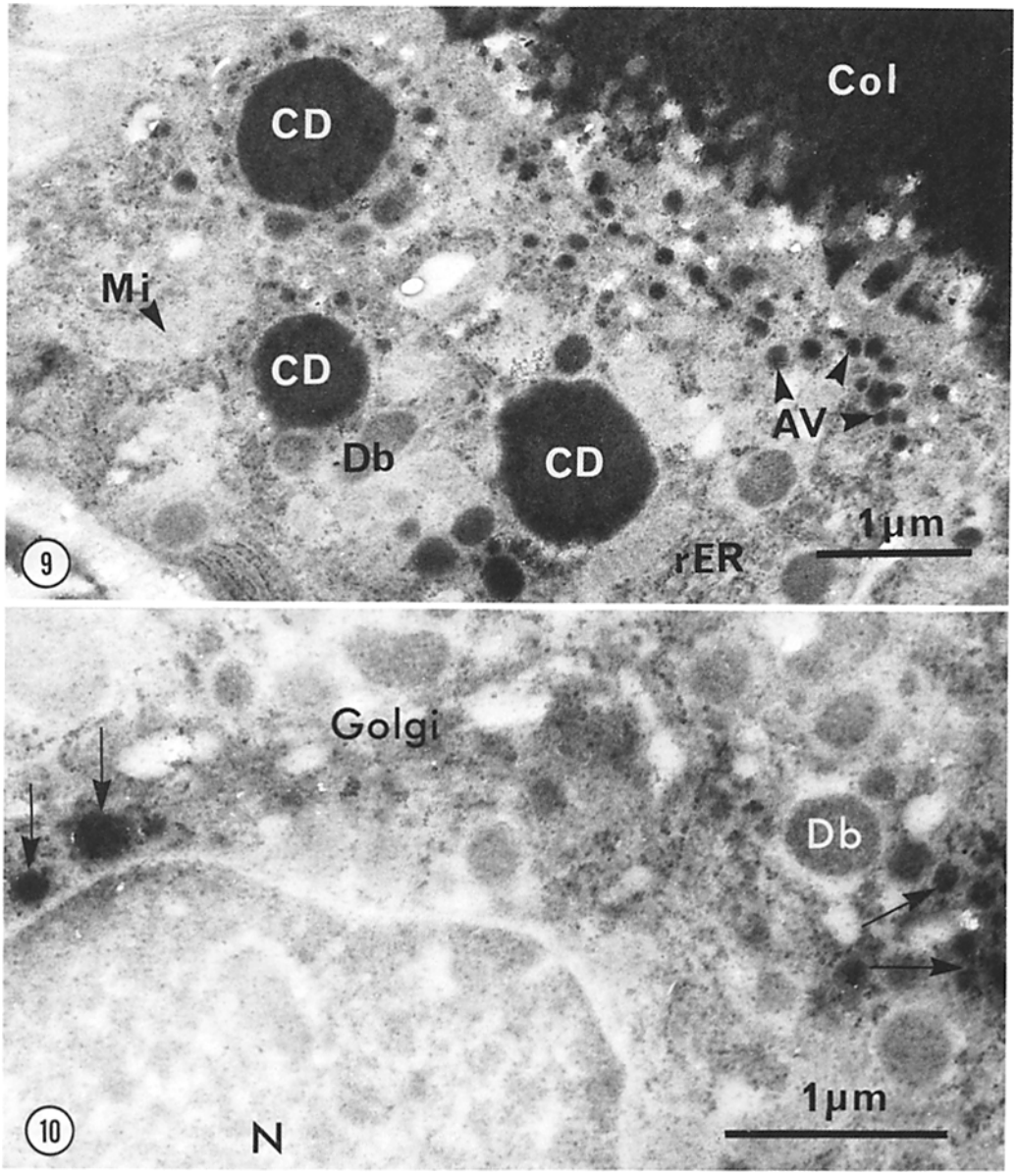


FIGURE 9 Apex of a thyroid follicular cell allowed to react with peroxidase-labeled antithyroglobulin antibodies and stained with DAB and osmium tetroxide. × 21,000. The staining intensities over colloid (*Col*), apical vesicles (*AV*), and colloid droplets (*CD*) are comparable. *Db*, dense body; *Mi*, mitochondrion; rER, rough endoplasmic reticulum showing stained ribosomes in places.

FIGURE 10 Perinuclear cytoplasm in a thyroid follicular cell treated as in Fig. 9. × 30,000. The reaction product is observed in small vesicles (arrows at right and left of center) associated with the Golgi apparatus. *N*, nucleus; *Db*, dense body.

the apical vesicles. Furthermore, the Golgi saccules were also overlaid by a few silver grains (Fig. 20).

Finally, silver grains were scattered over the rough endoplasmic reticulum throughout the cell (Fig. 21), and were distributed over both cisternal content and wall.

