

THE ROLE OF MYCOBACTERIA AND THE EFFECT OF
PROTEOLYTIC DEGRADATION OF THYROGLOBULIN
ON THE PRODUCTION OF AUTOIMMUNE
THYROIDITIS*

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Experimental autoimmune thyroiditis can be produced in rabbits by immunization with aqueous preparations of altered rabbit thyroglobulin (1). This apparent termination of immunological unresponsiveness to self is similar in all respects to the termination of acquired unresponsiveness to heterologous serum proteins following immunization with altered preparations of the tolerated protein (2). These observations suggest that at least some autoimmune diseases may result from stimulation of the immune mechanism by altered body components. However, this hypothesis does not explain why autoimmune thyroiditis can be easily produced by immunizing animals with unaltered homologous thyroglobulin incorporated into Freund's adjuvant, unless it is assumed that thyroglobulin is altered in the adjuvant. Such an assumption appears reasonable, since it is unlikely that a protein as labile (3-5) as thyroglobulin would reach antigen-sensitive units in an unaltered state after both being incorporated in a water-in-oil emulsion and persisting for even a short period of time in a granuloma. The present study was designed to investigate the effect of the in vivo environment established by adjuvant, including proteolytic enzymes, on both the in vivo behavior and antigenicity of thyroglobulin.

Materials and Methods

Animals.—New Zealand white rabbits weighing 2.5-3.0 kg were used throughout the study. Guinea pigs of the Hartley strain were used as the source of antibody to rabbit thyroglobulin.

Proteins.—Crystalline bovine serum albumin (BSA), lot D-71209, was purchased from Armour Pharmaceutical Co., Kankakee, Ill. Bovine hemoglobin (stock No. 303) was purchased from Reheis Chemical Co., Chicago, Ill.

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Rabbit thyroglobulin was isolated and purified as previously described (1). Fresh, unfrozen tracheas of New Zealand white rabbits were obtained from Pel-Freez, Rogers, Ark. The glands were removed, stripped relatively free of fat, minced with scissors, suspended in 0.15 M NaCl (100 g of tissue/150 ml), and passed through a tissue press. Debris was removed first by filtration through stainless steel mesh and then by centrifugation at 20,000 *g* for 30 min in a Spinco model L preparatory centrifuge. The thyroglobulin was isolated by repeated centrifugation at 105,000 *g* (6). The upper two-thirds of the fluid was aspirated and discarded, and the lower portion was decanted and retained.

Labeling of Proteins with Radioactive Iodine.—BSA and rabbit thyroglobulin were labeled with either ^{131}I or ^{125}I by the method described by McConahey and Dixon (7). Radioactivity measurement of duplicate 0.5 ml samples was made in a dual-channeled scintillation counter containing a NaI crystal monitored by two spectrometers. One spectrometer monitored for activity at the energy level of ^{131}I , and the other monitored at the energy level of ^{125}I . The spectrometer monitoring for ^{125}I activity also picked up some ^{131}I activity, while the spectrometer monitoring for ^{131}I activity did not detect ^{125}I activity. The correction factor by which the observed ^{125}I counts were adjusted to compensate for the ^{131}I overlap was obtained from the ratio given by the ^{131}I standard at the two different settings on the spectrometers. Whole-body ^{125}I activity was determined in a large scintillation counter containing a plastic crystal.

Injection and Bleeding of Rabbits.—Freund's adjuvant was injected in a total volume of 1 ml subcutaneously either beneath the footpads of all four feet or along both flanks. For both histological studies and determination of cathepsin activity at the site of injection, some rabbits were injected in the spleen with 0.05 ml of adjuvant. The spleen was exposed by a midline incision of the abdominal wall while the rabbits were under Diabotal anesthesia. A 22 gauge needle 2 inches long was inserted from the tip to the base of the spleen, and the adjuvant was deposited with a Gilmont micrometer syringe as the needle was withdrawn. The bleeding at the puncture was stopped by applying an absorbable gelatin sponge. The adjuvant was composed of 9 parts Bayol F (Humble Oil and Refining Co., Houston), 1 part Arlacel 83 (McKesson and Robbins, Los Angeles), and 10 parts of 0.15 M NaCl containing the antigens. Complete adjuvant contained 10 mg of mycobacteria/ml incorporated into the oil phase.

Twelve normal rabbits were injected with 1 ml of Freund's adjuvant containing 10 mg rabbit thyroglobulin and 10 mg mycobacteria. Five normal rabbits and five rabbits rendered immunologically unresponsive to BSA were injected with Freund's adjuvant containing 10 mg BSA and 10 mg mycobacteria. All rabbits were bled 7, 14, and 28 days after injection, and the sera were analyzed for precipitating antibody to the respective protein injected.

Rabbits injected with aqueous preparations of either native thyroglobulin or altered thyroglobulin received 15 mg of protein subcutaneously for 4 days. On the 5th day they received 15 mg intravenously. This series of injections was repeated 2 wk later, and the rabbits were bled 7 days after the last injection. 1 month after the last injection the rabbits received an intravenous injection of 15 mg of native thyroglobulin and were bled 7 days later.

Rabbits injected with either ^{125}I - or ^{131}I -labeled proteins were bled periodically, and the protein-bound radioactivity in the plasma was determined.

Induction of Immunological Unresponsiveness to BSA.—Rabbits were rendered immunologically unresponsive by neonatal injection of 500 mg of BSA during the first 5 days following birth. Rabbits treated in this manner when 3 and 5 months of age failed to show an immune elimination of 20 mg ^{131}I -BSA injected intravenously.

Antibody Analysis.—The level of circulating antibody to native thyroglobulin in rabbits was measured by hemagglutination (8). A 2.5% suspension of tannic acid-treated sheep cells was sensitized with 0.5 mg of native thyroglobulin/ml. Before use, sera were absorbed with

an equal volume of sheep erythrocytes and heated at 56°C for 20 min. Sera were also scanned for the presence of precipitating antibody by the Preer double diffusion in agar technique (9).

Guinea pig anti-rabbit thyroglobulin was analyzed by a quantitative precipitin test (10) involving the use of ^{125}I -labeled thyroglobulin. This test measures the amount of ^{125}I -thyroglobulin precipitated at equivalence by 1 ml of serum. At equivalence the antibody/antigen ratio with thyroglobulin is 1.0.

