THE INDUCTION OF AUTOIMMUNITY IN RABBITS FOLLOWING INJECTION OF HETEROLOGOUS OR ALTERED HOMOLOGOUS THYROGLOBULIN*

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It has been shown previously that immunological tolerance to bovine serum albumin (BSA) induced in rabbits by neonatal injection of BSA was terminated following injection of certain cross-reacting albumins (1). Similarly, the BSA-tolerant state was terminated following injection of certain preparations of BSA which were structurally altered but still cross-reacted with native BSA (2). On the basis that acquired tolerance induced to BSA in neonatal rabbits may be the same as tolerance to self constituents, it was postulated that the termination of tolerance to BSA in rabbits involves mechanisms similar to those involved in the induction of autoimmunity (2, 3). Thus, either an individual could contact a substance related to a body constituent or as a result of inflammation, trauma, *etc.*, a body constituent could become altered. In either event, an autoimmune response might result with accompanying disease.

The present study is an attempt to investigate the ability of injections of a homologous, altered tissue component to produce autoimmunity in the rabbit. Rabbit thyroglobulin was selected as the tissue component, since it is known to be involved in an autoimmune phenomenon in the rabbit (4) and it can be obtained in a relatively pure state. The thyroblogulin was altered by several different methods including coupling it to diazonium derivatives, since the injection of BSA-tolerant rabbits with similarly altered preparations of BSA resulted in the termination of the tolerant state (2). The immune response of rabbits to cross-reacting bovine thyroglobulin also was investigated.

Materials and Methods

Isolation and Purification of Thyroglobulin.—Rabbit and bovine thyroglobulin were isolated from thyroid extracts and purified by a modification of the method described by Edelhoch

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(5). Tracheae of New Zealand white rabbits were obtained from Pel-Freeze, Rogers, Arkansas. The lobes of the thyroids were removed and stripped relatively free of fat. The glands were minced with a scalpel, suspended in 0.15 M NaCl (100 gm of tissue/150 ml) and passed through a tissue press. Tissue debris was removed by filtration through glass wool and centrifugation at 20,000 *g* for 30 minutes, in a Spinco model L preparatory centrifuge. The thyroglobulin was isolated and purified by centrifugation at 105,000 *g*. Following centrifugation the thyroglobulin was present in the lower one-third of the tube. The upper two-thirds of the fluid was aspirated and the lower portion decanted. A small amount of heavy material forming a button at the bottom of the tube was discarded. The thyroglobulin was purified further by repeated centrifugation at 105,000 *g*. Bovine thyroids were obtained from a local slaughter house and the thyroglobulin isolated and purified as described above.

Modification of Rabbit Thyroglobulin.—Rabbit thyroglobulin was coupled to diazonium derivatives by the method described by Baker *et al.* (6). The diazonium derivatives were prepared by dissolving 0.069 gm of sulfanilic acid or 0.078 gm of arsanilic acid in a mixture of 0.9 ml of 1 N HCl and 0.72 ml of 0.5 N $NaNO_2$. The solutions were brought to 5 ml with distilled water and added dropwise, at 0°C with constant stirring, to 0.5 gm of thyroglobulin in 30 ml of phosphate buffer, pH 7.5 and $\mu = 0.1$. The pH was maintained at 7.5 to 7.8 by the addition of 0.1 N NaOH. The final pH was adjusted to 7.8 and the conjugate stored overnight at 0–3°C. The non-coupled derivative was removed from the modified thyroglobulin either with sephadex G-25 or by extensive dialysis. The number of azo linkages was estimated spectrophotometrically at 335 mμ employing an extinction coefficient of 26,000 (7). Sulfanil-thyroglobulin was prepared by adding 2.5 ml of the diazonium derivative of sulfanilic acid to 0.5 gm of thyroglobulin, while arsanil-sulfanil-thyroglobulin was prepared by simultaneously adding 2.5 ml of the diazonium derivative of arsanilic acid and 2.5 ml of the diazonium derivative of sulfanilic acid.

Picryl-thyroglobulin was prepared by coupling thyroglobulin to picryl chloride (8). A 10 per cent solution of picryl chloride was prepared in distilled acetone and 0.45 ml added to 30 ml of 0.15 M NaCl containing 300 mg of thyroglobulin and 15 mg of Na_2CO_3 at 0°C. The non-reactive picryl chloride was removed by extensive dialysis against 0.14 M NaCl containing 0.01 M phosphate buffer, pH 7.2. Under these conditions the picryl-thyroglobulin remained in solution. The extent of picrylation was determined spectrophotometrically at 350 mμ employing a molar extinction coefficient of 18,200. The extinction coefficient was calculated from data obtained from analysis of picryl–epsilon aminocaproic acid.

Heat dissociated thyroglobulin was prepared by heating a 0.3 per cent solution of thyroglobulin at 65°C in 0.01 M carbonate buffer, pH 9.6, for 15 minutes (9). The dissociated thyroglobulin was dialyzed for 48 hours against 0.14 M NaCl buffered at pH 7.2 with 0.01 M phosphate.

Analytical Centrifugation.—Both native and altered preparations of rabbit thyroglobulin were analyzed in the Spinco model E ultracentrifuge with an AnD rotor at 59,780 RPM (259,697 *g*). The temperature was controlled at 20°C. Schlieren optics and a diaphragm angle of 60 were used. The solvent was 0.15 M NaCl buffered at pH 7.2 with 0.01 M phosphate. Concentrations of the thyroglobulin ranged between 3.0 and 6.1 mg/ml.

Injecting and Bleeding of Rabbits.—New Zealand white rabbits weighing 2.5 to 3.0 kg were injected with either native or modified preparations of thyroglobulin. The injections were made with soluble preparations, preparations precipitated by alum or preparations incorporated into Freund's adjuvant. The adjuvant was composed of 1 part arlacel C (Atlas Powder Co., Wilmington, Delaware), 9 parts bayol F (ESSO Standard Oil Co. of New Jersey) and 10 parts of 0.15 M NaCl containing the thyroglobulin preparation. With the exception of one experiment mycobacteria were omitted from the adjuvant.

Preparation of Guinea Pig Anti-Rabbit Thyroglobulin.—Guinea pigs weighing 400 to 500 gm were injected subcutaneously each week for 3 weeks with incomplete Freund's adjuvant con-

taining 5 mg of native rabbit thyroglobulin. The guinea pigs were exsanguinated 14 days after the last injection and the antisera obtained from the bloods were pooled.