The immunocytological controls were run on

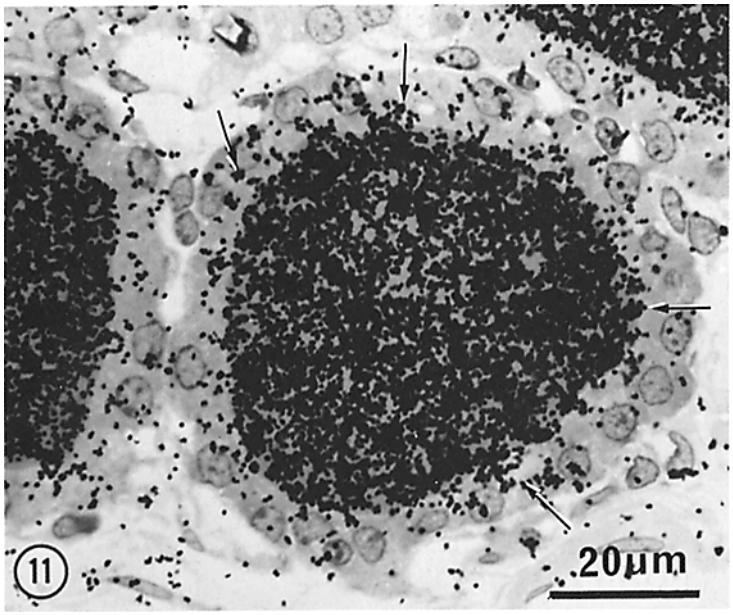


FIGURE 11 Light microscope radioautograph of a semithin section of rat thyroid embedded in Araldite, allowed to react with radiolabeled antithyroglobulin antibodies, processed for radioautography (21-day exposure), and poststained with toluidine blue. × 1,000. The follicular colloid shows a high concentration of silver grains, whereas the follicular epithelium shows a rather low concentration (arrows), although it exceeds the background reaction seen over perifollicular tissues. The structures overlaid by a concentration of silver grains are those binding the antithyroglobulin antibodies; they are interpreted as sites of thyroglobulin antigenicity.

serial sections of the same thyroid exposed to the various treatments; silver grains were then counted over corresponding colloids (Table III). The "method controls" showed no reaction above the background when the sections were treated with buffer or free radioactive iodide, and only a slight reaction with radiolabeled bovine serum albumin, amounting to 5.6% of that produced by radiolabeled antithyroglobulin antibodies. In the first specificity control, treatment with radiolabeled normal rabbit immunoglobulin produced a slight reaction (Table III). In the next test, treatment with a solution of labeled antithyroglobulin antibodies, previously adsorbed by thyroglobulin, produced no reaction above the background. In the third of these controls, the treatment of thyroid sections with unlabeled antithyroglobulin antibodies before treatment with labeled ones, blocked the reaction, although not completely (17.2% of the reaction produced by the experimental treatment). Therefore, although the unlabeled antibodies occupied most of the reactive sites, they had been displaced from some of them by the labeled antibodies. From these various tests, it was concluded that the reactions in thyroid sections occurred at sites specifically binding the radioiodinated antithyroglobulin antibodies.

In the sensitivity test, serial sections treated with different concentrations of ^{125}I-antithyroglobulin antibodies showed that doses ranging from 2 to 22 µg/ml of antibody solution produced a linear increase in the immunolabeling of the colloids (Fig. 22). Indeed, with doses between 8 and 16 µg/ml, the number of grains was sufficient to show the degree of labeling within each compartment, but not high enough to obscure their identity. Such doses were used for quantitation.

QUANTITATION OF RADIOAUTOGRAPHIC RESULTS: Direct counts of silver grains yielded their distribution over intracellular structures in thyroid sections exposed to ^{125}I-antithyroglobulin antibodies (experimental sections) or ^{125}I-normal rabbit immunoglobulin (control sections) (Fig. 23). Comparison of the two distributions by the chi-square method revealed that the experimental and control sections differed significantly ($P < 0.001$). Indeed, comparison of individual structures showed that colloid droplets, apical vesicles,

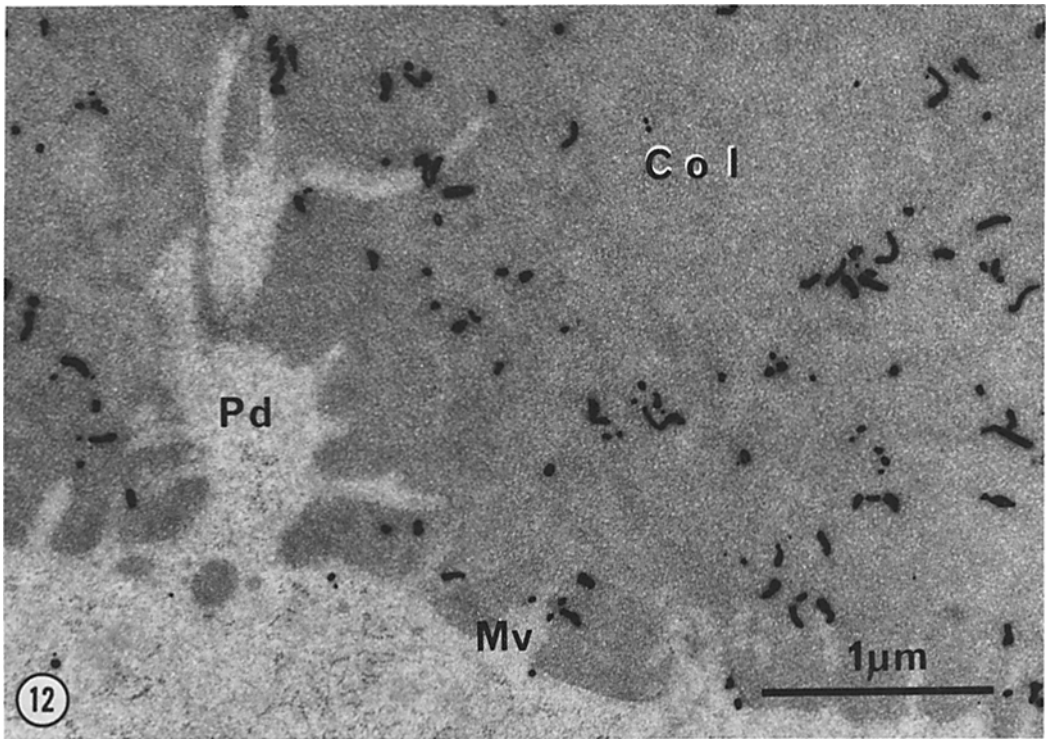


FIGURE 12 Electron microscope radioautograph of a thin section of rat thyroid embedded in Araldite, allowed to react with a solution containing 15 μg of radiolabeled antithyroglobulin per ml, process for radioautography, and exposed for 10 days. Contrast was enhanced by staining the sections after radioautography with aqueous uranyl acetate for 5 min, and lead citrate for 30 min. Cell-colloid interface with microvilli (*Mv*) and pseudopods (*Pd*). (Some of the silver grains are rods rather than spheres due to overdevelopment.) Almost all visible silver grains are over the colloid (*Col*). × 36,000.