Polyacrylamide Disc Electrophoresis.—Native thyroglobulin, pepsin-degraded thyroglobulin, frozen and thawed thyroglobulin, and thyroglobulin released from adjuvant after freezing and thawing were analyzed by polyacrylamide disc electrophoresis in gel columns formed in glass tubes (7×0.5 cm). Electrophoresis was carried out at 2.5 ma/tube for 2 hr at pH 8.3 with a gel concentration of 4%. A spacer gel was not used for application of the samples. The gel columns were stained for 1 hr with a solution of 0.2% Amido black in 7.5% acetic acid.

Analytical Centrifugation.—Both native and treated preparations of rabbit thyroglobulin were analyzed in the Spinco model E ultracentrifuge with an AnD rotor at 52,640 rpm (201,366 g). Schlieren optics and a diaphragm angle of 60 were used. The temperature was controlled at 20°C. The solvent was 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate.

Preparation of Reagent Antisera.—Anti-rabbit thyroglobulin was prepared by injecting 500 g guinea pigs once a week for 3 wk with incomplete Freund's adjuvant containing 0.5, 1.0, and 5 mg of thyroglobulin, respectively. The guinea pigs were bled 2 wk later, and the sera were pooled. The pool contained 0.44 mg antibody N/ml. Anti-BSA was prepared by periodic injections of rabbits with an aqueous preparation of BSA (totaling 360 mg). The rabbits were bled after the last injection. The pooled sera contained 0.92 mg antibody N/ml.

Determination of ^{125}I -BSA and ^{125}I -Thyroglobulin in the Circulation.—Circulating ^{125}I -BSA and ^{125}I -thyroglobulin were usually measured by determining the respective protein-bound radioactivities as described above. However, in one experiment, in which rabbits were injected with Freund's adjuvant containing both ^{125}I -BSA and ^{125}I -thyroglobulin, the proteins were coprecipitated from 0.5 ml of the serum with specific antibody and unlabeled antigen added in antibody excess. 0.5 ml of serum was added to a mixture of anti-rabbit thyroglobulin (0.1 ml serum) containing either 44 μg antibody N and 10 μg nonlabeled thyroglobulin N or 184 μg anti-BSA N (0.2 ml serum) and 10 μg nonlabeled BSA N. The mixtures were incubated at 37°C for 60 min and then overnight at 0–3°C. The precipitates which formed were washed and analyzed for their specific radioactivity.

Enzyme Studies.—Normal rabbit spleens and rabbit spleens injected 3 days previously with 0.5 ml of either incomplete or complete Freund's adjuvant were analyzed for intracellular cathepsins D and E. Spleens were removed from rabbits after exsanguination, and the cells were separated from the capsule with stainless steel rakes in 0.15 M NaCl buffered at pH 7.0 with 0.01 M phosphate. The cells from five spleens were pooled, centrifuged at 1000 rpm for 10 min, and resuspended in 2.0 ml of 0.01 M phosphate buffer, pH 8.0. The debris was removed by filtration through stainless steel mesh, and the cells were disrupted with a 10-kc Raytheon oscillator for 5 min. Large cell fragments were removed by centrifugation, and the cell-free supernatant was dialyzed for 1½ hr at 0–3°C against 0.01 M phosphate buffer, pH 8.0. The supernatant was then placed on a DEAE-cellulose column (2.5×40 cm), equilibrated with 0.01 M phosphate buffer, pH 8.0, and eluted with the same buffer at 0–3°C. After the preliminary elution with the starting buffer, protein was eluted with a salt gradient of 0.01–0.3 M. The gradient was pumped through the column at a rate of 100 ml/hr, and 9 ml fractions were collected. Protein remaining on the column was eluted with 0.5 M NaCl.

Each fraction was tested for its ability to degrade denatured bovine hemoglobin into non-trichloroacetic acid (TCA)-precipitable fragments. 0.2 ml of the fractions was added to 0.4 ml

of a solution of denatured bovine hemoglobin in 0.01 M citrate buffer, pH 3.0, containing 10 mg protein/ml. The mixture was adjusted to pH 3.0, and digestion was carried out for 1 hr at 37°C. An equal volume of 5% TCA was added to precipitate the protein. 0.5 ml of the supernatant was tested for degraded protein by the Folin procedure. One unit of enzyme activity was defined as the amount of activity required to raise the optical density (at 700 m μ) of the developed Folin reaction 0.001 unit/hr.

In order to test mycobacteria for proteolytic activity, 10 mg *Mycobacterium tuberculosis* var. *hominis* was added for each milliliter of 0.15 M NaCl containing 7.5 mg of ¹³¹I-rabbit thyroglobulin/ml. The mixtures were incubated at 37°C at both pH 2.5 and 7.0. Thyroglobulin was also incubated at 37°C (pH 2.5 and 7.0) in the absence of mycobacteria. Aliquots were removed periodically during a 6 hr period, and the non-protein-bound ¹³¹I activity was determined in a scintillation counter.

The ability of cathepsin D to degrade both rabbit thyroglobulin and BSA was determined simultaneously by incubation of 0.2 ml of solutions of ¹³¹I-thyroglobulin and ¹³¹I-BSA containing 10 mg protein/ml at 37°C, pH 3.0, for 1 hr. The non-protein-bound activities were determined simultaneously in a dual-channeled scintillation counter as described above.

Pepsin Degradation of Thyroglobulin.—Rabbit thyroglobulin was degraded with pepsin (twice crystallized, Worthington Biochemical Corp., Freehold, N. J.) at both pH 2.5 and 4.8 according to the procedure described by Kaminski and Tanner (11). Degradation at pH 2.5 was carried out at 37°C for 6 hr with 1×10^{-8} mg pepsin/mg thyroglobulin, while degradation at pH 4.8 was carried out at 37°C for 1 hr with 1×10^{-8} mg pepsin/mg thyroglobulin. Degradation was stopped by adjusting the pH to 7.0. The concentration of thyroglobulin was 7.5 mg/ml in 0.15 M NaCl. Higher concentrations resulted in gross precipitation between pH 4.7 and 3.5 during adjustment of the pH to 2.5. Although the precipitation was largely reversible, going back into solution at pH 2.5 or 7.0, some irreversible aggregation was noted with higher concentrations of thyroglobulin. All pH adjustments were carried out at 0°C.

Histology.—Thyroid tissue was removed by hemithyroidectomy during the experiment and when the rabbits were sacrificed after the terminal bleeding. The tissue was fixed in Bouin's solution and embedded in paraffin, and sections were cut through the long axis. The sections were mounted and stained with hematoxylin and eosin. Spleen tissue fixed in formaldehyde was prepared for histology in the same manner.

