The Properties of Thyroglobulin
VII. The Immunologic Activity of Thyroglobulin Fragments

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Received August 10, 1961

The antigenic (antibody-combining) activities of tryptic digests of beef thyroglobulin have been studied. At an early stage of digestion several components are produced which form precipitin lines with antiserum to native thyroglobulin. After prolonged digestion only traces of precipitating antigen fragments remain. The nonprecipitating digests are still capable of specifically combining with antibody, however, as shown by inhibition of precipitation. Some of the active fragments are quite small (average m.w. 700) and give only limited inhibition of precipitation, while larger ones (average m.w. 8,000) yield essentially complete inhibition. Physicochemical studies suggest that the antigenic activity of both fractions is related to the covalent structure of the peptides.

The nature of the antigenic determinants of proteins has been investigated by several approaches: (1) studying the changes in immunologic reactivity produced by chemical modification of the various amino acid side-chains (Cole, 1958) or by titration of the polar groups (Pepe and Singer, 1959) of proteins; (2) conjugating polyamino acids to a protein and observing the alterations in immunogenicity or investigating the immunogenicity of high molecular weight polypeptides themselves (Kabat and Mayer, 1961); and (3) hydrolytically cleaving the protein and examining the digest for active fragments. While much useful information has been gained by the first two methods, definitive answers must perforce depend on the isolation and characterization of the active antigenic sites. Progress in this latter area has been reported recently with silk fibroin (Cebra, 1961), lens crystallin (Hara, 1956), and oxidized ribonuclease (Brown and Delaney, 1961). Work with globular proteins has been limited, however, largely to the isolation of relatively high molecular weight fragments (Porter, 1957, 1959; Lapresle *et al.*, 1959; Nussenzweig *et al.*, 1960; Kaminski, 1960), though the separation of quite small, active fragments of serum albumin has recently been reported (Richard *et al.*, 1960).

We now wish to report the results of some recent studies on thyroglobulin fragments obtained by tryptic digestion. The course of digestion and the physicochemical and immunologic properties of these fragments will be described.

METHODS

Preparation of Thyroglobulin.—All the preparations of thyroglobulin were prepared by the ultracentrifugal method described by Edelhoch (1960). For immunization with beef thyroglobulin only materials corresponding to "Preparation III" were employed; all other thyroglobulins corresponded more closely to "Preparation II."

Preparation of Antisera.—The hind footpads of 3000-g NIH albino rabbits were injected with 3 parts antigen mixed with 1 part complete Freund's adjuvant. Animals were bled from the marginal vein of the ear and terminally from the femoral artery. After harvesting, the sera were inactivated at 56° for 30 minutes. The following sera were used:

1. Anti-beef thyroglobulin.

Serum	Animals in Pool	Total Antigen per Animal (mg)	Times of Injection (wk.)	Time Bled (wk.)
I	6	12	0, 2, 6	10
II	6	14	0, 2, 6, 10	11
AQ(1-6)	1	20	0, 2, 6, 10, 12, 24, 29	30

2. Anti-human thyroglobulin. Two animals were injected with 3 mg antigen at 0 and 6 weeks and bled at 8 weeks.

3. Anti-dog thyroglobulin. Two animals were injected with 2 mg antigen at 0 and 4 weeks and bled at 6 weeks.

4. Anti-chicken thyroglobulin. One animal was injected with 3 mg antigen at 0 and 3 weeks and bled at 5 weeks.

Absorption of Antisera.—Antisera were examined for antibodies against serum factors which might have contaminated the thyroglobulin preparations. Immunoelectrophoresis revealed a number of lines between anti-beef thyroglobulin and beef serum. Even large amounts of thyroglobulin failed to remove these antibodies, presumably because in any one preparation insufficient levels of these contaminants were present (though enough were present to be immunogenic). While these contaminants were too small in amount to interfere with the precipitin and inhibition tests, they might have interfered with the gel-diffusion studies; therefore, for these studies, sera were ab-

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sorbed with 10% v/v whole beef serum. Control tests indicated that complete absorption was achieved.

Purified Antibody.—Purified anti-beef thyroglobulin antibody was prepared according to a recently published technique (Metzger and Edelhoch, 1962). By ultracentrifugation this antibody has a sedimentation constant, $s^0_{20,w}$, of 7.2 *s*. The antibody was prepared from preabsorbed serum (see above).

Precipitin Tests.—Precipitin tests were run according to standard procedures (Kabat and Mayer, 1961) with only minor modification.

Precipitates were measured by dissolving the precipitate in 0.2 N NaOH and measuring the optical density of the solution at 287 mμ in a Beckman DU spectrophotometer. Under these conditions the optical density of a 1% solution of thyroglobulin is 10.5 and that of rabbit γ-globulin, 16.0 (McDuffie and Kabat, 1956).

Inhibition Tests.—Except as noted, the following procedure was used throughout: A standard amount of antibody (generally 0.1 or 0.2 ml serum) was incubated for 1 hour at room temperature and then for 2 hours at 4° with varying amounts of test material. After incubation a standard amount of thyroglobulin (see Results) was added to each tube and the mixture reincubated for 30 minutes at 37° and for 18–24 hours at 4°. Precipitates were measured as noted above. Activity was calculated as the percentage decrease in the amount of precipitate in the experimental tubes compared to that in control tubes.

Ouchterlony Gel-Diffusion.—These studies were performed on 7.5 × 3.8 cm glass slides; 1% Difco Noble Agar in 0.1 N NaCl containing 1:10,000 methyl orange and 1% sodium azide was used. Wells were arranged as in Figure 2. Each well was 3 mm in diameter, held 0.025 ml fluid, and was filled once. Plates were incubated at room temperature.

Immunoelectrophoresis.—Immunoelectrophoresis (Grabar, 1959) was kindly performed for us by Dr. Howard C. Goodman. It was carried out on a 12.5 × 17.5 cm plate in 1.0% agar dissolved in 0.025 N sodium barbital buffer, pH 8.2. Antigen solutions were placed in the wells, and electrophoresis was carried out for 2 hours at a constant current of 87 mamp. After the troughs were filled with the antibody solution, the plates were incubated at room temperature.