TABLE I

Mean Direct Counts of Silver Grains Per Unit Area over Five Adjacent Follicular Colloids ± Standard Deviation in Thyroid Sections Exposed to Radioiodinated Antithyroglobulin Antibodies, and then Radioautographed*

16.2 ± 1.7
15.6 ± 4.0
16.9 ± 1.9
14.9 ± 1.9
16.3 ± 2.6

* Over each colloid, silver grains were enumerated in 10 successive electron microscope frames measuring 10 μm^2. The radioautographic exposure lasted 5 days.

condensing vacuoles, Golgi saccules, and rough endoplasmic reticulum were substantially more labeled in experimental than control sections, but this was not the case for nuclei, mitochondria, and "other" structures (which included dense bodies, cytosol, lateral, and basal plasma membranes [Fig. 23]).

When the direct counts were expressed as concentrations, and the concentrations in the controls were deducted from those in the experimental sections (Table IV), the specific concentrations thus obtained confirmed that the label of the thyroglobulin antibodies was taken up by all compartments, with the exception of nucleus, mitochondria, and "other" structures. Furthermore, the label concentration increased from rough endoplasmic reticulum and Golgi saccules to Golgi condensing vacuoles, apical vesicles, and dense colloid droplets.

The distribution of the corrected counts obtained after recording exclusive and shared grains in the experimental micrographs, using resolution boundary circles with an 80-nm radius, was compared by the chi-square method to the random distribution of 80-nm circle hits (70). A highly significant different was obtained ($P < 0.001$),

TABLE II

Mean Direct Counts of Silver Grains Per Square Micrometer ± Standard Deviation over Follicular Colloid and Colloid Droplets of Various Densities in Thyroid Sections Exposed to Radioiodinated Antithyroglobulin Antibodies, and then Radioautographed

	Grains	
	Number counted	Mean number/μm^2*
Follicular colloid‡	187	11.5 ± 3.7
Colloid droplets		
As dense as follicular colloid‡	207	10.4 ± 2.3
Similar to ground cytoplasm	95	5.2 ± 2.4
Lighter than ground cytoplasm	44	2.4 ± 1.6

* Means of 18 counts over each type of structure in electron micrographs. All sections used were of the same block, and identically immunolabeled. The radioautographic exposure lasted 20 days.

‡ Grain counts made over follicular colloid and the dense colloid droplets showed no significant difference ($0.4 < P < 0.2$). Both differed significantly from the other counts ($P < 0.001$).

indicating that the distribution of silver grains was far from random. Repeating the test after recording grains with 160-nm circles gave the same result ($P < 0.001$).

In the next step, the 80- and 160-nm data were analyzed using Nadler's formula (46) to obtain corrected counts, and these counts, expressed per unit area, were recorded in Table V. Finally, these were used to calculate the corrected specific concentrations of radioactivity in the five labeled organelles (Table V).

With the three methods used (Table VI), the specific concentrations fell into three categories: the lowest were in rough endoplasmic reticulum and Golgi saccules, the intermediate ones in condensing vacuoles and apical vesicles, and the highest in the dark colloid droplets. The counts corrected using 160-nm circles (probably the most accurate) showed that the first two differed by a factor of 3 from condensing vacuoles and apical vesicles, and these in turn were about half the value in dark colloid droplets, presumed to be the same as the value of the follicular colloid (Table II).

DISCUSSION

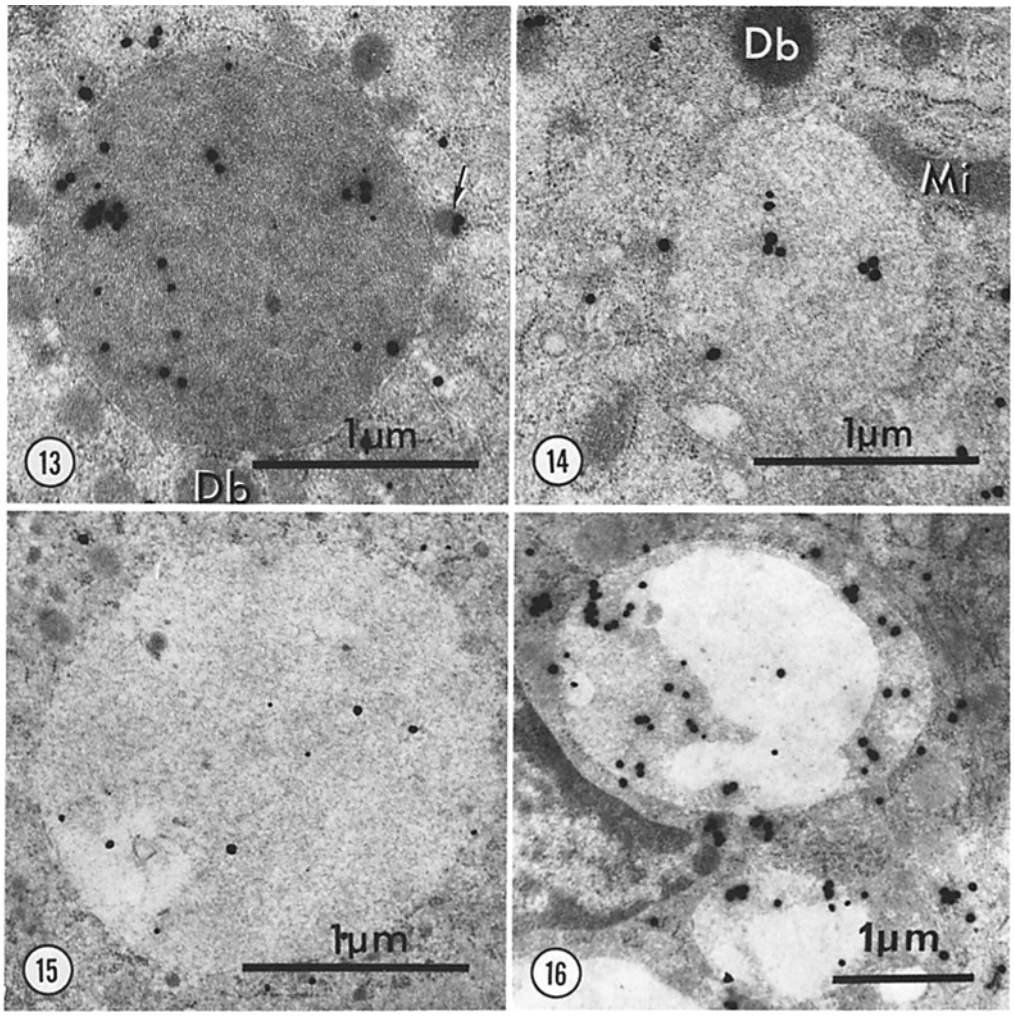
Thyroglobulin is known to be strongly antigenic, with as many as 40 determinants per molecule (65). When the rat thyroglobulin used in the present work was administered to rabbits, active antibodies were obtained, which immunodiffusion tests showed to consist of a single substance, without detectable contamination from the proteins of blood (Fig. 2) or thyroid (Figs. 2, 3).