Thyroiditis was graded according to the degree of infiltration of inflammatory cells. Lesions were graded + if at least five foci the size of one follicle or less of infiltrating cells were present in the longitudinal section of one thyroid lobe. Lesions were graded ++ if 10–20 foci were present, each of which occupied the area of several follicles. Lesions were graded +++ either if numerous foci were in each section, each of which occupied areas the size of a number of follicles, or if the entire lobe was involved with a more diffuse infiltration.

RESULTS

Serological Relationship between Mycobacteria and Thyroglobulin.—The sera of rabbits given three weekly injections of 1 ml of complete Freund's adjuvant contained neither precipitating nor hemagglutinating antibody to rabbit thyroglobulin when tested 20 days later. Furthermore, these rabbits failed to show an immune elimination of a subsequent injection of an aqueous preparation of ¹³¹I-thyroglobulin. Following injection the thyroglobulin equilibrated between intra- and extravascular spaces and was eliminated exponentially with a half-life of 2.5 days, similar to the process seen in normal rabbits (12).

Effect of Mycobacteria on Thyroglobulin.—Incubation of rabbit thyroglobulin (7.5 mg/ml) and mycobacteria (10 mg/ml) at either pH 3.0 or 7.0 and at 37°C did not result in any significant degradation of the thyroglobulin. At least 96% of the ^{131}I activity in samples taken between 30 min and 6 hr was protein-bound (precipitated with 10% TCA), the same as that observed with ^{131}I -thyroglobulin incubated in the absence of mycobacteria.

Properties of Thyroglobulin following Extraction from Adjuvant.—The incorporation of ^{131}I -labeled rabbit thyroglobulin into complete Freund's adjuvant

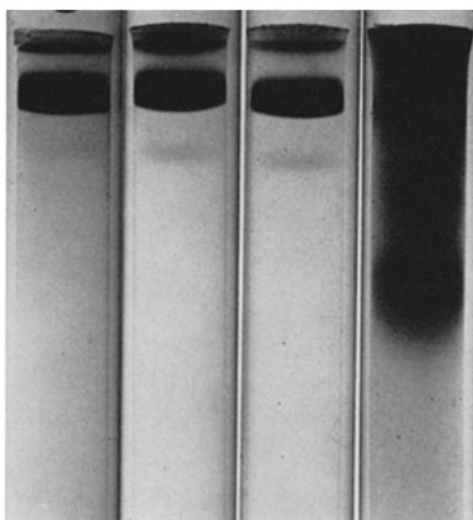


FIG. 1. Electrophoresis of rabbit thyroglobulin in polyacrylamide. Left to right: 0.1 mg native thyroglobulin; 0.1 mg frozen-thawed thyroglobulin; 0.1 mg thyroglobulin extracted from adjuvant; and 0.3 mg pepsin-degraded thyroglobulin.

did not significantly alter either its *in vivo* behavior or its migration on polyacrylamide gel. At least 90% of the ^{131}I -thyroglobulin incorporated into complete adjuvant was recovered from the aqueous phase after the emulsion was disrupted by freezing and thawing. The migration in acrylamide gel appeared identical for untreated thyroglobulin, thyroglobulin frozen and thawed, and thyroglobulin extracted from adjuvant after freezing and thawing (Fig. 1). One major band and two minor bands were detected on the gels. The band at the top of the column is the 27S thyroglobulin dimer. The heavy band is monomeric (18S) thyroglobulin, while the light band farther down the gel is probably the 12S split product. When injected intravenously into rabbits, all three preparations equilibrated between the intra- and extravascular spaces and were eliminated similarly at an exponential rate (Fig. 2).

Behavior of ^{125}I -BSA and ^{131}I -Thyroglobulin following In Vivo Release from Adjuvant.—Following subcutaneous injection of complete Freund's adjuvant containing 5 mg of ^{125}I -BSA and 5 mg of ^{131}I -thyroglobulin, a portion of both proteins escaped from the adjuvant and appeared in the serum. At maximal serum levels considerably more BSA was present than thyroglobulin (Fig. 3). Other rabbits were injected with complete adjuvant containing nonlabeled thyroglobulin and BSA subcutaneously and 1 mg of aqueous ^{125}I -BSA and ^{131}I -thyroglobulin intravenously. When similar serum levels of BSA and thyro-

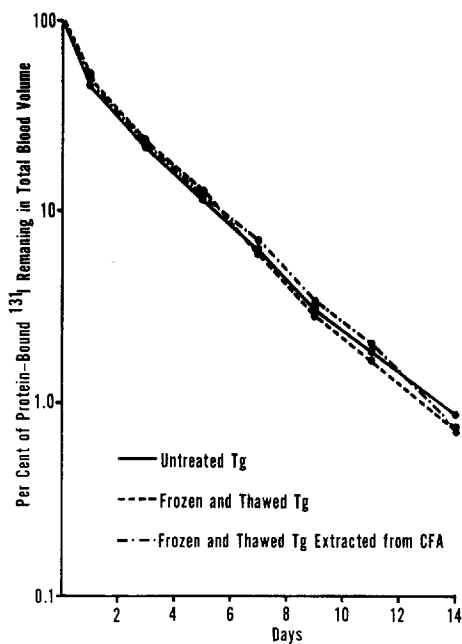


FIG. 2. Elimination of ^{131}I -labeled rabbit thyroglobulin (Tg) from the circulation of rabbits. CFA = complete Freund's adjuvant.

globulin were present in the rabbits injected intravenously, approximately 10 times more BSA than thyroglobulin was present in the circulation of rabbits injected with the labeled proteins in adjuvant. Immune elimination of both BSA and thyroglobulin appears to be initiated both in rabbits injected subcutaneously with the labeled proteins in adjuvant and rabbits injected intravenously with the labeled proteins between the 6th and 8th days. The data in Fig. 3 are the average of nine rabbits in each group from three different experiments.

The disappearance of ^{131}I activity from intact rabbits injected with either ^{131}I -BSA or ^{131}I -thyroglobulin is in agreement with the results obtained in the serum.