Histology.—Thyroid tissue was usually taken both by biopsy during the experiment and when the rabbits were sacrificed after the terminal bleeding. One lobe of the gland was usually fixed in Bouin's solution, embedded in paraffin, and sections cut through the long axis. The sections were stained with hematoxylin and eosin. At times the lobes were divided across the long axis and one-half frozen for fluorescent antibody studies and the other sectioned and stained with hematoxylin and eosin.

The grading of thyroiditis was determined by the degree of inflammatory cellular infiltration. The lesions were graded + if at least 5 foci (the size of one follicle or less) of infiltrated cells were present in the longitudinal section of one lobe. Lesions were graded ++ if 10 to 20 foci were present which occupied areas of the size of several follicles. Lesions were graded +++ if numerous foci were present in each section which occupied areas the size of a number of follicles (Figs. 1 *a* to 1 *c*).

The fluorescent antibody technique used for detecting tissue components was that described by Coons and Kaplan (10) with minor modifications (11).

Antibody Analyses.—The levels of circulating antibody were measured by both the quantitative precipitin (12) and hemagglutination (13) techniques. In the hemagglutination technique a 2.5 per cent suspension of tannic acid–treated sheep erythrocytes was sensitized with 0.5 mg of native thyroglobulin per ml. Before use, sera were absorbed with an equal volume of sheep erythrocytes and heated at 56°C for 20 minutes. Qualitative tests for the presence of precipitating antibody were performed by double diffusion in agar. Immunoelectrophoretic analyses were made with the agafor 1 apparatus (National Instrument Laboratories, Washington, D. C.) using the technique described by Scheidegger (14). Electrophoresis was performed in 2 per cent lonagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) at 40 v and 30 ma for 30 minutes.

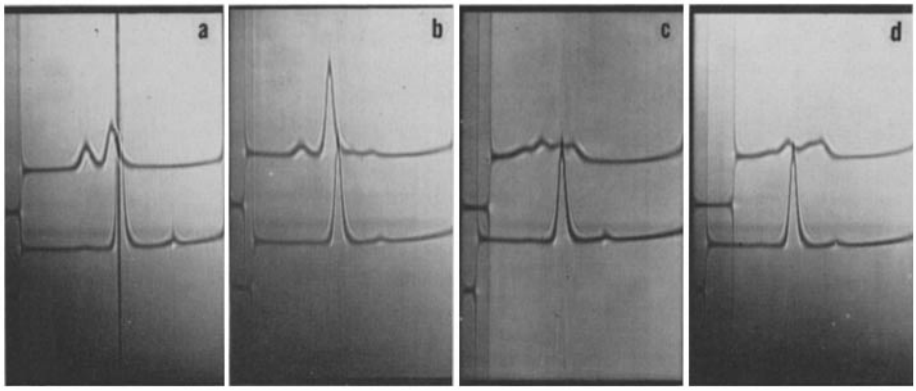
The measurement of cross-reacting guinea pig antibody to rabbit thyroglobulin was accomplished by both direct precipitation and inhibition of precipitation. In the inhibition test the guinea pig anti-rabbit thyroglobulin was first absorbed at equivalence with the cross-reacting protein. After removal of the precipitate a large excess of the cross-reacting protein was added and the mixture incubated for 30 minutes at 37°C. The amount of antibody remaining which could precipitate with native rabbit thyroglobulin was measured by direct precipitation.

In one experiment, hemagglutination tests for native thyroglobulin were performed both prior to and after treatment of the sera with 2-mercaptoethanol. The sera were diluted twofold with 0.15 M NaCl and dialyzed overnight at room temperature against 0.1 M 2-mercaptoethanol in 0.15 M NaCl. The sera were then dialyzed for 24 hours at 0–3°C against 0.01 M iodoacetamide in 0.15 M NaCl. The excess iodoacetamide was removed by dialysis against 0.15 M NaCl.

RESULTS

Properties of Native and Altered Thyroblogulin.—Ultracentrifuge patterns of purified native thyroglobulin of the rabbit in Text-figs. 1 *a* to 1 *d* show that the majority of the protein sedimented as a single component, accompanied by a slight trace of a component sedimenting slightly faster. Following modification of the thyroglobulin the sedimenting characteristics usually changed. Thyroglobulin heated at 65°C at pH 9.6 dissociated into 2 slower sedimenting components (Text-fig. 1 *a*) as described previously for bovine thyroglobulin (9). Extensive dissociation following the coupling of diazonium derivatives to thryo-

globulin apparently occurs. The ultracentrifuge patterns of arsanil-sulfanil-thyroglobulin (Text-fig. 1 *d*) and sulfanil-thyroglobulin (Text-fig. 1 *c*) show approximately 4 components. One of these components sedimented at approximately the same rate as native thyroglobulin and the others sedimented slower. Following picrylation of thyroglobulin a slower sedimenting component appeared (Text-fig. 1 *b*), however, the major portion of the preparation sedimented at a rate approximately the same as that of native thyroglobulin. Despite the presence of multiple components of the modified thyroglobulin preparations observed by analyses in the ultracentrifuge, these preparations showed only a single line following immunoelectrophoresis (Text-figs. 2 *a* to 2 *c*). Sulfanil-



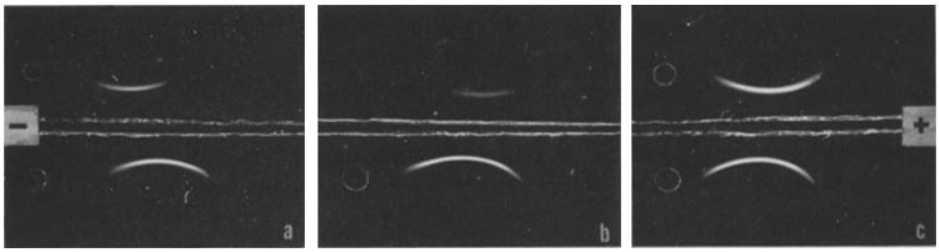
TEXT-FIGS. 1 *a* to 1 *d*. Ultracentrifuge patterns of native and altered thyroglobulin. 259,697 *g*. (*a*) 20.2 minutes. Top, 0.30 per cent heated thyroglobulin. Bottom, 0.61 per cent native thyroglobulin. (*b*) 16.2 minutes. Top, 0.31 per cent picryl-thyroglobulin. Bottom, 0.33 per cent native thyroglobulin. (*c*) 16.1 minutes. Top, 0.32 per cent sulfanil-thyroglobulin. Bottom, 0.31 per cent native thyroglobulin. (*d*) 16.2 minutes. Top, 0.31 per cent arsanil-sulfanil-thyroglobulin. Bottom, 0.36 per cent native thyroglobulin.

thyroglobulin and picryl-thyroglobulin migrated slightly faster than native thyroglobulin, while heated thyroglobulin migrated slower.