Physical Measurements.—All pH values were determined on a Radiometer TTT-1 pH meter with glass electrodes. pH-stat studies employed an Agla precision syringe and an Ole Dick Recorder. The number of peptide bonds cleaved at any time was calculated on the basis of base consumption and assuming a pK of 7.85 for the free peptide amino group.

Preparative ultracentrifugations were carried out in a Spinco Model L ultracentrifuge. Analytical ultracentrifugations were performed on a Spinco Model E ultracentrifuge fitted with a phase plate and a RITC temperature control. Sedimentation coefficients were calculated in the usual manner and corrected to water at 20°. Area measurements were made by weighing enlarged traces of the schlieren peaks. No corrections for Johnston-Ogston effects or radial dilution were made. Sedimentation coefficients and areas of low-molecular-weight components (<7 *s*) were determined in a synthetic boundary cell.

A standard double-sector cell was employed for "short-column" sedimentation equilibrium studies (Van Holde and Baldwin, 1958). Spinco FC 43 Flurochemical, 0.05 ml, was used to provide a liquid bottom, and 0.035 ml of protein (or sucrose) solution produced a column height of about 1 mm. The apparent refractive index of the solutions was determined by examining the same solutions in a synthetic boundary cell and determining the area under the schlieren peak. We wish to thank Dr. Frank Reithel for advice in these studies. Optical rotation studies were performed in a Rudolph Model 80 photoelectric polarimeter at room temperatures. Refractive index corrections were made by the Lorentz equation for the studies on urea solutions. Viscosities were measured in an Ostwald viscometer with average flow time of 88 sec. A constant-temperature bath was maintained at 25.00° ± 0.005°.

Thyroglobulin Digestion.—Beef thyroglobulin was used throughout. A water-jacketed cell connected to a constant-temperature bath was used for digestion of 1–5% thyroglobulin solutions. A stream of nitrogen was passed over the solution to prevent CO_2 absorption, and the pH was maintained by a pH-stat procedure. In all cases the initial enzyme concentration was 1% of the total protein. For prolonged digestions, an additional 1% was added after 12 hours. In general, 2 g of thyroglobulin was employed for preparative digestions.

Except as noted, two types of tryptic digests were employed:

1. "High-salt." The solvent was 0.1 N NaCl. The digestion was carried out at 30° and a pH of 8.0 was maintained with 1.0 N NaOH.

2. "Low-salt." The solvent was H_2O. The digestion was carried out at 37° and a pH of 9.0 was maintained with 2.0 M NH_4OH.

Digestion was stopped by addition of a quantity of trypsin inhibitor equal to three times the weight of added trypsin. pH-stat studies indicated that complete cessation of hydrolysis was achieved with this amount of inhibitor.

Fractionation of Thyroglobulin Digests.

1. Fractionation by Dialysis.—"Low salt" digests (above) were dialyzed by placing the digest in a 24/32 Visking membrane and dialyzing at 4° against 10 volumes of distilled water over 2 days, fresh solvent being added after the first day. Because of the high osmotic pressures that developed, care was required to assure that the bags were leak-proof. A single stock of membrane was used throughout this study. The dialyzable material (diffusate) was concentrated by pervapora-

tion at room temperature and stored frozen. The nondialyzable material (dialysate) was frozen as such and stored.

2. SEPHADEX FRACTIONATION.—The diffusate was fractionated further by use of a Sephadex G-25 column. Sephadex (Pharmacia, Uppsala, Sweden) was repeatedly washed with distilled water to free it of "fines" and poured into a glass column (150 × 2.3 cm I.D.) fitted with a glass-wool plug. The column was equilibrated with distilled water brought to pH 9.0 with NH_4OH. All runs were carried out at 4°. Fractions were collected in 4-ml aliquots. Flow rates of 0.5–1.0 ml per minute were used.

Elution patterns were determined by measuring the optical density at 280 mμ.

REAGENTS.—Trypsin (twice crystallized, salt free, Worthington Biochemical Corporation), soybean trypsin inhibitor (crystallized from ethyl alcohol, Worthington Biochemical Corporation), chymotrypsin (bovine, twice crystallized, salt free, Armour Research Division) and subtilisin (Nagarse) were dissolved in 0.005 N HCl and stored frozen until used. Pepsin (twice crystallized from ethyl alcohol, Worthington Biochemical Corporation) was dissolved in pH 5.0, 0.005 M sodium acetate buffer and used directly. Papain (twice crystallized, lyophilized, in 0.03 M cysteine, Mann Research Laboratories, Inc.) was used in the presence of 0.005 M cysteine and 0.001 M sodium ethylenediaminetetraacetate.

Urea was twice crystallized from H_2O. All other reagents were analytical grade. Glass-distilled water was used throughout.

RESULTS

Enzymatic Hydrolysis.

1. PH-STAT STUDIES.—Rapid hydrolysis of native thyroglobulin was produced by a variety of proteolytic enzymes: trypsin, chymotrypsin, papain, and subtilisin at pH 8.0 and pepsin at pH 5.2. The digestion was found to be quite sensitive to the ionic strength of the solvent. When an amount of trypsin equivalent to 1% of the total protein was added to thyroglobulin solutions of ionic strengths 0.03, 0.1, and 0.3, the number of peptide bonds cleaved per molecule after one hour at 30° was calculated to be 280, 210, and 100 respectively. Control studies using denatured hemoglobin as substrate showed on the contrary that digestion proceeded more rapidly with increasing salt concentrations.

In all cases there was an initial very rapid digestion followed by a prolonged slower phase.