Before thyroid sections were exposed to labeled antithyroglobulin antibodies, it was necessary to check whether the antigenicity of thyroglobulin was retained throughout the procedures used in preparing the sections. To examine the influence of fixation, the antigenicity of glutaraldehyde-treated thyroglobulin was estimated by precipitation with antithyroglobulin serum, and found to be slightly less than that of native thyroglobulin (Fig. 5). When the embedding procedures were considered, antigenicity was found to be lost in Epon, but retained in glycol methacrylate and Araldite. Of the latter two, methacrylate provided a much more complete retention of the antigenic sites than Araldite, and was used for the study with peroxidase-labeled antibodies. Yet, the distribution of the antigenic sites was the same with both embedding media and, since Araldite yielded better morphology, it was used in most of the work done with radiolabeled antibodies.

Validity of the Labeling Technique

PEROXIDASE-ANTIBODY CONJUGATE: Antibodies coupled by glutaraldehyde to peroxidase usually retain enzymatic activity and immunoreactivity (7, 9). In the present work, the DAB staining demonstrated retention of peroxidase activity by the conjugate, whereas the immunodiffusion tests showed retention of its immunoreactivity.

The significance of the dark reaction observed in thyroid sections exposed to the conjugate and stained with DAB was examined in a series of immunocytological controls. First, the peroxidase known to be present in follicular cells (12, 13, 66, 67) might be expected to react with DAB along with any bound peroxidase-antithyroglobulin antibody conjugate. However, the sections stained with DAB, without previous exposure to the conjugate, remained blank, indicating that the endogenous peroxidase was not retained in the course of the procedures used for fixation and embedding, and therefore, did not interfere with the reactions resulting from the binding of the conjugate. Sec-



FIGURES 13–16 Radioautographs of colloid droplets in sections treated as in Fig. 12, except that Figs. 13, 14, and 16 came from preparations which were block stained by uranyl acetate before embedding.

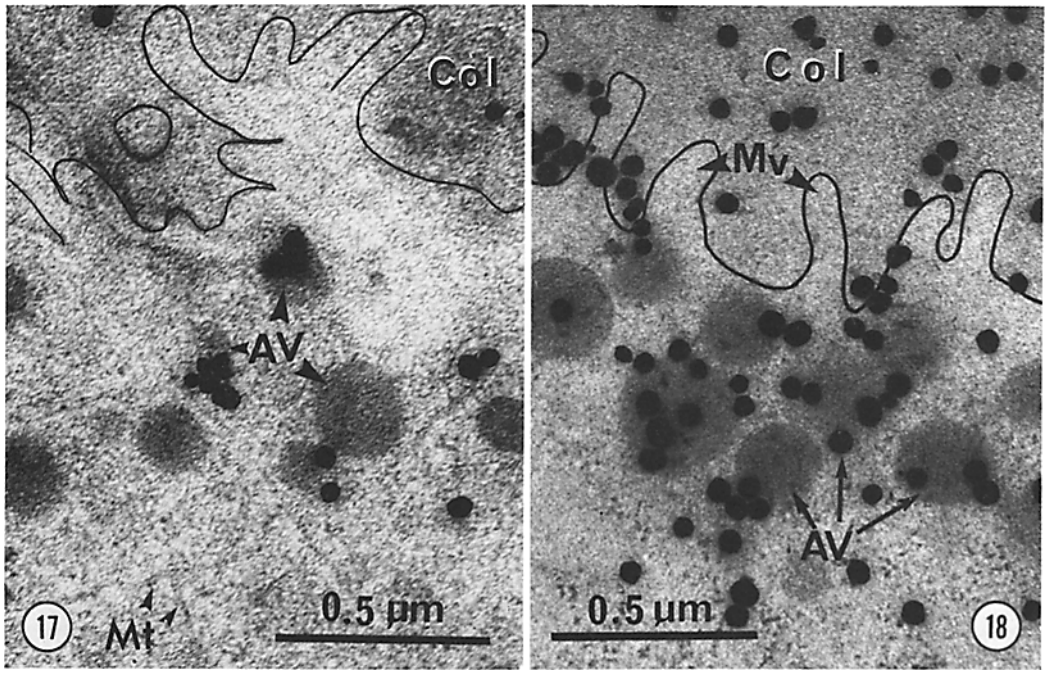
FIGURE 13 A colloid droplet showing an electron density similar to that of the follicular colloid, and greater than that of the cytoplasm. Silver grains are scattered over it. Some grains are also observed over a small vesicle, presumably an apical vesicle, at the periphery of the droplet (arrow). The larger peripheral vesicles are interpreted as dense bodies (*Db*). × 30,000.

FIGURE 14 Colloid droplet of electron density similar to that of the cytoplasm. Fewer silver grains are observed over it than over the colloid droplet in Fig. 13. *Db*, dense body; *Mi*, mitochondrion. × 30,000.