The arsanil-sulfanil-thyroglobulin and sulfanil-thyroglobulin contained approximately 50 azo linkages per molecule and the picryl-thyroglobulin contained 75 picryl groups per molecule of the original thyroglobulin.

All of the altered rabbit thyroglobulin preparations cross-reacted with guinea pig antibody to native rabbit thyroglobulin (Table I). Based on direct precipitation, heated and picrylated thyroglobulin cross-reacted about 60 per cent with anti-rabbit thyroglobulin. Sulfanil-thyroglobulin cross-reacted about 40 per cent, while arsanil-sulfanil-thyroglobulin cross-reacted only about 16 per cent. However, inhibition studies showed that all 4 preparations inhibited the precipitation of antithyroglobulin by native thyroglobulin 100 per cent. As

measured by inhibition bovine thyroglobulin cross-reacted 28 per cent with the guinea pig antibody to rabbit thyroglobulin.



TEXT-FIGS. 2 *a* to 2 *c*. Immunoelectrophoretic patterns. In all figures the arc at the bottom was formed with native rabbit thyroglobulin. The arc at the top in *a* was formed with heated thyroglobulin, in *b* with sulfanil-thyroglobulin (a similar pattern formed with arsanil-sulfanil-thyroglobulin), and in *c* with picryl-thyroglobulin. Guinea pig antibody to native rabbit thyroglobulin was placed in the troughs.

TABLE I

The Reaction of Guinea Pig Anti-Rabbit (Native) Thyroglobulin with Native Rabbit Thyroglobulin, Altered Rabbit Thyroglobulin and Bovine Thyroglobulin

Tg preparation tested	Antibody N precipitated/ ml	Cross-reaction	Inhibition
	mg	*per cent*	*per cent*
Rabbit-native	0.324		
Rabbit-picryl	0.201	62.2	100
Rabbit-dissociated	0.194	60.0	100
Rabbit-sulfanil	0.149	46.1	100
Rabbit-arsanil-sulfanil	0.053	16.4	100
Bovine-native	—	—	28

Inhibition tests were performed by adding 5 to 50 times the amount of antigen needed to precipitate the antibody at equivalence. (Fifty times the equivalent amount of bovine thyroglobulin was used.)

Tg, thyroglobulin.

Antibody and Thyroiditis Following Injection of Altered Thyroglobulin.—

Experiment 1: Rabbits were injected once a week for 4 weeks with 4 ml of incomplete Freund's adjuvant containing 6 mg of either native or arasnil-sulfanil-thyroglobulin (batch 1) and bled periodically. In the rabbits injected with arsanil-sulfanil-thyroglobulin precipitating antibody to both native and arsanil-sulfanil-thyroglobulin appeared in the sera by 14 days after the first injection and reached a maximum between 21 and 34 days (Table II). Precipitating antibody to arsanil-sulfanil-thyroglobulin was still present on day 55,

but precipitating antibody to native thyroglobulin could not be detected. The level of precipitating antibody to arsanil-sulfanil-thyroglobulin was approximately 5 times as high as the level of precipitating antibody to native thyroglobulin (Table III). Precipitating antibody was not detected in the sera of the rabbits injected with native thyroglobulin. The sera of rabbits injected with arsanil-sulfanil-thyroglobulin contained high levels of hemagglutinating antibody to native thyroglobulin, while hemagglutinating antibody to native thyroglobulin usually could not be detected in the sera of rabbits injected with native thyroglobulin. One lobe of the thyroid gland was removed on day 34 after the first injection and the other lobe was removed on day 55, when the

TABLE II

Production of Precipitating Antithyroglobulin in Rabbits Injected with Arsanil-Sulfanil-Thyroglobulin

Rabbit No.	Days after the first injection*							
	14		21		34		55	
	A.S.‡	Nat.§	A.S.	Nat.	A.S.	Nat.	A.S.	Nat.
1	+	+	+	+	+	+	Died	
2	−	−	−	−	−	−	−	−
3	+	+	+	+	+	+	+	−
4	+	−	+	+	+	+	+	−
5	−	−	+	+	+	+	Died	
6	+	−	+	+	+	+	+	−
7	−	−	−	−	−	−	Died	
8	−	−	−	−	−	−	−	−

* Injected on days 0, 7, 14, and 21.

‡ A.S., arsanil-sulfanil-thyroglobulin.

§ Nat., native thyroglobulin.

rabbits were sacrificed. Histological examination of the tissue taken on either day failed to reveal any significant thyroiditis. One rabbit (No. 6) injected with arsanil-sulfanil-thyroglobulin contained several small foci of inflammation in the thyroid tissue taken on day 34 but no lesions were present in the tissue taken on day 55.

Examination of arsanil-sulfanil-thyroglobulin in the ultracentrifuge revealed that it was, in large part, dissociated. In order to study the effect of dissociation alone on the antigenicity of rabbit thyroglobulin, rabbits were injected with thyroglobulin dissociated by heat and with picryl-thyroglobulin and sulfanil-thyroglobulin which on examination in the ultracentrifuge appeared to be dissociated. The preparation of thyroglobulin (batch 1) was the same as that used in the above experiment. The rabbits were injected once a week for 3

weeks with 4 ml of incomplete adjuvant containing 6 mg of protein and bled 28 days after the first injection. The sera of some of the rabbits injected with each preparation contained both precipitating and hemagglutinating antibody to native thyroglobulin (Table IV). Sera of the rabbits injected with either heated or picrylated thyroglobulin usually contained higher levels of antibody than did sera of the rabbits injected with sulfanil-thyroglobulin. The antibody levels in sera of rabbits injected with heated or picrylated thyroglobulin were similar to those in sera of rabbits injected with arsanil-sulfanil-thyroglobulin (Table III). Three of the rabbits injected with picryl-thyroglobulin contained

TABLE III

Production of Antithyroglobulin in Rabbits Injected with Arsanil-Sulfanil-Thyroglobulin (Tg)*

Material injected	Rabbit No.	Precipitating antibody N		Hemagglutinins‡ (native Tg)
		Arsanil-sulfanil	Native	
		μg	μg	
Arsanil-sulfanil-Tg	1	76.3	18.7	16,384
	2	0	0	16
	3	67.0	12.9	8,192
	4	69.0	10.9	32,768
	5	Trace	Trace	4,096
	6	90.0	16.9	32,768
	7	0	0	4,096
	8	0	0	0
Native Tg	1	0	0	8
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0

* Sera obtained 34 days after the first of a series of 4 weekly injections.