2. SEDIMENTATION ANALYSIS.—When digests produced by each of the enzymes listed above were examined in the ultracentrifuge a remarkably similar pattern obtained. Early in digestion a fast-moving peak with the identical appearance and sedimentation constant of native thyroglobulin ($s^0_{20,w}$ = 19.2) was completely separated from a more diffuse boundary which never separated fully from the meniscus. The line between the two boundaries

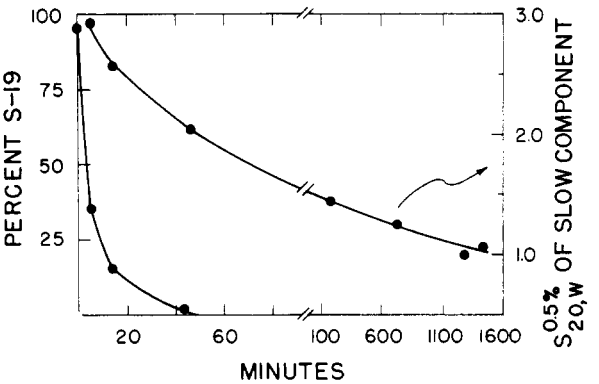

FIG. 1.—Course of "low-salt" tryptic digestion of thyroglobulin. Aliquots of digestion mixture reacted with trypsin inhibitor at indicated times and made 0.1 N in NaCl. Abscissa: reaction time. Left ordinate: percent *s*-19 component remaining. Right ordinate: sedimentation coefficient of slow component.

coincided with the baseline, indicating that no large intermediates were formed.

When examined in a synthetic-boundary cell this slow peak was quite broad and not entirely symmetrical. The total sedimentation pattern was qualitatively the same whether the digestion had been carried out in the presence of high or low salt, as long as the sedimentation analysis was carried out in 0.1 N salt. Consistent with the pH-stat studies the rate of disappearance of the *s*-19 component was greatly increased when digestion took place at low ionic strengths. For example, the initial "half-life" of the *s*-19 component in 0.1 N salt was about 30 minutes, about 5% still being present after 4 hours. In a digestion carried out in an essentially salt-free medium under the same conditions the early "half-life" was about 5–6 minutes, and after 1 hour only a trace of the heavy component remained. Figure 1 illustrates the results obtained when successive aliquots of a "low-salt" (see Methods) tryptic digest were examined. Under the conditions listed the *s*-19 peak disappeared rapidly, with negligible amounts remaining after 45 minutes. Digestion of the slow component was evidenced by the continual fall in its sedimentation coefficient and is consistent with the pH-stat and immunologic (see below) studies.

While the *s*-19 component appeared identical to native thyroglobulin by sedimentation and immunologic criteria (see below), some evidence was obtained which indicates that these molecules have in fact been modified. A "high-salt" tryptic digest was allowed to proceed to a point where 40% of the *s*-19 component had disappeared. The reaction was stopped with trypsin inhibitor. The heavy component was isolated in a single spin with a Spinco Model L ultracentrifuge and proved to be pure *s*-19. When heated in low salt its behavior was quite different from that of native control, which was handled exactly like the experimental sample except that trypsin inhibitor was added before addition of trypsin.

When native thyroglobulin is heated at pH 9.6

TABLE I
SEDIMENTATION PROPERTIES OF THYROGLOBULIN DIGEST FRACTION

Component	Digest $s_{20,w}$	Digest Amount	Control $s_{20,w}$	Control Amount
	A. Before Heating			
s-19	17.6[a]	100%	17.6	100%
	B. After Heating			
s-19	17.6	13%	17.6	59%
s-17	—	—	15.0[a]	14%
s-12	10.8[a]	50%	11.7[a]	27%
Small components	7.5 and less	37%	—	—

[a] These sedimentation coefficients agree with the expected values for the amount of each component present in a 1% total protein solution (See Fig. 1 in Edelhoch and Metzger, 1961).

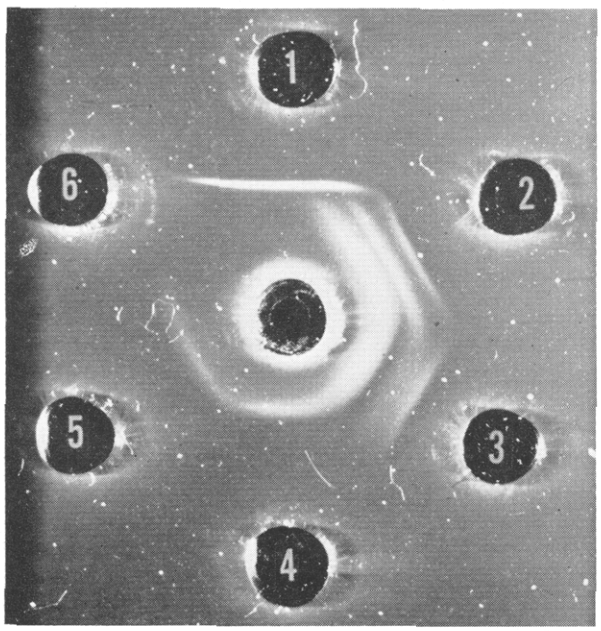

FIG. 2.—Ouchterlony gel-diffusion study. Digestion aliquots are the same used for the study shown in Figure 1. Well no. 1, zero time; no. 2, 15 minutes; no. 3, 45 minutes; no. 4, 12 hours; no. 5, 24 hours; no. 6, 0.1 M NaCl. Wells no. 1–5, total protein = 1%. Center well, purified antibody = 0.43%. Photographed after 18 hours' incubation.

in 0.01 M salt, fragmentation occurs, leading to two new components with sedimentation constants of 17 and 12 (Edelhoch and Metzger, 1961). As seen in Table I, the *s*-19 component from the partial "high-salt" digest was considerably more labile and breakdown was much more complete. In addition, new slower-sedimenting components were observed. Comparable findings were obtained with the isolated *s*-19 component of a "low-salt" digest. The *s*-19 components from both digests were examined by paper electrophoresis. The electrophoretic mobilities (pH 8.6, 0.1 M Na barbital buffer, 4 mamp. for 24 hours) of the *s*-19 components of both digests were indistinguishable from that of native thyroglobulin.