FIGURE 15 Colloid droplet of electron density slightly lower than that of the cytoplasm. Very few silver grains are present. × 30,000.

FIGURE 16 A polymorphic colloid droplet. Silver grains predominate over the electron-dense material displaced to the sides of the structure. This preparation was exposed for 60 days, that is, six times as long as those depicted in Figs. 13–15. × 18,000.

ondly, the possibility that antibodies were nonspecifically bound was eliminated by showing that thyroid sections placed in contact with peroxidase coupled with normal rabbit immunoglobulin (Fig. 8), or free peroxidase, showed no reaction. Hence, the positive results obtained with the per-



FIGURES 17, 18 Two radioautographs of the cell-colloid interface after the sections were allowed to react with the same concentration of radiolabeled antithyroglobulin antibodies. The emulsion was exposed briefly (10 days) for Fig. 17, or over a long period (60 days) for Fig. 18. The specimens were block stained by uranyl acetate, but were otherwise treated as in Fig. 12. A line was drawn to outline the interface between cell and follicular colloid (*Col*).

FIGURE 17 After a 10-day exposure, silver grains were few over follicular colloid and apical vesicles (*AV*). *Mt*, microtubules. × 54,000.

FIGURE 18 After a 60-day exposure, silver grains were numerous over follicular colloid (*Col*) and apical vesicles (*AV*). *Mv*, microvilli. × 54,000.

oxidase-antithyroglobulin antibody conjugate were attributed to its binding to the sections. Indeed, previous adsorption of the conjugate with thyroglobulin prevented the reaction. Furthermore, when exposure to the peroxidase-conjugated antibodies (0.05 mg) was preceded by exposure to a large dose (3 mg) of unconjugated antibodies, no DAB staining appeared. Presumably, the unconjugated antibodies remained bound to the antigenic sites, or were not displaced enough by the conjugated antibodies to be detected by DAB.

RADIOIODINATED ANTIBODIES: When the antithyroglobulin antibodies were labeled with radioiodine, the immunoreactivity was retained, because the precipitation band produced by carrier antibodies (Fig.4, top) coincided with that observed by radioautography of the radioiodinated antibodies (Fig. 4, bottom).

Immunocytological controls were run as in the peroxidase studies, but with the added advantage of quantitation of silver grains over the colloid in serially cut thyroid sections. The first test showed that, in contrast to fresh thyroid slices which were known to take up radioiodide in vitro (27, 71), the Araldite sections of thyroid tissue exposed to free ^{125}I-iodide did not collect radioactivity (Table III). Secondly, radioiodinated bovine serum albumin and normal rabbit immunoglobulin showed only slight binding to the colloid (Table III) or to cell components (Fig. 23, controls). Finally, the binding of the radioiodinated antithyroglobulin antibodies to the sections was completely prevented by absorbing the antibodies with excess thyroglobulin, and partially prevented by pretreatment of the sections with unlabeled antibodies (which here was not done with a large excess, as in the peroxidase control experiments, but with a small dose,

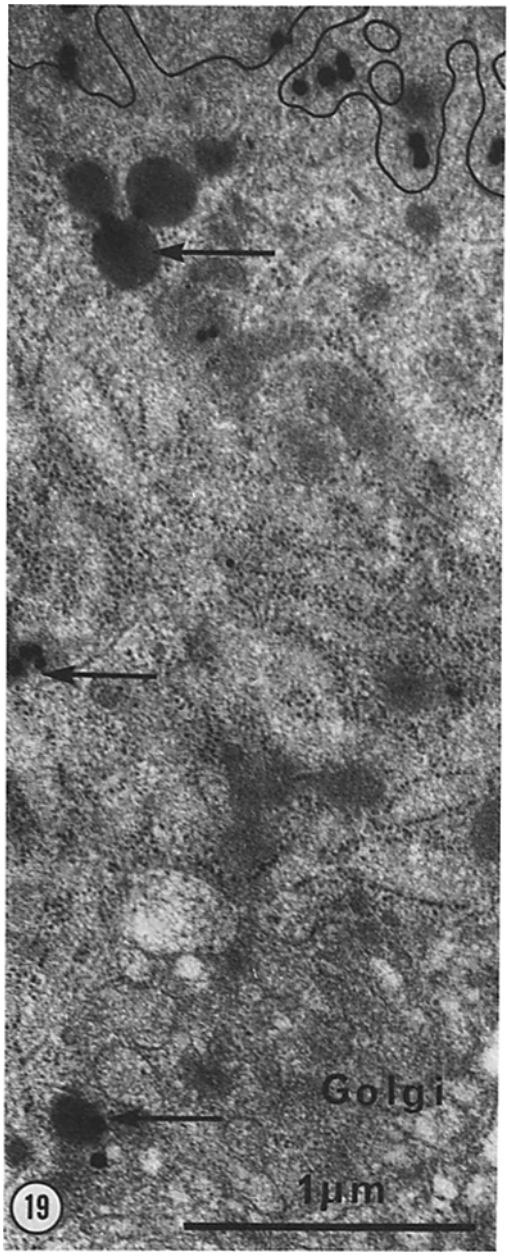


FIGURE 19 Radioautograph of a thyroid follicular cell treated with radiolabeled antithyroglobulin antibodies, as in Fig. 12, and exposed for 10 days. The interface between colloid and the microvillar surface is outlined in black. From top to base, the colloid shows a few silver grains. The top arrow indicates labeled apical vesicles. The arrow at center left points to grains which seem to be over an apical vesicle midway between the colloid and the Golgi apparatus. The arrow at bottom left points to a labeled vesicle identified as a condensing vacuole. × 38,500.

so that the labeled antibodies displaced a small proportion, 17.2% of the unlabeled ones; Table III). Therefore, the binding of the peroxidase or radioiodine label to the sections was attributed to a specific binding of labeled antithyroglobulin antibodies by sites of thyroglobulin antigenicity.