‡ Reciprocal of highest serum dilution giving complete agglutination.

thyroid lesions on day 28 after the first injection. There was no correlation between level of antibody and presence or absence of thyroid lesions. One rabbit which produced no detectable precipitating antibody had thyroid lesions. There also was no correlation between the ability of an altered thyroglobulin to induce antibody production and its degree of cross-reaction with antibody to native thyroglobulin (Table I).

Experiment 2: Rabbits were injected once a week for 3 weeks with 4 ml of incomplete adjuvant containing 6 mg of either native or arsanil-sulfanil-rabbit thyroglobulin (batch 2) and bled 14, 21, and 28 days after the last injection. By day 21 precipitating antibody to native thyroglobulin appeared in the sera

of 8 of 10 rabbits injected with arsanil-sulfanil-thyroglobulin. Precipitating antibody to native thyroglobulin appeared in the sera of the remaining 2 rabbits by day 28. The level of precipitating antibody to native thyroglobulin is given in Table V. Seven of the 10 rabbits injected with arsanil-sulfanil-thyroglobulin had thyroiditis as evidenced by lesions present in thyroid tissue taken at biopsy on day 17 following the first injection. The thyroid of one rabbit which had lesions on day 17 was normal on day 28. The lesions (Figs. 1 *a* to 1 *c*)

TABLE IV

Production of Antibody and Lesions in Rabbits Injected with Altered Thyroglobulin (Tg)*

Altered Tg injected	Rabbit No.	Antinative Tg		Thyroid lesions
		Precipitating N	Hemagglutination‡	
		μg		
Sulfanil-Tg	1	6.2	2,048	−
	2	Trace	256	−
	3	0	32	−
	4	0	0	−
	5	6.2	2,048	−
Dissociated Tg	1	26.5	2,048	−
	2	62.1	32,768	−
	3	28.2	16,384	−
	4	51.0	16,384	−
	5	0	2	−
	6	0	8	−
Picryl-Tg	1	28.0	2,048	−
	2	0	16	+
	3	33.8	32,768	−
	4	45.3	65,536	+
	5	5.0	1,024	+

* Sera and tissue obtained 28 days after the first of a series of 3 weekly injections.

‡ Reciprocal of highest dilution of sera giving complete agglutination.

were similar to those described by Rose and Witebsky in rabbits injected with thyroid extract incorporated into complete Freund's adjuvant (4).

The sera of 2 of the rabbits injected with native thyroglobulin contained precipitating antibody to native thyroglobulin on day 14 after the first injection, however, precipitating antibody did not appear in the sera of the remaining rabbits in this group during the next 14 days. Only 1 rabbit injected with native thyroglobulin developed thyroiditis. Again there was no correlation between the level of circulating antibody and the presence of thyroiditis.

Experiment 3: The experiment with picrylated thyroglobulin was repeated with a different batch of thyroglobulin (batch 3). Rabbits were injected once a week for 3 weeks with 4 ml of incomplete Freund's adjuvant containing 6 mg of either native or picrylated thyroglobulin. The results were similar to those

TABLE V

Production of Antibody and Lesions in Rabbits Injected with Native Thyroglobulin or Arsanil-Sulfanil-Thyroglobulin

Tg preparation injected	Rabbit No.	Precipitating antinative Tg			Antinative Tg precipitated/ml‡	Thyroid lesions	
		Day* 14	Day 21	Day 28		Day 17	Day 28
					μg		
Arsanil-sulfanil	1	−	+	Died		+	Died
	2	−	+	Died		++	Died
	3	−	−	+	28.6	+	−
	4	−	+	+	30.5	+	+++
	5	−	−	+	27.9	+	+
	6	−	+	+	39.2	−	−
	7	−	+	Died		−	Died
	8	−	+	Died		++	Died
	9	−	+	+	16.5	−	−
	10	−	+	+	24.4	++	++
Native	1	−	−	Died	0	−	Died
	2	−	−	−	0	−	−
	3	−	−	−	0	−	−
	4	+	+	+	44.7	−	−
	5	−	−	−	0	−	−
	6	−	−	−	0	++	++
	7	−	−	−	0	−	−
	8	+	+	+	28.9	−	−
	9	−	−	−	0	−	−
	10	−	−	−	0	−	−

Tg, thyroglobulin.

* Days after the first of a series of 3 weekly injections.

‡ Sera obtained on day 28 following the first of a series of 3 weekly injections.

observed in Experiment 1. Four of the 9 rabbits injected with picryl-thyroglobulin contained precipitating antibody to native thyroglobulin in their sera and, with the exception of 1, the sera of all of them contained hemagglutinating antibody to native thyroglobulin (Table VI). Two of the 6 surviving rabbits possessed a moderate to marked degree of thyroiditis on day 28. The sera of rabbits injected with native thyroglobulin contained no precipitating antibody to native thyroglobulin, but did contain small amounts of hemagglutinating

antibody. The thyroids of these rabbits showed no evidence of thyroiditis on day 28.

Experiment 4: This experiment was designed to study the effect of both a small amount of mycobacteria and an alteration of the thyroglobulin on the production of antibody and thyroiditis in rabbits injected with homologous

TABLE VI

Production of Antibody and Lesions in Rabbits Injected with Native and Picryl-Thyroglobulin*

Tg preparation injected	Rabbit No.	Antinative Tg		Thyroid lesions
		Precipitating	Hemagglutinating	
Picryl	1	−	256	−
	2	−	512	−
	3	−	1024	−
	4	+	4096‡	Died
	5	−	2	−
	6	−	512§	Died
	7	+	512‡	Died
	8	+	65,536	+++
	9	+	2,048	++
Native	1	−	16	−
	2	−	16	−
	3	−	32	−
	4	−	32	−
	5	−	128	−
	6	−	64	−
	7	−	16	−
	8	−	64	−
	9	−	2	−

Tg, thyroglobulin.