3. OUCHTERLONY AND IMMUNOELECTROPHORETIC STUDIES.—The course of digestion was also followed by the Ouchterlony technique. The samples

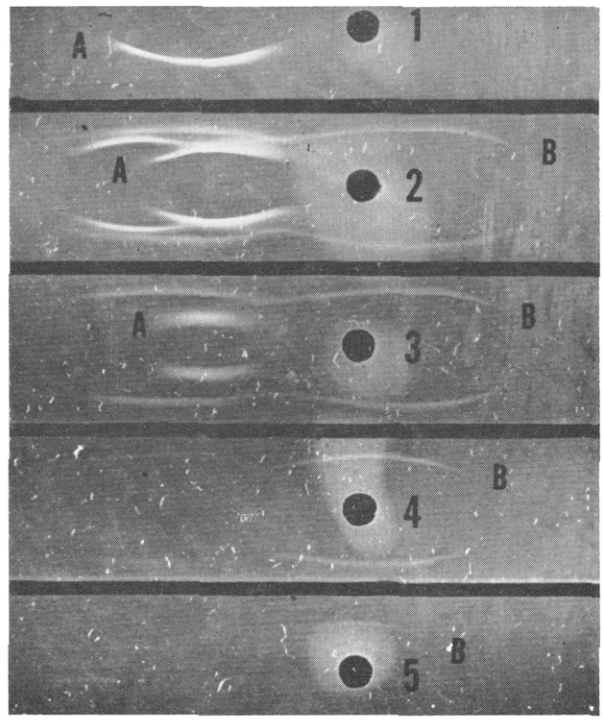

FIG. 3.—Immunoelectrophoretic study. Digest preparations and antibody identical to those used for the study shown in Figure 2. Well no. 1, zero time; no. 2, 15 minutes; no. 3, 45 minutes; no. 4, 12 hours; no. 5, 24 hours. Photographed after 18 hours' incubation. Anode is at left.

used for the plate shown in Figure 2 were from the digestion shown in Figure 1. As can be seen, a number of new components became evident early during digestion. Some of these disappeared rather rapidly again, while one persisted and was still faintly evident after 24 hours. These components form precipitin lines which are concave toward the antibody well, indicating that they have a higher diffusion constant than 7 *s* γ-globulin (Korngold and Van Leeuwen, 1957). The remaining *s*-19 component (wells 2 and 3) forms a line of identity with native thyroglobulin.

When examined by immunoelectrophoresis a number of noteworthy features of the digestion became evident. The preparations used in the study shown in Figure 3 were the same as those shown in Figure 2. It is interesting that the lines representing the *s*-19 component in the digest (line A, wells 2 and 3) have a slower electrophoretic mobility than the undigested sample (line A, well 1). On the other hand, line B, representing a fragment of thyroglobulin, is seen to increase in mobility during the course of digestion (compare line B in wells 2, 3, 4, and 5). Ouchterlony and immunoelectrophoretic patterns of both "low-salt" and "high-salt" digestions were very similar.

Fractionation of Whole Digest.—Preliminary studies (Metzger and Sharp, 1961) had indicated that the thyroglobulin digests contained low-molecular-weight, non-precipitating fragments which were still capable of specifically binding antibodies to

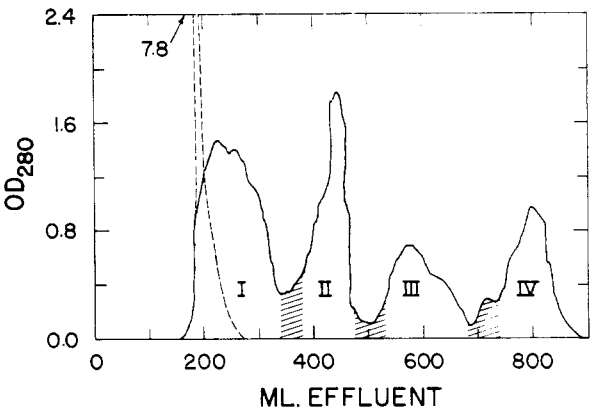

FIG. 4.—Elution pattern obtained when 600 mg (in 10 cc) of diffusate applied to a 150 × 2.3 cm I.D., G-25 Sephadex column equilibrated with H_2O. Material shown as shaded areas were discarded. The dashed line indicates the pattern obtained when an equal quantity of bovine serum albumin was run on the column under identical conditions.

native thyroglobulin. It was decided to examine further these smaller active fragments. Twenty-four-hour, low-salt digests were dialyzed (Methods). About 30% of the digests was dialyzable in 48 hours. Under conditions approximating as closely as possible those used for the dialysis of the thyroglobulin digest, about 1% of ribonuclease (m.w. 13,700) and 15% of glucagon (m.w. 4000) were dialyzable in 24 hours.

The dialyzable material (diffusate) and non-dialyzable material (dialysate) were then examined further.

Fractionation of Diffusate.—Further fractionation of the diffusate was obtained by applying this material to a 150-cm G-25 Sephadex column. As can be seen in Figure 4, four major peaks were obtained. Much of the material was well removed from the "front" and hence may be presumed to be of molecular weight less than 2,000–3,000.[1] The fractions obtained from one such run were lyophilized and extinction coefficients at 280 mμ of 1% solutions of each fraction obtained. They were F-I = 5.7, F-II = 4.8, F-III = 24.8, F-IV = 55.0. It can be seen therefore that the material under peaks III and IV in Figure 4 represents only a very small amount of the total on a weight basis. The high extinction coefficients of these latter fractions is consistent with previous observations which indicate that Sephadex gels tend to retard substances rich in aromatic groups (Porath, 1960).

Physical Properties.

1. DIALYSATE.—The dialysate had a sedimentation constant, $s^0_{20,w}$, of 1.25. When examined in 0.1 N NaCl at pH 6.5 the intrinsic viscosity was 0.05 deciliters/g. With the Flory-Mandelkern equation for random-chain polymers (Flory, 1953) an average molecular weight of 8,000 was calculated. ($\bar{v}$ was taken as equivalent to that for native thyroglobulin: 0.715 [Edelhoch, 1960].)