The whole-body ^{131}I activity was measured periodically in rabbits injected with complete Freund's adjuvant containing 10 mg of either ^{131}I -BSA or ^{131}I -thyroglobulin. From day 2 until day 6 following injection, the percentage of ^{131}I activity in the rabbits injected with ^{131}I -BSA was significantly higher than in rabbits injected with ^{131}I -thyroglobulin (Table I). On day 9 the levels of BSA and thyroglobulin activity were similar. It appears that the difference between BSA and thyroglobulin before day 9 results from the BSA which is released

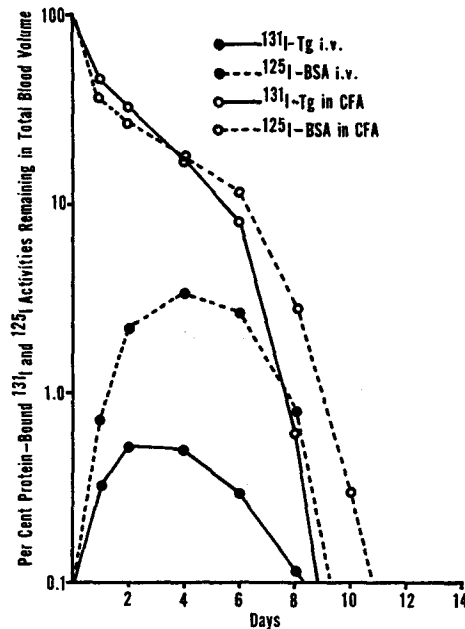


FIG. 3. In vivo behavior of rabbit thyroglobulin (Tg) and BSA following subcutaneous injection after either incorporation into complete Freund's adjuvant (CFA) or intravenous injection of aqueous preparations.

from the adjuvant persisting in the circulation. After release from the adjuvant, most of the thyroglobulin apparently does not circulate. On the 9th day the levels of BSA and thyroglobulin were approximately the same, probably as the result of an immune response to BSA and subsequent removal of the immune complexes from the circulation. The data in Table I are averages of three experiments totaling nine rabbits for each protein.

Histological Studies following Splenic Injections of Complete Freund's Adjuvant.—The histological changes were studied in spleen sections of rabbits 3 and 7 days following splenic injections of both complete and incomplete Freund's adjuvant. Spleens removed from rabbits 3 days after injection of complete

Freund's adjuvant contained discrete areas of dense infiltration of neutrophils (Fig. 9). The dense infiltration was usually seen around vacuoles presumably containing dispersed droplets of the adjuvant. By the 7th day the areas of infiltration became necrotic, resulting in apparent disruption of the neutrophils (Fig. 10). In contrast, spleen sections removed either 3 (Fig. 11) or 7 days after

TABLE I
Percentage of ^{131}I -Thyroglobulin and ^{131}I -BSA Remaining in Rabbits after Injection in Complete Freund's Adjuvant

Protein injected	Days after injection			
	2	4	6	8
	%	%	%	%
BSA	94.9	85.3	72.9	59.3
Thyroglobulin	79.8	68.7	64.2	61.4

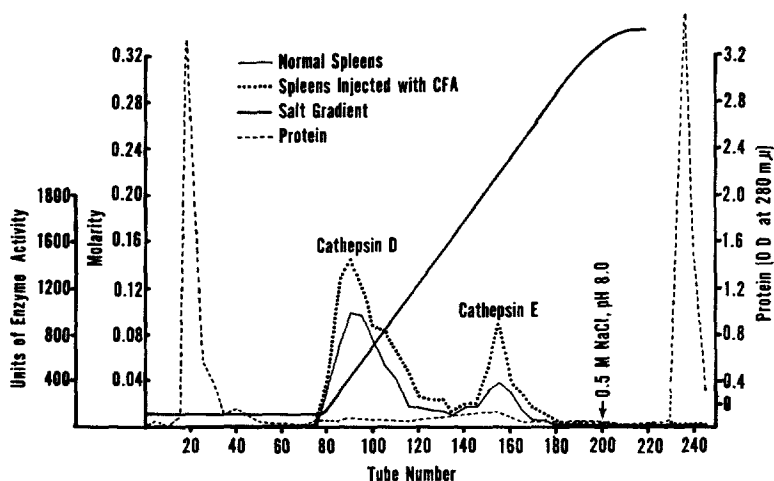


FIG. 4. Isolation of cathepsins D and E by DEAE-cellulose chromatography from extracts of spleens obtained from either normal rabbits or rabbits injected in the spleen with complete Freund's adjuvant (CFA).

splenic injections of incomplete adjuvant did not contain dense infiltration of neutrophils. A few neutrophils were observed diffusely scattered throughout the spleen, but the number was not much greater than those observed in normal spleens.

Cathepsin Activity in Spleens Injected with Adjuvant.—Extracts of both normal spleens and spleens injected 3 days previously with either complete or incomplete Freund's adjuvant were analyzed for cathepsin D and E activities.

The cathepsins were eluted from DEAE-cellulose using a salt gradient as described above. The eluted fractions were assayed for enzyme activity according to their ability to degrade denatured hemoglobin (Fig. 4). Significantly more of both cathepsin D and cathepsin E activity was isolated from spleens injected 3 days previously with complete Freund's adjuvant than from uninjected spleens. The data in Fig. 4 are an average of three experiments in which the extracts of a pool of five spleens were assayed. The average total activities per spleen injected with complete adjuvant were 3.24×10^6 units for cathepsin D and 1.10×10^6 units for cathepsin E, while the average activities per normal spleen were 2.18×10^5 units for cathepsin D and 0.64×10^6 units for cathepsin E. Two experiments were carried out in which the pooled extracts of five spleens

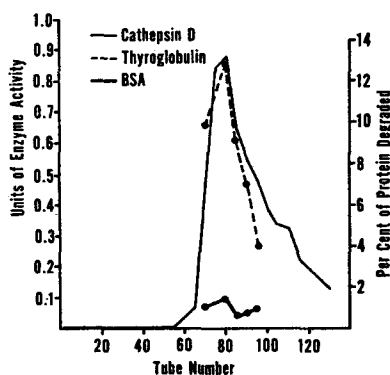


FIG. 5. Degradation of rabbit thyroglobulin and BSA by cathepsin D isolated by DEAE-cellulose chromatography from spleen extracts.

injected with incomplete adjuvant were assayed, and the activities of both cathepsins D and E were not significantly different from those obtained with normal spleens. There was no overlap in the eight experiments between the results obtained with either normal spleens or spleens injected with incomplete adjuvant and spleens injected with complete adjuvant. The average weights of normal spleens, spleens injected with complete adjuvant, and spleens injected with incomplete adjuvant were 1.53, 1.55, and 1.47 g, respectively.