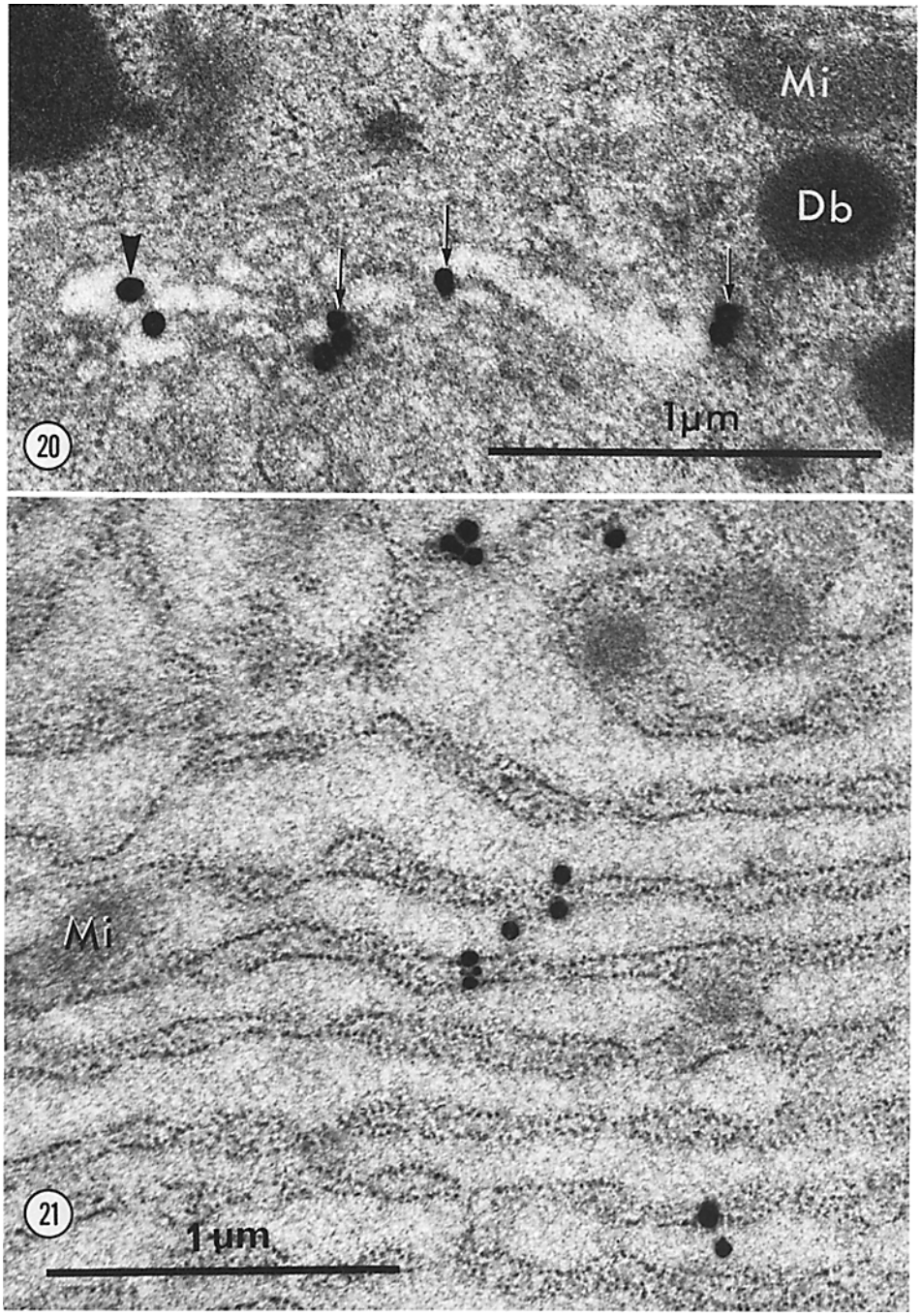
Comparison of the peroxidase and radioiodine techniques showed that the first one provides a good localization of reactive sites, except for an occasional diffusion of reaction product. Furthermore, the sensitivity of this procedure is limited because it does not identify regions of low antibody binding. As for the radiodinated antibody technique, the pictorial quality is less satisfactory, and the localization of reactive sites requires correction by elaborate quantitation. Once this is done, the technique provides a precise assessment of antibody binding, even in regions of low activity.

Significance of the Results

The information as summarized in the introduction provides evidence of the presence of thyroglobulin in the follicular colloid, but little precise information on the distribution of this substance in the cells. In the present work, a direct method was used to demonstrate the binding of antithyroglobulin antibodies not only to the colloid, but also to certain cell organelles, and thus confirms the presence of thyroglobulin antigenicity in these sites. These results were first presented in abstract form, using both peroxidase (52) and radioiodine labeling (53). Similar results were recently obtained by an indirect immunocytochemical approach, using the peroxidase-anti-peroxidase complex (55).

Within follicular cells, a strong reaction was given by dark colloid droplets (Figs. 9, 13), apical vesicles (Figs. 7, 17, 18), and the condensing vacuoles associated with the Golgi apparatus (Figs. 10, 19). The Golgi saccules and the rough endoplasmic reticulum showed a doubtful reaction with the peroxidase conjugate, but were clearly labeled with the iodinated material (Fig. 23). This labeling of the endoplasmic reticulum was compatible with the finding of thyroglobulin antigenicity in microsomes (43, 50, 63). In our radioautographs, the resolution was not sufficient to establish whether the ribosomal or cisternal component, or both, were involved; examination of centrifuged thyroid fractions of ribosomes (14, 33, 59) showed that they contained thyroglobulin antigenicity.

However, the mitochondria were definitely un-



FIGURES 20, 21 Radioautographs of cytoplasmic regions of thyroid follicular cells after treatment with radiolabeled antithyroglobulin antibodies as in Fig. 12.

FIGURE 20 A section through a Golgi apparatus showing saccular elements. Silver grains appear to be mainly distributed along the edges of the saccules (arrows). *Db*, dense body; *Mi*, mitochondrion. × 54,000.