[1] Pharmacia, Uppsala, Sweden (1960), "Sephadex in Gel Filtration."

The dialysate became largely insoluble at pH 5.2. Unlike acid-denatured thyroglobulin, which regains its solubility only very gradually and at pH values above 6.0, the dialysate was instantaneously and reversibly soluble when the pH was raised above 5.2. This finding suggests that the precipitation at acid pH values merely reflected a solubility property and not a denaturation reaction. Neither the pH at which precipitation occurred nor the rapid reversibility of solubility was affected by heating the solution to 80° for 10 minutes at neutral pH, or bringing it to pH 1.5 or 12.5.

When a solution of native thyroglobulin is titrated to alkaline pH levels and the optical density followed at 300 mμ the phenolic hydroxyl groups of the tyrosine residues are found to ionize with an apparent pK of 11.5 (Edelhoch and Metzger, 1961). (It has recently been determined that about 80% of the tyrosines are abnormal in thyroglobulin [Edelhoch, 1961].) The back titration curve is displaced about 0.6 pH units at the midpoint. However, in 8.0 M urea or 5.0 M guanidine solutions the tyrosyl groups are normalized. When a 0.12% aqueous solution of dialysate was spectrophotometrically titrated an apparent pK of 10.3 was obtained, which agrees with values obtained for tyrosyl peptides (Cohn and Edsall, 1943; Katchalski and Sela, 1953). On back titration a small (0.1 pH units) hysteresis occurred. However, the curve was parallel to the forward curve throughout and did not return to baseline values. The most likely explanation of this shift is that a small amount of turbidity had developed at higher pH levels.

When the difference spectrum between 270 and 310 mμ at neutral pH is determined for thyroglobulin in an aqueous solvent and in 9 M urea a "blue shift" is obtained, with principal peaks at 286 and 292.5 mμ (see Edelhoch and Metzger, 1961, Fig. 9). When dialysate was similarly studied only a small red shift was seen, comparable to that found when thyroglobulin was dissolved in a concentrated sucrose solution. These red shifts arise from the larger refractive index of the solvent (Bigelow and Geschwind, 1960).

Native thyroglobulin shows a decrease of 40° in specific (optical) rotation in 8.5 M urea over that found in water (Edelhoch and Metzger, 1961). No difference was found, however, for the specific rotation (measured from 600–400 mμ) of the dialysate between water and 9 M urea.

These studies suggested, therefore, that the fragments lacked any secondary or tertiary structure.

2. DIFFUSATE.—The diffusate had an $s_{20,w}$ of 0.5. Assuming it to have the same intrinsic viscosity as the dialysate (since in the Flory-Mandelkern equation the molecular weight is proportional to the square root of the intrinsic viscosity this assumption cannot cause a large error) a molecular weight of 2,800 was calculated.

The diffusate became partially insoluble at

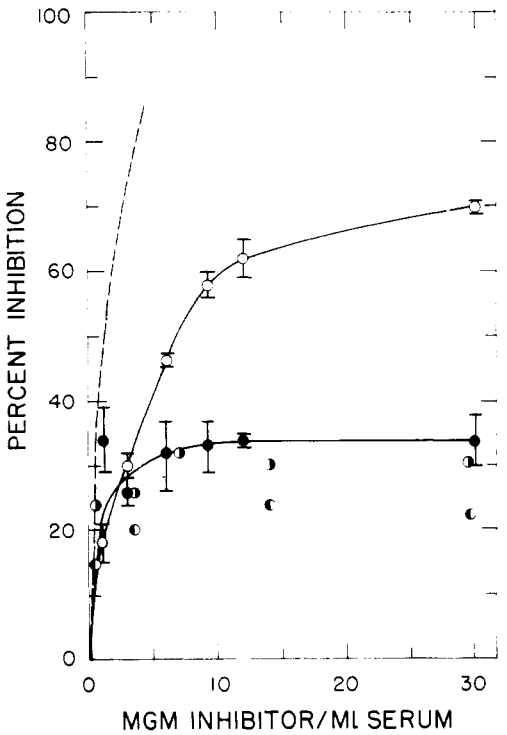

FIG. 5.—Comparison of activities of: ○ = dialysate; ● = diffusate; ◑ = diffusate, fraction I; ◐ = diffusate, fraction II. Points from dialysate and diffusate are averages of duplicates with ranges shown. Points from fraction I and fraction II are single determinations. Dashed line = inhibition by native thyroglobulin (see text). Serum: Pool I.

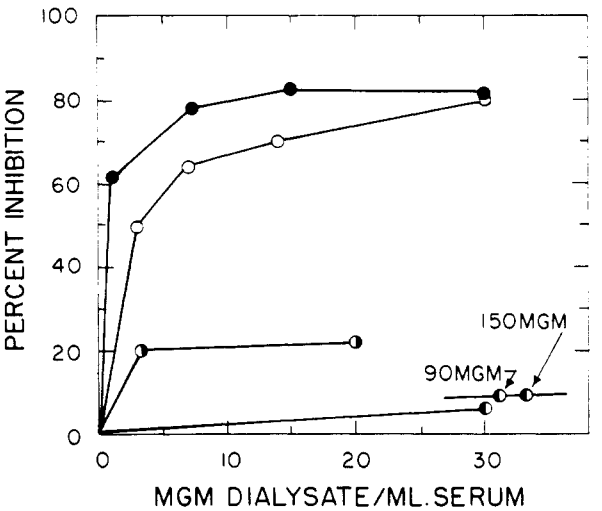

FIG. 6.—Comparison of activity of beef thyroglobulin fragments (dialysate) in homologous and heterologous systems. ○ = beef thyroglobulin–anti-beef thyroglobulin (Pool II); ● = beef thyroglobulin–anti-dog thyroglobulin; ◑ = beef thyroglobulin–anti-human thyroglobulin; ◐ = chicken thyroglobulin–anti-chicken thyroglobulin. All points are averages of closely agreeing duplicate determinations. That the curve for beef thyroglobulin–anti-dog thyroglobulin is displaced above the curve for the homologous system is best explained by the sensitivity of the inhibition test to the antigen/antibody ratio when small amounts of inhibitor are used (see text). If in the experiment the homologous reaction had been conducted somewhat further in the antigen excess zone, the curves might well have been reversed. This phenomenon tends to disappear with higher levels of inhibitor as is seen in the figure. For the heterologous, cross-reacting systems, inhibitor/serum ratios were normalized so that the inhibitor/cross-reacting antibody ratios were equivalent to those present in the homologous reaction.

around pH 4.0, but again this was instantaneously reversible on back titration. As with the dialysate, heating the diffusate to 80° for 10 minutes at neutral pH, or titrating it to pH 1.5 or 12.5, caused no change in these solubility properties.