Fractions of spleen extracts eluted from DEAE-cellulose and containing cathepsin D activity were tested for their abilities to degrade a mixture of ^{131}I -labeled rabbit thyroglobulin and ^{125}I -BSA at pH 3.0 into fragments not precipitable by TCA. Cathepsin D degraded the ^{131}I -thyroglobulin much more extensively during a 1 hr incubation at 37°C than it did the ^{125}I -BSA (Fig. 5).

Effect of Pepsin Degradation on the Autoimmune Response to Rabbit Thyroglobulin.—Since cathepsins D and E rapidly lose their activity and relatively large amounts are difficult to obtain in an active form, rabbit thyroglobulin was

degraded with pepsin, which has a specificity similar to that of the cathepsins in its points of cleavage (13). Rabbits given two series of injections of aqueous rabbit thyroglobulin produced no antibody to thyroglobulin and failed to develop thyroid lesions, while most of the rabbits receiving similar injections of an aqueous preparation of thyroglobulin degraded at pH 2.5 for 6 hr with 1×10^{-5} mg pepsin/mg thyroglobulin produced hemagglutinating antibody to thyroglobulin and developed lesions (Table II and Fig. 12). Some rabbits also responded to injections of thyroglobulin incubated at pH 2.5 without pepsin, but the level of antibody was considerably lower and the lesions were both less severe and less frequent. Some rabbits injected with thyroglobulin degraded at pH 4.8 at 37°C for 1 hr with 1×10^{-3} mg of pepsin/mg thyroglobulin also produced antibody to thyroglobulin and developed lesions, but injection of the

TABLE II
Production of Antibody to Thyroglobulin and Thyroiditis in Rabbits Injected with Aqueous Preparations of Pepsin-Degraded Thyroglobulin

Pepsin concn	pH	Time of incubation	Antibody*	Lesions		
				0	+	++
<i>mg/ml thyroglobulin</i>						
1×10^{-5}	2.5	6	5.3	3	6	3
0	2.5	6	1.6	7	4	0
1×10^{-3}	4.8	1	3.4	6	4	0
0	4.8	1	0.9	7	1	1
0	7.0	6	0	12	0	0

* Log_2 of hemagglutinin titer.

latter preparation was not as effective as the injection of thyroglobulin degraded for 6 hr with 1×10^{-5} mg of pepsin/mg protein. Although a few rabbits made a weak response to injections of thyroglobulin incubated at pH 4.8 without pepsin, this treatment was not very effective.

1 month after the last injection of thyroglobulin degraded with pepsin at pH 2.5, the rabbits were bled again and then given an intravenous injection of native thyroglobulin. Serum obtained 10 days later from seven of the rabbits showed an increase in the level of hemagglutinating antibody to native thyroglobulin (Table III). Six of the seven rabbits had thyroid lesions of approximately the same severity as those produced by the injections of pepsin-degraded thyroglobulin.

Effect of pH and Pepsin Degradation on Rabbit Thyroglobulin.—Analytical ultracentrifugation revealed structural alterations in rabbit thyroglobulin following either pepsin degradation or merely incubation at acid pH. Thyroglobulin alone showed one major peak of monomeric thyroglobulin (18S) and a

minor peak, apparently of thyroglobulin dimers (27S). Analysis of thyroglobulin incubated at 37°C and pH 2.5 for 6 hr without pepsin revealed four fractions (Fig. 6 A). Two fractions, apparently composed of aggregated material, sedi-

TABLE III
Effect of Injection of Native Thyroglobulin in Rabbits following an Immune Response to Pepsin-Degraded Thyroglobulin*

Rabbit	Injection of pepsin-degraded thyroglobulin		Injection of native thyroglobulin antibody†		
	Antibody	Lesion	Before	After	Lesions
1	32	+	0	8	+
2	512	—	16	1024	—
3	128	+	0	8	+
4	64	++	0	8	++
5	32	++	0	16	++
6	512	+	4	32	++
7	256	+	4	512	+

* Injection of 0.15 mg native thyroglobulin given 5 wk after the last injection of pepsin-degraded thyroglobulin.

† Reciprocal of the highest dilution of serum showing complete agglutination of sheep red cells coated with native rabbit thyroglobulin.

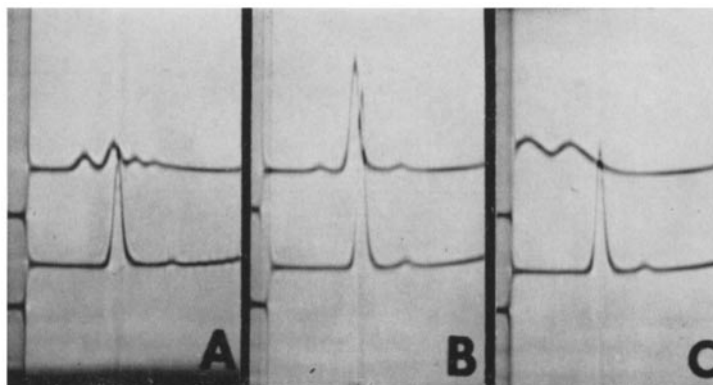


FIG. 6. Ultracentrifugal patterns of preparation of rabbit thyroglobulin at 201,366 *g*. A. 24 min: top, 0.7% thyroglobulin incubated at pH 2.5 for 6 hr at 37°C; bottom, 0.3% untreated thyroglobulin. B. 32 min: top, 0.3% thyroglobulin incubated at pH 4.8 for 1 hr at 37°C; bottom, 0.3% untreated thyroglobulin. C. 28 min: top, 0.7% thyroglobulin digested with 1×10^{-5} mg pepsin/mg at pH 2.5 for 6 hr at 37°C; bottom, 0.3% untreated thyroglobulin.

mented faster than native thyroglobulin, while one fraction sedimented considerably more slowly. The fourth fraction sedimented at a slightly slower rate than the native thyroglobulin. Thyroglobulin incubated under identical conditions, however, in the presence of 1×10^{-5} mg pepsin/mg protein, showed two

broad peaks sedimenting more slowly than native thyroglobulin (Fig. 6 C). The leading edge of the fastest sedimenting peak contained a small amount of material sedimenting as fast as, or even slightly faster than, native thyroglobulin. Polyacrylamide gel analysis demonstrated that this preparation contained at least four different fractions (Fig. 1). The majority of the protein from thyroglobulin incubated at pH 4.8 without pepsin for 1 hr sedimented in the ultracentrifuge at a rate similar to that of native thyroglobulin (Fig. 6 B). A