FIGURE 21 A few silver grains are distributed over the rough endoplasmic reticulum. *Mi*, mitochondrion. × 48,000.

TABLE III

*Direct Counts of Silver Grains over Rat Follicular Colloids after Treatment of Thyroid Sections by Different Control Procedures in Comparison with the Treatment by ^{125}I-Antithyroglobulin Antibodies**

Treatment	Grains	
	Number counted	Mean number/10 μm^2
Method controls		
Buffer alone	43	0.3‡
Free ^{125}I-iodide	58	0.4‡
^{125}I-Bovine serum albumin	292	1.8
Specificity controls		
^{125}I-Normal rabbit immunoglobulin	293	1.8
Thyroglobulin-adsorption of ^{125}I-antithyroglobulin antibodies	54	0.3‡
Unlabeled antithyroglobulin antibodies before ^{125}I-antithyroglobulin antibodies	879	5.5
Experimental		
^{125}I-Antithyroglobulin antibodies	5,122	32.0

* Counts on 100 frames at × 20,000; the radioautographic exposure lasted 10 days.
‡ Values equal to background.

reactive, as observed by the immunoperoxidase (Fig. 7) and radioiodine techniques (Fig. 23). The dense bodies presumed to be lysosomes, showed a doubtful immunoperoxidase reaction (Figs. 9, 10), but, with radioiodine labeling, it became clear that these organelles did not react specifically. The reactions of the nucleus and its envelope in treated sections (Fig. 7) did not exceed those in control sections (Figs. 8, 23). With reference to the cytosol, Pelletier et al. (55) reported some staining by the indirect technique. They interpreted this observation as an indication that thyroglobulin migrates through the cytosol, as in the proposal by Rothman (57) that, in pancreatic acinar cells, the intracellular enzymes bound for secretion migrate to the lumen by way of the cytosol. Our preparations did not show a definite reaction to the cytosol, except for an occasional diffuse gradient at the periphery of heavily reactive compartments (e.g., at the periphery of the luminal colloid in Fig. 9). This was interpreted as a diffusion of the DAB reaction product, a possibility proposed by Kraehenbuhl and Jamieson (36), and noted when tissue blocks were incubated with substrate (49). We concluded that there was no detectable thyroglobulin antigenicity in the cytosol.

At this point, it must be made clear that the thyroglobulin antigenicity encountered in the organelles of thyroid cells was not necessarily due to fully formed thyroglobulin. There was evidence that incomplete, nascent thyroglobulin contains antigenic determinants similar to those of the fully formed molecule (58). Even fragments as small as 700 daltons may inhibit the precipitation of native thyroglobulin by antithyroglobulin antibodies (42). There is evidence that, in the endoplasmic reticulum, thyroglobulin is not in a completed form, and at least the carbohydrate side chains are unfinished (30, 63, 69). Therefore, the local antigenicity is attributed to incomplete precursors. In the Golgi apparatus, glycosylation is completed (3), and some of the antigenicity of condensing vacuoles is due to fully formed thyroglobulin. This substance should also be responsible for the antigenicity of apical vesicles. As for the luminal colloid, the site of iodination of thyroglobulin, it is likely that the antigenic material includes both the iodinated and noniodinated forms of this substance. Finally, in colloid droplets, where proteolytic processes were known to occur by fusion with

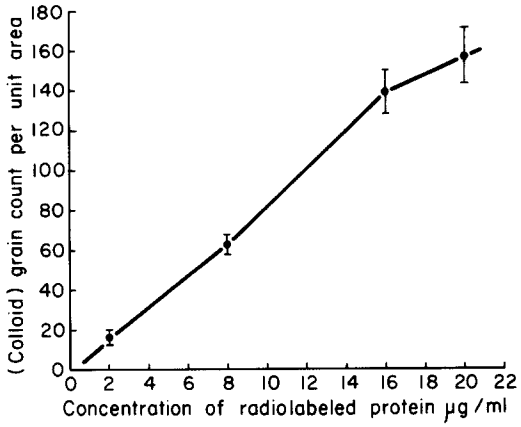


FIGURE 22 Immunolabeling of the follicular colloid as a function of the amount of radiolabeled antibodies used. Serial sections from the same block were treated with different concentrations of radiolabeled antibodies. Vertical bars represent standard deviations for the average grain counts obtained at each dilution. Grain counts were made at 20,000 magnification and expressed per microscope frame. Adjacent frames were counted in a linear sequence over the colloid surface. A total of 50 frames were counted at each dilution.

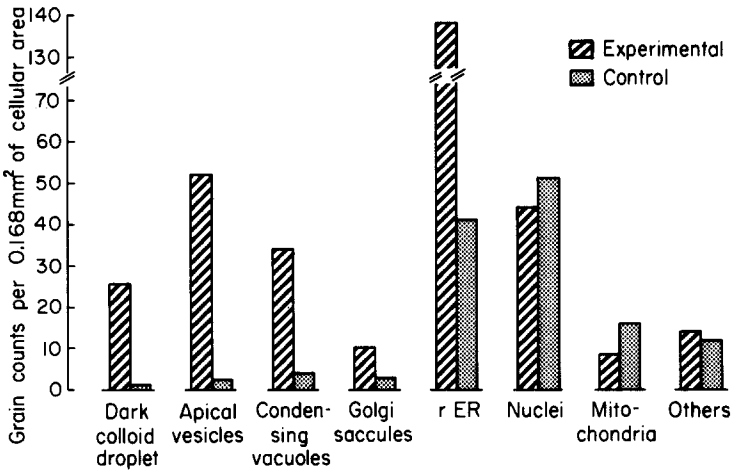


FIGURE 23 Histogram showing direct counts of silver grains (expressed per 0.168 mm² of cellular area) over the organelles of follicular cells in sections treated with radiolabeled antithyroglobulin antibodies (experimental sections), or radiolabeled normal rabbit immunoglobulin (control sections). Experimental and control sections were processed identically with respect to immunolabeling, concentration of radiolabeled proteins, and specific activity of the material used. Both were then radioautographed in an identical manner. (The microvilli were not included in the counts, and the narrow areas of cytosol between rough endoplasmic reticulum cisternae and Golgi saccules were considered to belong to these structures). To assess the errors, the photographs were examined in six groups of eight experimentals, and an equal number of control groups, and *t* tests were carried out. The experimental values were significantly higher than controls ($P < 0.001$) in the case of apical vesicles, condensing vacuoles, Golgi saccules, and rER. There was borderline significance for the dark colloid droplets, and no significant difference for nuclei, mitochondria, and "other" structures.