* Sera and tissue usually obtained 28 days after the first of a series of 3 weekly injections

‡ Sera obtained day 21.

§ Sera obtained on day 14.

thyroglobulin in adjuvant. Rabbits were injected once a week for 3 weeks with 4 ml of Freund's adjuvant containing 1 mg of mycobacteria (*Mycobacterium tuberculosis* var. *hominis*) and 6 mg of either native thyroglobulin or sulfanil-thyroglobulin. Eight of 10 rabbits injected with sulfanil-thyroglobulin produced precipitating antibody to native thyroglobulin and 5 developed thyroiditis (Table VII). Four of the 9 rabbits injected with native thyroglobulin produced precipitating antibody to native thyroglobulin, but the levels produced by 3 of the 4 were quite small. One of the 9 rabbits developed a thyroiditis.

Experiment 5: Nine rabbits were injected once a week for 3 weeks with 4 ml of incomplete Freund's adjuvant containing 6 mg of bovine thyroglobulin and bled on days 21, 28, and 40 after the last injection. All of the rabbits produced appreciable amounts of precipitating antibody to bovine thyroglobulin (Table VIII). The antibody in the sera of some of these rabbits also precipitated with

TABLE VII

Production of Antibody and Lesions in Rabbits Injected with Complete‡ Adjuvant Containing Thyroglobulin*

Tg preparation injected	Rabbit No.	Precipitating antibody to native Tg	Thyroid lesions
		μg	
Sulfanil	1	3.0	−
	2	9.0	+
	3	5.0	−
	4	13.0	+
	5	27.0	+
	6	Trace§	+
	7	5.0	−
	8	0	−
	9	74.0	+
	10	0	−
Native	1	0	−
	2	0	−
	3	8.0	−
	4	0	−
	5	Trace	−
	6	Trace	−
	7	Trace	+
	8	0	−
	9	0	−

Tg, thyroglobulin.

* Sera and tissue obtained 28 days after the first of a series of 3 weekly injections.

‡ 0.8 mg of mycobacteria per injection.

§ Detectable by double diffusion in agar but too small to quantitate.

rabbit thyroglobulin. On day 28 the sera of 6 of the 9 rabbits contained precipitating antibody which reacted with rabbit thyroglobulin. The antibody was transient and by day 40 the sera of only 3 rabbits contained precipitating anti-rabbit thyroglobulin. The sera of all rabbits contained hemagglutinating antibody to rabbit thyroglobulin on both day 28 and day 40. Absorption of the sera with an excess of bovine thyroglobulin removed all precipitating and hemagglutinating antibody to rabbit thyroglobulin. Lesions were not observed in thyroid tissue taken on either day 28 or day 40.

Experiment 6: Rabbits were injected with either native or arsanil-sulfanil-thyroglobulin either in soluble form or after precipitation by alum. Eight rabbits were given a series of injections of soluble arsanil-sulfanil-thyroglobulin and 7 rabbits were given an identical series of injections of soluble native thyroglobulin. The rabbits were injected subcutaneously each day for 4 days with 8 mg of protein and on the 5th day they received 8 mg intravenously. Two weeks later the series of injections was repeated, the rabbits bled, and their thyroid gland removed 7 days after the last injection. Similarly, 8 rabbits received a

TABLE VIII

Production of Antibody in Rabbits Injected with Bovine Thyroglobulin

Rabbit No.	Antibovine* Tg N	Antibody to native rabbit Tg				
		Precipitating			Hemagglutinating§	
		Day‡ 21	Day 28	Day 40	Day 21	Day 40
	μg					
1	200.0	+	+	−	1024	1024
2	283.0	+	+	−	4096	1024
3	96.0	−	−	−	256	256
4	101.2	−	+	+	128	256
5	208.0	−	+	+	256	2048
6	177.0	+	+	+	512	2048
7	144.0	+	+	−	512	1024
8	114.0	−	−	−	256	512
9	180.0	−	−	−	256	512

Tg, thyroglobulin.

* Sera obtained on day 40 after the last injection.

‡ Days after the first of a series of 3 weekly injections.

§ Reciprocal of highest dilution of sera giving complete agglutination.

series of injections of alum-precipitated arsanil-sulfanil-thyroglobulin and 7 rabbits received a series of injections of alum-precipitated native thyroglobulin. The rabbits received 4 injections each week for 4 weeks. A total of 3 mg of protein was given the 1st week, 6 mg the 2nd week, 12 mg the 3rd week, and 18 mg the 4th week. The rabbits were bled and the thyroid gland removed 7 days after the last injection. The sera of rabbits injected with native thyroglobulin either in soluble form or precipitated by alum contained no hemagglutinating or precipitating antibody to either native or arsanil-sulfanil-thyroglobulin. Likewise, histological examination of the thyroid glands revealed no lesions. On the other hand, the sera of 7 of 8 rabbits injected with soluble arsanil-sulfanil-thyroglobulin contained hemagglutinating antibody to native thyroglobulin (Table IX). The sera of 4 of these rabbits contained precipitating

antibody to both native and arsanil-sulfanil-thyroglobulin and the serum of an additional rabbit contained precipitating antibody to arsanil-sulfanil-thyroglobulin, but not to native thyroglobulin. Four of the rabbits injected with arsanil-sulfanil-thyroglobulin possessed thyroid lesions which appeared identical

TABLE IX

*Production of Antibody and Lesions in Rabbits Injected with Soluble Arsanil-Sulfanil-Thyroglobulin**

Rabbit No.	Tg injected	Anti-Tg			Lesions
		Hemagglutinating	Precipitating		
		Native	Native	Arsanil-sulfanil	
1	Arsanil-sulfanil-soluble	64	−	−	−
2		1024	+	+	+
3		0	−	−	−
4		128	+	+	+
5		4096	+	+	−
6		1024	+	+	−
7		256	−	+	+
8		64	−	−	++
1	Arsanil-sulfanil-in alum	256	−	+	−
2		0	−	−	−
3		16	−	−	−
4		8	−	−	−
5		4	−	−	−
6		0	−	−	−
7		8	−	−	−
8		0	−	−	−

Tg, thyroglobulin.