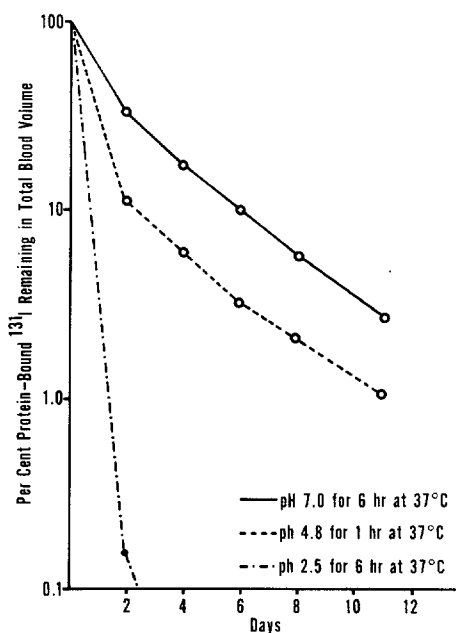


FIG. 7. The effect of pH on the elimination of rabbit thyroglobulin from the circulation of rabbits.

small amount of material sedimented more slowly. The thyroglobulin dimers were present in native thyroglobulin preparations and in preparations incubated at pH 4.8, but were absent in preparations incubated at pH 2.5 either with or without pepsin.

The effect of acid pH on thyroglobulin is further illustrated by the *in vivo* behavior of rabbit thyroglobulin after incubation at either pH 2.5 for 6 hr (37°C) or pH 4.8 for 1 hr (37°C). ¹³¹I-labeled thyroglobulin degraded with pepsin at either pH 2.5 or 4.8 was completely eliminated from the circulation of rabbits during the first 24 hr following intravenous injection. Furthermore, ¹³¹I-thyroglobulin incubated at either pH 2.5 or 4.8 without pepsin behaved differently following intravenous injection than native thyroglobulin (Fig. 7).

^{131}I -Thyroglobulin incubated at pH 7.0 for 6 hr at 37°C following equilibration between intra- and extravascular spaces was eliminated at an exponential rate with a half-life of 2.5 days (Fig. 8), similar to that of unincubated thyroglobulin (12). Over 98% of ^{131}I -thyroglobulin incubated at pH 2.5 for 6 hr at 37°C was eliminated during the first 2 days following intravenous injection. Approximately 50% of ^{131}I -thyroglobulin incubated at pH 4.8 for 1 hr at 37°C was eliminated shortly after injection, while the remaining portion was eliminated at a rate with a half-life similar to that of native thyroglobulin. The 50% that was

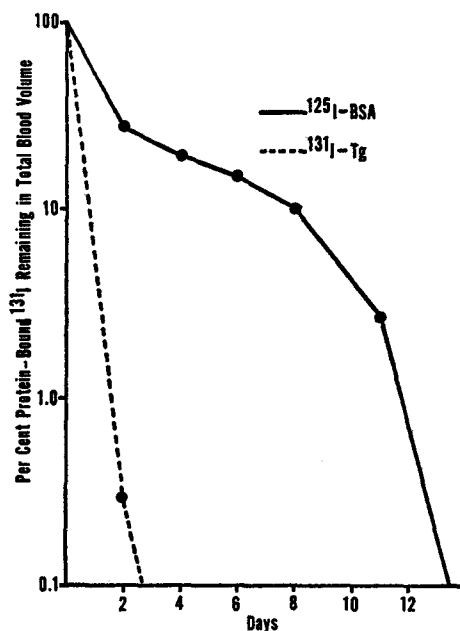


FIG. 8. The effect of incubation of thyroglobulin (Tg) and BSA at pH 2.5 for 6 hr at 37°C on their elimination from the circulation.

rapidly eliminated is considerably greater than the degree of alteration indicated by analytical centrifugation (Fig. 6 B).

In contrast to thyroglobulin, BSA was resistant to incubation at acid pH. A mixture of ^{125}I -BSA and ^{131}I -thyroglobulin was incubated at 37°C for 6 hr at pH 2.5 and after being adjusted to pH 7.0 was injected intravenously into rabbits. The ^{131}I -thyroglobulin was rapidly eliminated from the circulation, while the ^{125}I -BSA equilibrated between intra- and extravascular spaces and was eliminated at an exponential rate similar to that observed with untreated BSA (Fig. 8). A characteristic immune elimination of the ^{125}I -BSA occurred after the 8th day.

Immune Response to Rabbit Thyroglobulin and BSA in Complete Adjuvant.—The production of precipitating antibody in normal rabbits injected with thyroglobulin in adjuvant and in both normal rabbits and rabbits unresponsive to BSA injected with BSA in adjuvant is shown in Table IV. Normal rabbits immunized with rabbit thyroglobulin contained precipitating antibody in their sera on days 7, 14, and 28, with the maximal level on day 14. Normal rabbits immunized with BSA contained precipitating antibody to BSA in their sera on days 7, 14, and 28, with the maximal level on day 28. The level of antibody in the normal rabbits injected with BSA was considerably higher than in the normal rabbits injected with rabbit thyroglobulin. All of the rabbits unresponsive to BSA retained their unresponsive state following injection of BSA in adjuvant, failing to produce any precipitating antibody.

TABLE IV
*Production of Precipitating Antibody in Rabbits Immunized with Either Rabbit Thyroglobulin or BSA in Complete Freund's Adjuvant**

Rabbit Type	No.	Protein injected	Antibody		
			7 days	14 days	28 days
			<i>μgN/ml serum</i>		
Normal	12	Tg	4.9	7.7	5.7
Normal	5	BSA	35.2	86.9	206.8
Unresponsive to BSA	5	BSA	0	0	0

* 1 ml containing 10 mg protein and 10 mg mycobacteria.

DISCUSSION

The above data lend support to the contention that the initial event responsible for autoimmune thyroiditis in rabbits injected with homologous thyroglobulin in complete Freund's adjuvant is *in vivo* alteration of the thyroglobulin following injection. It is suggested that the alteration is the result of the local environment in the granuloma, caused by the mycobacteria in the adjuvant. The most significant events that probably lead to alteration are infiltration of neutrophils with subsequent release of proteolytic enzymes, and an increase in hydrogen ion concentration at extracellular foci. The available data suggest the same conditions do not develop following injection of thyroglobulin in incomplete Freund's adjuvant.