3. DIFFUSATE-FRACTION II.—The average molecular weight of fraction II of the diffusate (see above) was determined by "short-column" sedimentation equilibrium. The schlieren gradient appeared linear throughout, without any suggestion of heavier components. A molecular weight of 710 was calculated. (The value obtained for sucrose [m.w. 342] under the same conditions was 300.)

Immunologic Studies.—Studies performed with the partial digests have already been described above. It was shown that in a 24-hour "low-salt" digest only traces of precipitating material remained. While a definite precipitation line was obtained on gel diffusion (Fig. 2 and 3), only negligible and poorly reproducible amounts of precipitate were measured by standard precipitin analysis with either whole digest or dialysate. (No traces of precipitate were ever detected with the diffusate.)

Also mentioned above was the finding that a low-molecular-weight nonprecipitating fraction effectively inhibited the precipitation between native thyroglobulin and its antibody. It was determined that the inhibition was very rapid, being complete at 30 minutes and probably earlier. Similarly the amount of inhibition was not influenced by the length of time the native thyroglobulin was incubated with the inhibited antibody. Even incubation periods as long as 5 days failed to displace the inhibitory fragments. No large differences were obtained in the amount of inhibition given by the various pools of sera or by individual sera. It was found, however, that the percentage inhibition obtained was very sensitive to exactly where on the precipitin curve one conducted the inhibition experiment. We generally chose a point in the region of antigen excess at which the amount of precipitate formed with native thyroglobulin was one third less than at equivalence.

1. INHIBITION BY VARIOUS FRACTIONS.—Figure 5 shows the amount of inhibition obtained when dialysate, diffusate, and fractions I and II of diffusate were compared. At higher levels essentially complete inhibition of precipitation was achieved with dialysate. With diffusate, however, the inhibition was never more than partial and showed a plateau at around 30–35% inhibition (with serum AQ-6 up to 50% inhibition was demonstrated with diffusate). Fraction II showed slightly less inhibition (with serum AQ-6, 35%

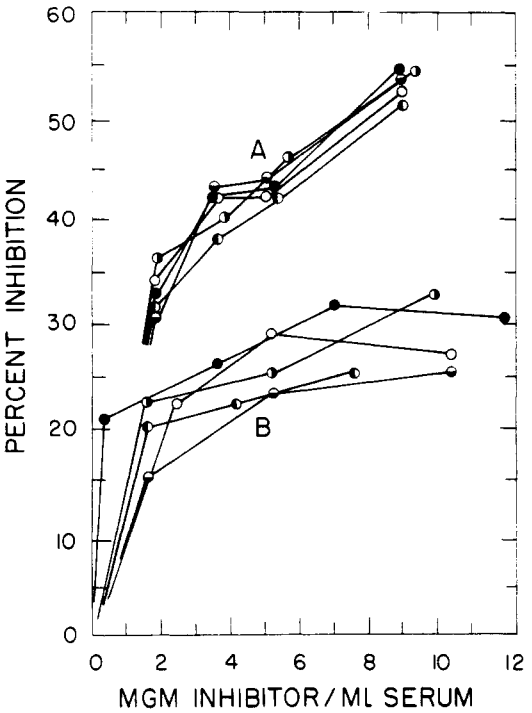

FIG. 7.—Effect of denaturants on activity of: A. Dialysate: ● = native; ◐ = pH 1.5/30 min.; ◑ = pH 12.5/30 min.; ◒ = 8 M urea; ◑ = 100°/5 min. Serum: AQ-6. All points are averages of closely agreeing duplicates. Ranges omitted for purposes of clarity. B. Diffusate: ● = native; ◐ = pH 1.5/30 min.; ◑ = pH 12.5/30 min.; ◒ = 8 M urea plus 0.3 M β-mercaptoethanol; ○ = 80°/5 min. Serum: Pool II.

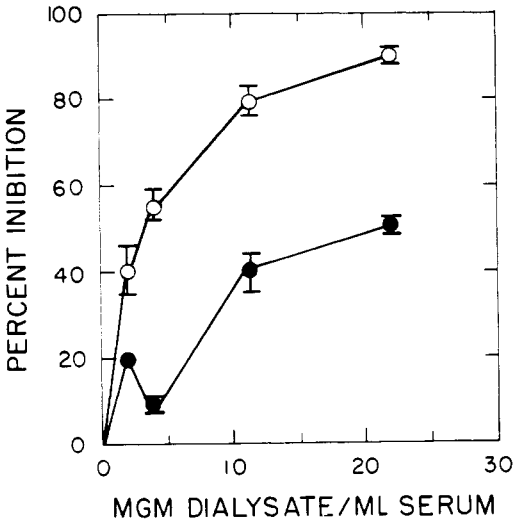

FIG. 8.—Effect of peptic hydrolysis on inhibitory activity of dialysate. ○ = unhydrolyzed control; ● = 24-hour peptic hydrolysate. Serum: AQ-6. Points are averages of duplicates with ranges shown.

inhibition was obtained with fraction II). Only limited testing of fractions III and IV was performed, and these fractions seemed to have little or no activity. The dashed line in Figure 5 represents the relative activity of native thyroglobulin. It was computed by taking the precipitin curve of thyroglobulin in the region where the inhibition experiments were conducted and plotting it as an inhibition curve. It is seen that roughly ten times as much dialysate is required to give comparable amounts of inhibition.