TABLE IV

Analysis of Direct Grain Counts in Thyroid Sections

	Counts/0.168 mm² of cellular area		Point hits		Concentrations		Specific concentrations (experimentals minus controls)
	Controls	Experimentals	Controls	Experimentals	Controls	Experimentals	
			%		*counts/% point hits*		
Dark colloid droplets	0.97	25.52	0.64	1.34	1.51	19.05	17.54
Apical vesicles	2.71	52.36	1.64	4.58	1.65	11.43	9.78
Condensing vacuoles	3.99	34.36	2.28	3.04	1.75	11.30	9.55
Golgi saccules	3.35	9.82	2.39	3.07	1.40	3.20	1.80
rER	41.34	137.76	48.68	50.49	0.85	2.73	1.88
Nucleus	50.92	44.18	29.34	27.12	1.73	1.63	−0.10
Mitochondria	16.60	8.51	9.39	5.76	1.77	1.48	−0.29
"Others"	11.97	13.74	5.63	4.58	2.13	3.00	0.87

lysosomes (2, 72), degradation products, as well as thyroglobulin itself, could be involved in the antigenicity.

Relation of Thyroglobulin Antigenicity to the Sequence of Events in the Thyroid Follicle

Radioautographic studies, after injection of labeled leucine, revealed that protein material presumed to be thyroglobulin was synthesized in the rough endoplasmic reticulum, and migrated to the Golgi apparatus (48). This conclusion was supported by our finding of thyroglobulin antigenicity in both structures. The view of Nadler et al. (48), Haddad et al. (25), and Ekholm et al. (15), that condensing vacuoles leave the Golgi apparatus in the form of apical vesicles to transfer thyroglobulin to the colloid, was in accord with the presence of antigenicity in all these locations. The

TABLE V

Analysis of Corrected Grain Counts in Thyroid Sections

	Corrected counts/0.168 mm² of cellular area		Point hits		Corrected concentrations		Corrected specific concentrations (experimental minus controls)
	Controls	Experimentals	Controls	Experimentals	Controls	Experimentals	
			%		counts/% point hits		
Correction using circles with a 80-nm radius							
Dark colloid droplets	0.01	21.84	0.78	1.34	0.01	16.30	16.29
Apical vesicles	1.30	35.56	2.13	4.58	0.61	7.76	7.15
Condensing vacuoles	1.36	21.66	2.94	3.04	0.46	7.13	6.67
Golgi saccules	0.47	11.61	3.00	3.07	0.16	3.78	3.62
rER	18.79	171.43	48.08	50.49	0.39	3.40	3.01
Correction using circles with a 160-nm radius							
Dark colloid droplets	0.98	19.11	0.78	1.34	1.26	14.26	13.00
Apical vesicles	1.64	33.88	2.13	4.58	0.77	7.40	6.63
Condensing vacuoles	1.40	17.60	2.94	3.04	0.48	5.78	5.30
Golgi saccules	1.04	6.61	3.00	3.07	0.35	2.15	1.80
rER	23.03	144.66	48.08	50.49	0.48	2.87	2.39

TABLE VI

Comparison of the Specific Concentrations Measured by Three Methods

		Corrected	
	Direct	80-nm circles	160-nm circles
Dark colloid droplets	17.5	16.3	13.0
Apical vesicles	9.8	7.2	6.6
Condensing vacuoles	9.6	6.7	5.3
Golgi saccules	1.8	3.6	1.8
rER	1.9	3.0	2.4

observation that colloid droplets were small portions of luminal colloid, removed by endocytic processes extending from the cell apex (47), was in accord with the similar antigenicity of these two types of colloid material. Finally, the digestion of the thyroglobulin contained in the colloid droplets (72), resulting in a decrease of their density (2) with release of the amino acid components, was associated with a lesser antigenicity in the more pale droplets (Figs. 13–15).

In short, antigenicity was observed in the structures involved in thyroglobulin synthesis and degradation, but was absent in nucleus, mitochondria, and other structures. When the various cell compartments were compared by measuring the specific concentrations of thyroglobulin antigenicity (Table VI), this was found least in the rough endoplasmic reticulum and Golgi saccules, was sharply increased in condensing vacuoles, and to a greater amount in apical vesicles, and reached a maximum occurrence in the colloid. These results were in accord with the prevalent view that secretory processes include a concentration of the elaborated molecules in condensing vacuoles (54).

An adequate survey of the distribution of thyroglobulin antigenicity in thyroid sections was provided by combining the pictorial qualities of the peroxidase-antibody conjugate reactions, and the quantitative assessment of the iodinated antibody reactions. Briefly, antigenicity was present along the path of thyroglobulin elaboration in follicular cells, with concentration taking place mainly in the Golgi apparatus, where the secretion was packaged for transfer to the luminal colloid. As the colloid droplets, collected from the lumen by cellular processes, were digested, their decrease in antigenicity indicated a loss of thyroglobulin.

We wish to acknowledge the assistance of Dr. John Bergeron and Dr. E. Preddie for assistance in the biochemical work, Dr. B. Kopriwa for radioautography, and Dr. N. J. Nadler for advice in the quantitation of radioautographs. This work was done with the support of the Medical Research Council of Canada. The senior author received fellowships from the Quebec Ministry of Education.

Received for publication 12 October 1976, and in revised form 5 May 1977.

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