* Injected subcutaneously with 8 mg per day for 4 days and then injected intravenously with 8 mg on the 5th day. Two weeks later the series of injections were repeated and the rabbits bled 7 days after the last injection.

to the lesions observed in rabbits injected with arsanil-sulfanil-thyroglobulin incorporated into incomplete adjuvant. There was no correlation, however, between the presence of circulating antibody and lesions. In fact, the rabbit with the highest level of hemagglutinating antibody contained no lesions in its thyroid, while the rabbit with a low level of hemagglutinating antibody and which contained no precipitating antibody possessed the most severe lesions. Five of 7 rabbits injected with arsanil-sulfanil-thyroglobulin, precipitated by alum, contained hemagglutinating antibody to native thyroglobulin, but with

the exception of 1 rabbit, the antibody was present in only trace amounts. None of the rabbits contained either thyroid lesions or precipitating antibody to native thyroglobulin.

Fluorescent Antibody Studies.—The globulin fractions of sera, containing anti-rabbit thyroglobulin, which were obtained from a number of the rabbits in the above experiments were labeled with fluorescent isocyanate and applied to thyroid sections. The fluorescent globulin was found localized in the colloid of the thyroid follicles of both autologous and homologous thyroid tissue, but did not stain either the epithelial cells lining the follicles or the connective tissue. Sheep anti-rabbit γ-globulin labeled with fluorescent isocyanate did not stain sections of normal thyroid tissue, but did localize in foci of inflammation in tissue containing lesions. A similar localization of fluorescent labeled sheep antibody to rabbit serum albumin was not observed.

DISCUSSION

Antibody to homologous thyroglobulin and in some cases thyroiditis was produced in rabbits injected with either soluble thyroglobulin or thyroglobulin incorporated into incomplete adjuvant provided the thyroglobulin was altered prior to injection. Both precipitating and hemagglutinating antibody to native thyroglobulin was produced in rabbits injected with incomplete Freund's adjuvant containing homologous thyroglobulin coupled to the diazonium derivatives of sulfanilic acid (sulfanil-thyroglobulin), thyroglobulin coupled to the diazonium derivatives of both arsanilic and sulfanilic acids (arsanil-sulfanil-thyroglobulin), thyroglobulin coupled to picryl chloride (picryl-thyroglobulin), or thyroglobulin dissociated by heating. The hemagglutinating antibody to native thyroglobulin was probably 7S γ-globulin, since it was not affected by treatment with 2-mercaptoethanol. Mild to severe thyroid lesions were observed in some rabbits injected with incomplete Freund's adjuvant containing either arsanil-sulfanil-thyroglobulin or picryl-thyroglobulin. The injection of native thyroglobulin in incomplete adjuvant resulted in production of little if any circulating antibody, although in some experiments low levels of hemagglutinating antibody were produced and an occasional rabbit produced precipitating antibody. Only very rarely were thyroid lesions observed in rabbits injected with incomplete adjuvant containing native thyroglobulin. A series of injections of soluble arsanil-sulfanil-thyroglobulin was an effective way of inducing both thyroiditis and production of circulating antibody to native thryoglobulin. It was not possible to decide whether injection of arsanil-sulfanil-thyroglobulin in soluble form or incorporated into incomplete adjuvant was most effective. However, the injection of either preparation was more effective than the injection of arsanil-sulfanil-thyroglobulin precipitated by alum. Very little circulating antibody and no thyroiditis was produced in rabbits injected with arsanil-sulfanil-thyroglobulin precipitated by alum. Similarly, injection of either

soluble arsanil-sulfanil-BSA or BSA incorporated in Freund's adjuvant was more effective in terminating immunological tolerance to BSA than the injection of alum-precipitated preparations of arsanil-sulfanil-BSA (15).

The ability to produce experimental autoimmunity to thyroglobulin by injection of soluble thyroglobulin (altered) without incorporation into adjuvant offers new approaches to the study of this phenomenon. It should be possible to study the progress of both lesions and antibody formation following a relatively rapid clearance of the offending antigen. Furthermore, if the lesions and antibody levels subside after disappearance of the altered thyroglobulin, the effect of booster injections of both altered and native thyroglobulin can be investigated. Also, the stimulation by soluble (altered) thyroglobulin may be more natural than the prolonged stimulation given by antigens in adjuvant.

The thyroid lesions produced in rabbits injected with either soluble arsanil-sulfanil-thyroglobulin or altered thyroglobulin incorporated into incomplete adjuvant (Figs. 1 *a* to 1 *c*) appear to be identical with the lesions produced following the injection of native thyroglobulin incorporated into complete Freund's adjuvant. In the present study the lesions are characterized by marked infiltration of inflammatory cells with replacement of some of the follicles. Similar histological changes in the thyroid of rabbits injected with presumably native thyroid extract incorporated into complete Freund's adjuvant were previously reported by Rose and Witebsky (4). Rabbit γ-globulin was localized in the thyroid lesions, but not in the normal thyroid tissue of the experimental rabbits in the present study. The γ-globulin of antisera obtained from rabbits injected with altered thyroglobulin localized in the colloid of the follicles but not in the epithelial cells or connective tissues. The localization occurred in the colloid of autologous as well as homologous tissue, demonstrating that the antibody was autoimmune in character. Beutner *et al.* (16) reported similar findings in rabbits injected with native thyroid extract incorporated into complete Freund's adjuvant. As in the autoimmune phenomenon produced by injection of native thyroglobulin incorporated into complete Freund's adjuvant (17), the data in the present study show no correlation between the level of circulating antithyroglobulin and severity of lesions.

The nature of the alteration responsible for the induction of antibody and thyroiditis following the injection of rabbits with altered thyroglobulin is not clear. With all preparations an apparent dissociation occurred resulting in fragments which sedimented slower in the ultracentrifuge than native thryoglobulin. However, the ultracentrifuge patterns of the fragments resulting from the various treatments were usually different and not related to production of either antibody or thyroiditis following their injection into rabbits. Likewise, no correlation between the electrophoretic mobility of a preparation and its ability to produce autoimmunity upon injection was observed, although all of the various alterations were accompanied by changes in electrophoretic mo-

bility. Furthermore, the extent of cross-reaction of altered or heterologous thyroglobulin preparations with antisera to native rabbit thyroglobulin was not related to their ability to induce either antibody production or lesions. More detailed studies of the structures of the altered and cross-reacting thyroglobulins would have to be made to arrive at any relationship between the nature of alteration and immunogenicity.