Since thyroglobulin contains small, though significant amounts of iodoamino acids which might function as antigenic determinants, the predominant iodinated tyrosine derivatives were tested as inhibitors. Monoiodotyrosine (1.0×10^{-3} M), diiodotyrosine (1.0×10^{-3} M), and thyroxine (1.0×10^{-5} M) failed to give any inhibition.

2. ACTIVITY IN HETEROLOGOUS SYSTEMS.—The immunospecificity of the inhibitory fragments was checked with one non-cross-reacting and two cross-reacting systems. As shown in Figure 6 a chicken thyroglobulin *vs.* anti-chicken thyroglobulin system was not significantly inhibited by beef thyroglobulin fragments. This is in keeping with previous investigations (Hektoen *et al.*, 1927) and our own observations, which failed to show cross reactions between chicken and mammalin thyroglobulins. On the other hand, two systems which do cross react when tested by precipitin tests were at least partially inhibited.

The Polypeptide Nature of the Active Fragments.

1. DENATURATION STUDIES.—Physical studies (above) had failed to reveal any evidence for secondary or tertiary structure in the thyroglobulin digests. As a further test the active fragments were subjected to a variety of conditions which ordinarily are effective in disrupting globular proteins. The following preparations were tested:

Dialysate and diffusate were (1) titrated to pH 12.5 and neutralized after 30 minutes, (2) titrated to pH 1.5 and neutralized after 30 minutes, (3) heated to 100° for 5 minutes, (4) maintained in 8 M urea and 0.3 M β-mercaptoethanol at pH 8 for 4 hours at room temperature. The reagents were removed either by dialysis (dialysate) or by passing the mixture through a G-25 column (diffusate). In addition, dialysate was (1) maintained in 8 M urea for 4 hours at room temperature and then dialyzed for 12 hours against 0.1 N NaCl, and (2) maintained in 0.3 M β-mercaptoethanol for 4 hours at pH 8.5 at room temperature and 18 hours at 4°, the solution being then diluted thirtyfold. The final concentration of reducing agent in the test and control samples was the same. As seen in Figure 7, no significant reduction in activity was produced by any of the conditions, suggesting that the antigenic activity was related to primary, covalent structure rather than to a labile conformation of a peptide chain or a loose association of two or more peptide chains. Not shown are the results of reacting dialysate with reducing agent either with or without urea. In the former case, *i.e.*, with reducing agent alone, a small (*ca.* 8%) though consistent drop in inhibitory activity was noted. In the reaction of dialysate with β-mercaptoethanol plus urea some fragmentation

seems to have occurred, since the concentration fell during dialysis. The 25% drop in activity observed with this preparation must therefore be accepted with some caution.

2. Effect of Pepsin and Chymotrypsin.—If the immunologic activity of the thyroglobulin digests was related to peptide fragments it might be expected that the activity would be reduced if the tryptic digests were subjected to hydrolytic cleavage by proteolytic enzymes of different specificity than trypsin. The results with a peptic digest of dialysate are shown in Figure 8. It should be noted that the control solution showed normal activity, though it had been maintained at pH 1.6 for 24 hours at 37°. Peptic digestion of the diffusate similarly caused about a 40% drop in inhibitory activity. Chymotryptic digestion of the dialysate and diffusate also caused consistent though somewhat smaller (*ca.* 20%) reductions in activity.

Discussion

The Proteolysis of Thyroglobulin.—Thyroglobulin was rapidly hydrolyzed by a variety of proteolytic enzymes, in agreement with previous findings (Robbins and Rall, 1960). Trypsin was chosen for our studies mainly because of its greater specificity. Though a detailed analysis was not performed, pH-stat data indicated that the majority of susceptible bonds were broken after 24 hours. Since the average molecular weight of the dialysate was 8,000, it is doubtful that significant amounts of an unhydrolyzed "core" remained. Sedimentation and gel-diffusion studies had originally suggested that the digestion was, at least initially, of the "one-by-one" type (Lindstrøm-Lang, 1952), but more detailed analysis revealed that this was not the case. The *s*-19 component isolated from a partial digest was found to be more labile and when "stressed" readily fragmented not only into the *s*-12 and *s*-17 components, observed when native thyroglobulin is dissociated, but also into smaller pieces. This suggests that a number of bonds have been split and that parts of the molecule are held together by secondary and tertiary forces. A somewhat similar phenomenon has been described in the digestion of myosin (Szent-Gyorgyi and Borbiro, 1956).

The immunoelectrophoresis suggested, however, that additional changes had ensued. If the only modification had been the breaking of peptide bonds, then at pH 8.2 the molecule would be more negatively charged. The slower negative mobility seen in gel electrophoresis (though not demonstrated on paper electrophoresis) suggests that the molecules had a net charge more positive than thyroglobulin and may mean that a negatively charged peptide had been released. A considerable number of amino acid residues could have been released without having produced a detectable fall in the sedimentation coefficient of a molecule as large as thyroglobulin.

The Immunologic Properties of Thyroglobulin Digests.—On the basis of a molecular weight of 670,000 for thyroglobulin (Edelhoch, 1960) and of 170,000 for rabbit anti-thyroglobulin antibody (Metzger and Edelhoch, 1962) the extrapolated valency for beef thyroglobulin in the region of extreme antibody excess was found to be 30 in experiments not described here. This agrees well with the recalculated data of Stokinger and Heidelberger (1937). It was interesting to note, therefore, that only a limited and reproducible number of precipitin lines were formed when a partial digest was studied by immunoelectrophoresis. It may be pertinent that some evidence suggests that a subunit one fourth the size of thyroglobulin exists (Edelhoch *et al.*, 1961). Thus the number of antigenic determinants may be as low as 7 or 8 per subunit. The uniqueness of each of these is of course an open question.