Thyroglobulin apparently is not altered significantly either during incorporation into complete Freund's adjuvant or by proteolytic enzymes present in lyophilized mycobacteria after incorporation. When the water-in-oil emulsion of complete Freund's adjuvant was broken by freezing and thawing, the thyroglobulin could be aspirated from the aqueous phase in an apparently unaltered state. The recovered thyroglobulin behaved both *in vivo* and on polyacrylamide

gel as did native thyroglobulin. Furthermore, incubation of thyroglobulin at 37°C and pH 3.0 or 7.0 with high concentrations of mycobacteria did not result in degradation of the thyroglobulin.

It seems most likely that alteration of thyroglobulin injected in complete Freund's adjuvant occurs *in vivo* and is initiated shortly after injection. Significant amounts of both bovine serum albumin and rabbit thyroglobulin were released from complete Freund's adjuvant within the first 24–48 hr following injection; however the BSA, but not the thyroglobulin, persisted in the circulation. Apparently the thyroglobulin was sufficiently altered in the developing granuloma so that it either did not enter the circulation or was rapidly eliminated shortly after it did enter the circulation. The difference between the *in vivo* stability of thyroglobulin and BSA is further evidenced by the *in vitro* effect of pH on the *in vivo* behavior of these proteins. Rabbit thyroglobulin incubated at 37°C for 6 hr at pH 2.5 was sufficiently altered as to be almost completely eliminated from the circulation within 48 hr after injection, whereas BSA treated in an identical manner remained unaltered and, following intravenous injection, behaved identically with native BSA. Thyroglobulin incubated at 37°C for 1 hr at pH 4.8 was largely altered, in that 50% was rapidly eliminated following injection. It is well known that thyroglobulin is rapidly denatured at pH 4.5 and below (14, 15). A slower denaturation also occurs at pH 4.8 (14). The relative instability of thyroglobulin at alkaline pH, in neutral salt at temperatures above 53°C, and in low ionic strength solvents is well established (see ref. 3.).

Alteration of thyroglobulin *in vivo* following injection is most likely the result of infiltration of neutrophils around depositions of the adjuvant. It is likely that proteolytic enzymes (cathepsins) released from the lysosomes of neutrophils are involved in the *in vivo* alteration of thyroglobulin by partial degradation. Since the cathepsins are active only at relatively high hydrogen ion concentration, the pH at the site of degradation would have to be low. Rous (16) obtained evidence that the pH within leukocyte granules was 3.0 or less following phagocytosis of particles. Several groups of workers subsequently demonstrated that a heightened production of lactate occurs under the anaerobic conditions of phagocytosis (17–19). In addition, McCarty et al. (20) showed that joint fluid in which neutrophils were occurring showed a fall in pH over a period of time which was a function of the number of neutrophils present. In the present study, rabbit spleens injected with complete Freund's adjuvant within 3 days contained a marked infiltration of neutrophils around the adjuvant deposit, whereas infiltration of neutrophils in spleens injected with incomplete adjuvant (without mycobacteria) was not significant. Likewise, rabbits injected with thyroglobulin incorporated into complete adjuvant produce autoantibodies and develop thyroiditis, whereas neither is produced in rabbits injected with thyroglobulin in incomplete adjuvant. Furthermore, significantly greater amounts of cathepsins

D and E were isolated from extracts of rabbit spleens injected with complete adjuvant than from extracts of either normal rabbit spleens or spleens injected with incomplete adjuvant.

Cathepsin D was much more effective in the degradation of thyroglobulin than BSA at pH 2.5. Since thyroglobulin is denatured at this pH, preferential degradation of thyroglobulin may be the result of cathepsin D being more effective in the degradation of denatured than of native proteins. A similar situation may occur *in vitro* when thyroglobulin is denatured at local sites or within cells where a high concentration of hydrogen ions exists.

Partial degradation of rabbit thyroglobulin with pepsin, which has a specificity of cleavage similar to that of the cathepsins, renders the thyroglobulin antigenic in rabbits. Rabbits given two series of injections of aqueous preparations of rabbit thyroglobulin partially degraded at acid pH with pepsin produced antibody to native thyroglobulin and developed thyroiditis. Injection of thyroglobulin at acid pH without pepsin also resulted in production of antibody and thyroiditis, but the incidence and severity were less than when pepsin was added. Once the natural unresponsive state to thyroglobulin was terminated following injection of pepsin-degraded thyroglobulin, the rabbits responded to a subsequent injection of native thyroglobulin. These observations with enzyme- and pH-altered thyroglobulin are similar to those made previously with chemically altered thyroglobulin, where injections of rabbits with aqueous preparations of rabbit thyroglobulin coupled to the diazonium derivatives of arsanilic and sulfanilic acids resulted in both antibody and thyroiditis (1). Rabbits treated in the latter manner also responded to a subsequent injection of native thyroglobulin given 1 month later. Stylos and Rose (21) previously reported that injection of rabbits with aqueous preparations of extracts of rabbit thyroid degraded with papain resulted in antibody to native thyroglobulin. However, it was not noted whether thyroid lesions also developed.

Mycobacteria probably play other roles in the induction of autoimmune thyroiditis in the rabbit than indirectly causing an alteration of thyroglobulin. Once alteration occurs and the altered thyroglobulin is recognized as an antigen, the mycobacteria undoubtedly cause an enhanced immune response. In addition, mycobacteria in adjuvant would be expected to institute a state of delayed hypersensitivity. However, there is no evidence that delayed hypersensitivity is responsible for autoimmune thyroiditis in the rabbit (22). The role that mycobacteria play may be different for other experimental autoimmune diseases. It is possible that some tissue antigens may be sufficiently altered during isolation and/or purification and the role played by mycobacteria may be only the enhancement of the immune response and establishment of cellular sensitivity.

The inability of injections of BSA incorporated into complete Freund's adjuvant to terminate acquired immunological unresponsiveness to BSA may be the result of the relative stability of BSA in the *in vivo* environment established

by the mycobacteria. Rabbits rendered unresponsive to BSA by neonatal injections of BSA did not respond to BSA incorporated into complete Freund's adjuvant, while normal rabbits injected with thyroglobulin in complete adjuvant lost their unresponsive state and developed antibody to thyroglobulin by the 7th day, and severe thyroiditis was observed by the 14th day. This difference could be explained by a difference in the mechanisms involved in acquired immunological unresponsiveness to heterologous serum proteins and those involved in natural immunological unresponsiveness to self. However, it seems more likely that the difference is the result of the differential *in vivo* stability of BSA and thyroglobulin following injection in complete adjuvant. The unresponsive state to BSA is readily terminated following injections of certain altered preparations of BSA, with or without the use of Freund's adjuvant (2, 23).