Freund's adjuvant when used as a carrier for thyroglobulin may have some direct effect on the structure of the thyroglobulin. In the present study an occasional rabbit produced precipitating antibody and some rabbits produced low levels of hemagglutinating antibody following injection of incomplete Freund's adjuvant containing native thyroglobulin. One rabbit injected with incomplete adjuvant containing native thyroglobulin developed thyroiditis. Rose *et al.* (17) more consistently observed the production of low levels of circulating antibody and thyroiditis following injections of native thyroglobulin incorporated into incomplete Freund's adjuvant, although native thyroglobulin incorporated into complete adjuvant was much more effective. The thyroglobulin employed by these workers was prepared by fractionation with ammonium sulfate, a procedure which since has been shown to alter the thyroglobulin and greatly increase its antigenicity in rabbits (18). In the experiments reported above multiple injections of either soluble native thyroglobulin or native thyroglobulin precipitated by alum never resulted in the production of circulating antibody or lesions. The small amount of antibody produced by some of the animals injected with native thyroglobulin incorporated in incomplete adjuvant may have been the result of alteration produced in either the adjuvant or the granuloma arising following injection. The thyroglobulin certainly would not be expected to remain native after emulsion into adjuvant and exposure to protelytic enzymes undoubtedly present in such granuloma. The incorporation of tubercle bacilli into the adjuvant may be effective, in part, by enhancing the degree of alteration.

The mechanism involved in the production of autoantibody following injection of heterologous thyroglobulin may be similar to that involved in the induction of autoimmunity following injection of altered thyroglobulin. However, the injection of rabbits with bovine thyroglobulin resulted in the production of antibody reactive with rabbit thyroglobulin, but not in the induction of lesions. Rose and Witebsky (19) injected rabbits with complete adjuvant containing extracts of hog, beef, human, and dog thyroid tissue and observed a thyroid specific antibody in the sera, but this antibody would not react with rabbit thyroid extract. However, later they were able to induce the production of antibody in rabbits, following injection of complete adjuvant containing beef or hog thyroid extracts, which reacted with rabbit thyroid extract (17). Only an intensive injection schedule gave lesions with heterologous thyroglobulin. There are two possible explanations for the failure of injections of

heterologous thyroglobulin to readily induce lesions in the rabbit. *First*, the serological relationship between heterologous thyroglobulin and rabbit thyroglobulin may not be optimal. In the present study, bovine thyroglobulin, which did not induce lesions upon injection, cross-reacted only 28 per cent with guinea pig anti-rabbit thyroglobulin. *Second*, the antigenic determinants shared by rabbit and heterologous thyroglobulin may not be those primarily associated with induction of tissue damage.

The mechanisms involved in the immune and/or autoimmune phenomenon produced following injection of altered thyroglobulin may be similar to those involved in the termination of acquired immunological tolerance. In the latter case, immunological tolerance to serum protein antigens was terminated following injections of these antigens after they were coupled to certain diazonium derivatives (2). Following the termination of tolerance to BSA the rabbits made an immune response to subsequent injections of native BSA for a limited period of time (20). Experiments are in progress to determine if rabbits will respond to subsequent injections of native thyroglobulin following the induction of autoimmunity by injections of altered thyroglobulin.

The induction of an autoimmune phenomenon in rabbits following injection of altered homologous thyroglobulin may have a bearing on some of the autoimmune phenomena in humans. It often has been suggested that autoimmunity may result from either malfunction of the immune mechanism which enables it to be stimulated by a normal body constituent or stimulation of a normal immune mechanism by an altered body constituent. That the latter of these two possibilities is feasible is emphasized by the present results. Furthermore, Deodhar, Haas, and Goldblatt (21) have been able to induce antibody to native renin following injection of rats, rabbits, and dogs with complete Freund's adjuvant containing acetylated, homologous renin. Injections of adjuvant containing native renin was ineffective. Numerous other reports demonstrate that injection of heterologous (cross-reacting) tissue antigens can initiate autoimmunity and in some cases disease (cited in reference 3).

SUMMARY

Experimental autoimmunity was produced in rabbits following injection of altered homologous thyroglobulin. The thyroglobulin was altered by coupling to chemically defined haptens and by heating. With some preparations antibody to native thyroglobulin as well as thyroid lesions were produced. Injections of thyroglobulin coupled to the diazonium derivatives of arsanilic acid and sulfanilic acid were effective when given in either soluble form or incorporated into incomplete Freund's adjuvant, while injections of the same preparations precipitated by alum had relatively little effect on production of antibody or induction of lesions. The injection of native thyroglobulin in soluble form, incorporated into incomplete adjuvant or precipitated by alum usually resulted

in production of little or no antibody and only rarely in the formation of lesions. The injection of a heterologous thyroglobulin into rabbits resulted in the production of antibody reacting with both the heterologous and rabbit thyroglobulin, but no thyroid lesions were observed.