Two recent papers have suggested the presence of "hidden determinants" in proteins, which could be revealed during hydrolytic breakdown or dissociation of the native molecule (Bartel and Campbell, 1959; Ishizaka *et al.*, 1960). We looked for evidence of this, care being taken to remove any low-molecular-weight fragments which might inhibit such reactions. However, no evidence for such newly uncovered determinants was found, and when absorbed with whole thyroglobulin (and beef serum to eliminate antibodies to serum components) our sera failed to show any precipitin lines with the digests, on gel-diffusion plates.

After 24 hours of tryptic digestion only traces of antibody precipitating material remained in the dialysate. This fraction was a potent inhibitor, however, being able to inhibit the precipitation of thyroglobulin with its antibody essentially completely. Only about five to ten times as much (by weight) of this material as of whole thyroglobulin was necessary to give equivalent activity in inhibition of precipitation studies. It is therefore unlikely that mere traces of much heavier fragments can account for the observed activity. That activity remained even in very small fragments is confirmed by the studies on whole diffusate and fraction II of diffusate. Fraction II of the diffusate was well removed from the "front" on Sephadex "chromatography" and should certainly have been free of high-molecular-weight contaminants. Significantly, in both cases a strictly limited amount of inhibition of precipitation was found, and raising the concentration of inhibitor many fold failed to yield higher levels of activity. Had the activity of these fractions merely reflected contamination with dialysate a continuous rise in the level of inhibition to that observed with the dialysate should have occurred.

Assuming complete digestion, the limited activity of the diffusate can be explained by postulating that certain of the antigenic determinants were located on small, dialyzable peptides while others were present on larger nondialyzable fragments. Since dialysate was always "contaminated" with diffusate, the former would contain the full comple-

ment of antigenic sites surviving tryptic digestion.

It is worth noting that the fact that dialysate was able to inhibit the precipitation of thyroglobulin practically completely does not mean that all of the antigenic sites remained intact—only enough of them to interfere with the process of gross precipitation.

The Structure of the Active Fragments.—A problem that has long confronted immunologists in studying the antigenic determinants of proteins is that during the process of analysis these sites may be destroyed. If an antigenic site is composed of parts of two or more (non-covalently linked) peptide chains (see Boyd, 1956, Fig. 3-2, p. 115), or if a site is based on a labile conformation of a peptide chain, then a detailed description of the determinants would be precluded with presently available methods.

The basis for thinking that the antigenic determinants are in fact based on such labile configurations stems mainly from the observation that "denatured" proteins show reduced activity when studied by the precipitin technique. We have observed (unpublished observations) that when heat and alkaline-denatured thyroglobulin are reacted with antiserum to native thyroglobulin they are able to precipitate only 80% and 60% of the available antibody respectively. However, when studied in the region of antibody excess the antigenic valency of the two modified thyroglobulins was indistinguishable from that of the native form. Moreover, the alkaline-denatured molecules were able to completely inhibit the precipitation between native thyroglobulin and its antibody. Obermayer and Pick (1904) and later Spiegel-Adolph (1926) showed that a heat-denatured non-precipitating form of albumin was able to inhibit the precipitation of native albumin. Ram and Maurer (1959) have studied a modified albumin in which the carboxyl groups were esterified. Though these molecules no longer precipitated with antibody, antibody binding was evidenced by passive cutaneous anaphylaxis and complement-fixation tests, as well as by (apparently reversible) inhibition of precipitation. A decrease in precipitating ability may therefore not necessarily imply destruction of antigenic determinants and may merely result from such factors as alteration of the accessibility of the sites or changes in the solubility properties or in the state of aggregation.

That the antigenic activity we observed was indeed due to thyroglobulin polypeptides is suggested by the following: The activity was directly related to the size of the tryptic fragments (compare dialysate and diffusate). When further peptide bond cleavage was produced by hydrolyzing the digests with pepsin or chymotrypsin, significant reductions in activity occurred. The limited data obtained with reagents that cleave disulfide bonds is similarly consistent. Had the activity been due only to the presence of oligosaccharides (significant amounts of glucosamine, hexoses, and sialic acid have been detected in various thyroglobulin preparations [Robbins and Rall, 1960]) or free amino acids these findings would not be readily explicable. That the activity was not artifactual was confirmed by the immunospecificity exhibited by the fragments when tested in a series of heterologous precipitin systems and further by positive passive cutaneous anaphylaxis tests (not reported here)—a strictly immunologic phenomenon.

While one study exists which suggests that the iodinated amino acid residues may be in and of themselves important antigenic determinants in native thyroglobulin (Clutton *et al.*, 1938), the investigations of others as well as ourselves failed to confirm this (Stokinger and Heidelberger, 1937; Snapper and Grunbaum, 1953). It seems curious that there should be a substantial degree of species specificity in thyroglobulin anti-thyroglobulin systems if the iodinated tyrosines (by themselves) were of great importance antigenically. Moreover, highly iodinated thyroglobulin shows only the same or less antibody-precipitating ability than the native molecule, depending on the degree of iodination (Edelhoch *et al.*, 1961).

The configurational properties of the thyroglobulin fragments were examined. Because of the small size of these fragments, hydrodynamic measurements were not expected to yield useful answers and hence more indirect means were resorted to. With native thyroglobulin, as mentioned above, as well as with many other macromolecules, conformational changes are accompanied by modifications in optical rotation, tyrosyl spectral titration curves, and spectral difference curves. To the extent that these measurements are sensitive to such structural innovations in globular proteins our failure to observe alterations in these parameters with digest fractions implies that either the peptides had a "random" configuration or that their conformation was unusually stable. Our inability to alter the solubility properties or the activity of the fragments by any procedures short of breaking additional covalent links is consistent with such an interpretation.

Insofar as the observations presented in this study are valid for other proteins, it appears therefore that at least some of the antigenic determinants of globular proteins are based on the covalent structure. If this is so then one may hope that, as has been begun with fibrous proteins, the antigenic determinants of globular proteins will be amenable to detailed chemical analysis.

Acknowledgement

We wish to express our gratitude to Dr. Falconer Smith for extending his facilities to us and for helpful discussions during the course of this work.

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