SUMMARY

Data are presented which suggest that the initial event involved in experimental autoimmune thyroiditis following injection of rabbits with homologous thyroglobulin in complete Freund's adjuvant is alteration of the thyroglobulin. Alteration of the thyroglobulin does not occur during incorporation into the adjuvant or *in vitro* storage in the adjuvant, and the mycobacteria in the adjuvant have no direct effect on the thyroglobulin. Most likely, the alteration results from an increase in hydrogen ion concentration within cells or local areas in the granuloma and the subsequent action of proteolytic enzymes. These conditions are probably established in the granuloma as the result of neutrophilic response to the mycobacteria in the adjuvant.

Rabbits injected with aqueous preparations of homologous thyroglobulin partially degraded *in vitro* with pepsin at acid pH produced antibody to native thyroglobulin and developed thyroiditis. Most of these rabbits responded to a subsequent injection of native thyroglobulin given 1 month later.

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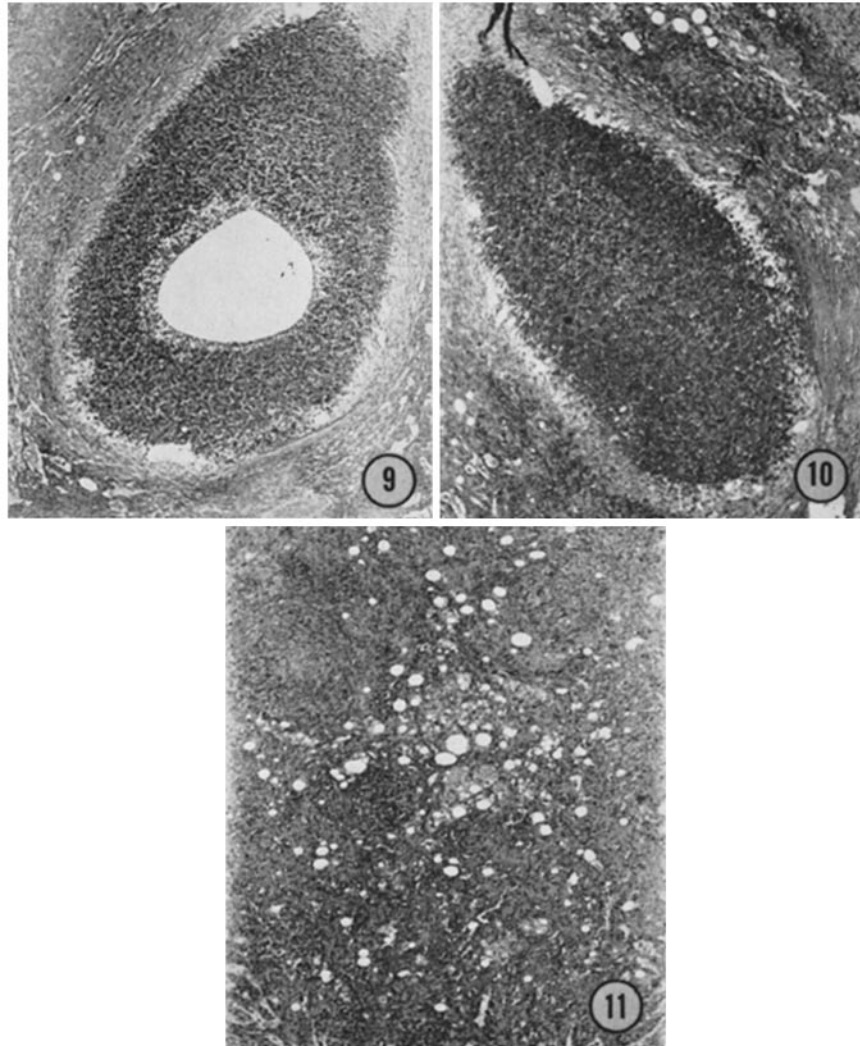


FIG. 9. Section of spleen taken from a rabbit 3 days after injection of complete Freund's adjuvant into the spleen. Hematoxylin and eosin, $\times 36$.

FIG. 10. Section of spleen taken from a rabbit 7 days after injection of complete Freund's adjuvant into the spleen. Hematoxylin and eosin, $\times 36$.

FIG. 11. Section of spleen taken from a rabbit 3 days after the injection of incomplete Freund's adjuvant into the spleen. Hematoxylin and eosin, $\times 36$.

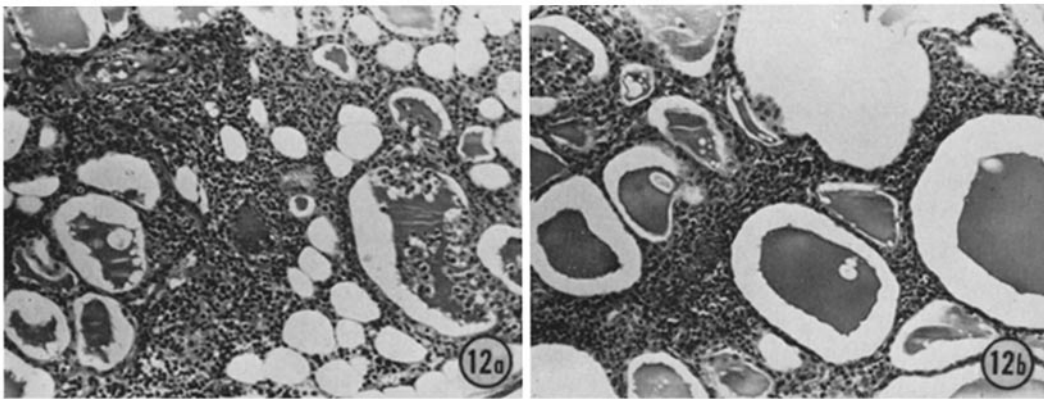


FIG. 12. Sections of thyroid taken from a rabbit 7 days after the last of a series of injections of an aqueous preparation of pepsin-degraded thyroglobulin. Hematoxylin and eosin. a, $\times 68$; b, $\times 137$.