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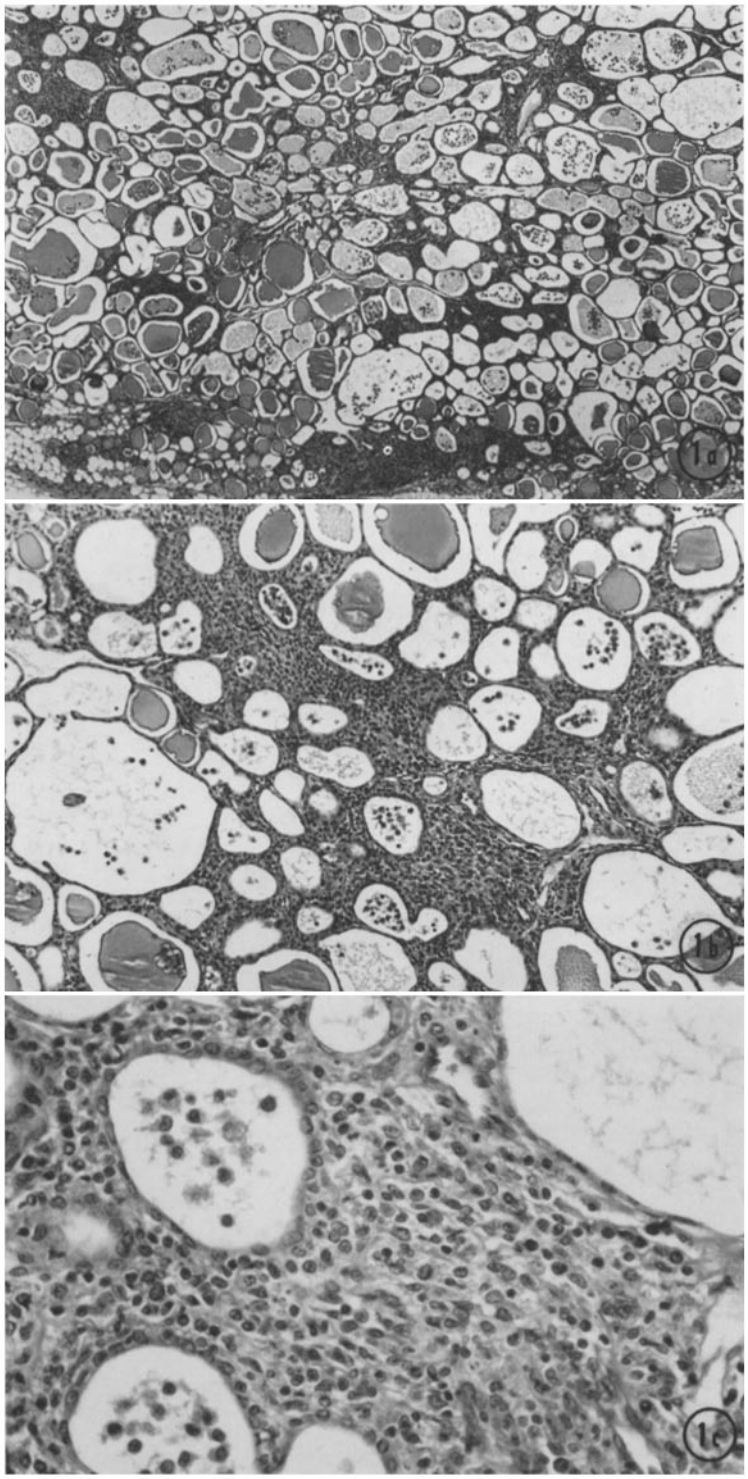
BIBLIOGRAPHY

1. Weigle, W. O., The immune response of rabbits tolerant to bovine serum albumin to the injection of other heterologous serum albumins, *J. Exp. Med.*, 1961, **114,** 111.
2. Weigle, W. O., Termination of acquired immunological tolerance to protein antigens following immunization with altered protein antigens, *J. Exp. Med.*, 1962, **116,** 913.
3. Weigle, W. O., Autoimmunity and termination of acquired immunological tolerance, *in* IIIrd International Symposium on Immunopathology, (P. Grabar and P. Miescher, editors), Basel, Benno Schwabe and Co., 1964.
4. Rose, N. R., and Witebsky, E., Studies on organ specificity. V. Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts, *J. Immunol.*, 1956, **76,** 417.
5. Edelhoch, H., The properties of thyroglobulin. I. The effects of alkali, *J. Biol. Chem.*, 1960, **235,** 1326.
6. Baker, M. C., Campbell, D. H., Epstein, S. I., and Singer, S. J., Physical chemical studies of soluble antigen-antibody complexes. VII. Thermodynamics of the reaction between benzenearsonic acid-azo-bovine serum albumin and rabbit antibodies to benzenearsonic acid, *J. Am. Chem. Soc.*, 1956, **78,** 312.
7. Gelewitz, E. W., Riedeman, W. L., and Klotz, I. M., Some quantitative aspects of the reaction of diazonium compounds with serum albumin, *Arch. Biochem. and Biophysics*, 1954, **53,** 411.
8. Benacerraf, B., and Gell, P. G. H., Studies on hypersensitivity. I. Delayed and Arthus-type skin reactivity to protein conjugates in guinea pigs, *Immunology*, 1959, **2,** 53.
9. Metzger, H., and Edelhoch, H., The properties of thyroglobulin. IV. Denaturation kinetics, *J. Am. Chem. Soc.*, 1961, **83,** 1423.
10. Coons, A. H., and Kaplan, M. H., Localization of antigen in tissue cells. II. Improvements in method for the detection of antigen by means of fluorescent antibody, *J. Exp. Med.*, 1950, **91,** 1.
11. Cochrane, C. G., and Weigle, W. O., The cutaneous reaction to soluble antigen-antibody complexes, *J. Exp. Med.*, 1958, **108,** 591.
12. Kabat, E. A., and Mayer, M., Experimental Immunochemistry, Springfield, Illinois, Charles C Thomas, 2nd edition, 1961.
13. Boyden, S. V., Adsorption of proteins on erythrocytes treated with tannic acid and subsequent hemagglutination by antiprotein sera, *J. Exp. Med.*, 1951, **93,** 107.

14. Scheidegger, J. J., Une micro-méthode de l'immune-électrophorèse, *Internat. Arch. Allergy and Appl. Immunol.*, 1955, **7**, 103.
15. Weigle, W. O., unpublished data.
16. Beutner, E. H., Witebsky, E., Rose, N. R., and Gerbasi, J. R., Localization of thyroid and spinal cord autoantibodies by fluorescent antibody technique, *Proc. Soc. Exp. Biol. and Med.*, 1958, **97**, 712.
17. Rose, N. R., Kite, J. H., and Doebbler, T. K., Experimental autoimmune thyroiditis, *in* Mechanism of Cell and Tissue Damage Produced by Immune Reactions, IInd International Symposium on Immunopathology, (P. Grabar and P. Miescher, editors), Basel, Benno Schwabe and Co., 1961.
18. Rose, N. R., Autoimmunity. Clinical and Experimental Aspects, *Ann. New York Acad. Sc.*, in press.
19. Rose, N. R., and Witebsky, E., Studies on organ specificity. II. Serological interrelationships among thyroid extracts of various species, *J. Immunol.*, 1955, **75**, 282.
20. Weigle, W. O., The immune response of BSA tolerant rabbits to injections of BSA following the termination of the tolerant state, *J. Immunol.*, 1964, **92**, 791.
21. Deodhar, S. D., Haas, E., and Goldblatt, H., Production of antirenin to homologous renin and its effect on experimental renal hypertension, *J. Exp. Med.*, 1964, **119**, 425.

EXPLANATION OF PLATE 27

FIGS. 1 *a* to 1 *c*. Sections of thyroid taken 28 days after the first of a series of 3 weekly injections of incomplete Freund's adjuvant containing arsanil-sulfanil-thyroglobulin. Hematoxylin and Eosin. +++ reaction. Fig. 1 *a*, × 35. Fig. 1 *b*, × 96. Fig. 1 *c*, × 250.



(Weigle: Autoimmunity in rabbits)