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Splitting of human thyroglobulin

IV. THE ANTIGENICITY OF THE PEPSIN-DERIVED FRAGMENTS

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SUMMARY

Purified human thyroglobulin (Tg) was hydrolysed by pepsin. After completion of hydrolysis the pepsin hydrolysate was passed through a Sephadex G-200 column to remove undigested Tg. Further isolation of the enzymatic fragments was effected by passage through a Sephadex G-75 column. Two discrete fragments, termed pep I and pep II, were separated. The two fragments had sedimentation coefficients of 1·0 and 0·6, respectively. These fragments retained antigenic determinants reactive with both hetero- and auto-antibodies to Tg. The larger fragment, pep I, possessed all antigenic determinants to intact Tg while pep II lacked some determinants. Neither fragment contained novel determinants resulting from proteolytic degradation.

INTRODUCTION

Papain-derived fragments of rabbit thyroglobulin are immunogenic in the rabbit. Even when injected intravenously without adjuvant, these fragments elicit circulating autoantibodies (Stylos & Rose, 1968) and thyroiditis (Anderson & Rose, 1971). Several reports on trypsin-derived fragments of thyroglobulin from various species (Metzger, Sharp & Edelhoch, 1962; Mates & Shulman, 1967, 1968; Stylos & Rose, 1969; Ghayasuddin & Shulman, 1970; Salabe, Davoli & Fontana, 1973) have suggested that such peptides, although fully reactive with heteroantiserum, are antigenically deficient in their reactivity toward autoantibodies.

In this paper we describe the results of studies on the pepsin-derived fragments of human thyroglobulin. These fragments were separated from intact thyroglobulin by gel filtration. Further separation and isolation were effected by additional gel filtration utilizing smaller pore gels. The peptic fragments were tested for purity by immunochemical techniques. Their antigenicity was determined using hetero- and autoantisera specific for thyroglobulin and their immunogenicity tested by injection into rabbits.

MATERIALS AND METHODS

Saline extracts. Human thyroid saline extracts were prepared as previously described (Rose & Stylos, 1969).

Thyroglobulin (Tg). Saline extracts of human thyroid glands were precipitated between 1·60 M and 1·70 M ammonium sulphate and passed through DEAE-cellulose columns as previously described (Rose & Stylos, 1969). The material eluted with the fourth buffer contained mainly intact Tg. This was the starting material for the enzymatic degradation experiments.

Enzyme. Pepsin, twice crystallized, was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Thyroid antisera. The human thyroiditis sera ('autoantiserum') and rabbit antisera ('heteroantiserum') to human thyroglobulin have been previously described (Rose & Stylos, 1969).

Anti-peptic fragment rabbit sera. The production of antisera in the rabbit to the peptic fragments has been carried out in the same manner described in a previous publication (Stylos & Rose, 1969).

Enzyme proteolysis. A 10 mg/ml solution of purified human Tg was mixed with 1/100 (w/w) pepsin using isotonic phosphate

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buffer, pH 5·5. The Tg–pepsin mixture was incubated at 37°C for 18 hr. Proteolytic action was stopped by re-adjusting the pH to 7·5. The digestate was then exhaustively dialysed against phosphate buffered saline, pH 7.2.

DEAE-cellulose chromatography. DEAE-cellulose chromatography was used to purify Tg. Stepwise elution of phosphate buffers of decreasing pH and increasing molarity were used (Rose & Stylos, 1969).

Gel filtration. The enzymatic products of the pepsin digestion of Tg were separated by filtration on dextran gels (Sephadex G-200 and G-75, Pharmacia, Stockholm) according to the method of Flodin & Killander (1962) and Killander (1964). Appropriate fractions were pooled and concentrated by dialysis against sucrose.

Immunodiffusion and immunoelectrophoresis. Double diffusion in 1·2% agarose (Bausch and Lomb) were carried out as described by Ouchterlony (1962), and immunoelectrophoresis (2% agarose) was conducted following the procedure of Scheidegger (1955).

Acrylamide gel electrophoresis. Disc electrophoretic experiments were conducted using both 5% and the standard 7·5% concentrations of acrylamide according to Ornstein (1964).

Tanned cell haemagglutination. Tanned cell haemagglutination or haemagglutination inhibition tests were carried out according to the technique of Boyden (1951) as modified by Witebsky & Rose (1956).

Analytical ultracentrifugation. Analytical ultracentrifugal studies were carried out in a Spinco Model E analytical ultracentrifuge.

RESULTS

After purified thyroglobulin (Tg) was digested with pepsin in the manner described in Materials and Methods, the immunoelectrophoretic patterns of the purified Tg and the whole Tg–pepsin digest were tested with anti-normal human thyroid extract rabbit serum (heteroantiserum). The results are shown in Fig. 1. The rabbit heteroantiserum produced one precipitin arc with the purified Tg, and same major precipitin arc, plus an additional, more rapidly migrating arc, with the Tg–pepsin digest. Gel diffusion analysis (Fig. 2) of the whole Tg–pepsin digest showed two precipitin lines that merged with intact Tg when reacted with the heteroantiserum. By disc electrophoresis in 5% acrylamide gel (Fig. 3), several faster migrating components not present in the purified intact Tg are seen with the Tg–pepsin digest.

The Tg–pepsin digest was then subjected to gel filtration on a Sephadex G-200 column to separate undigested Tg (Fig. 4). The major protein fraction, eluted in the void volume, contained undigested Tg. The subsequent fractions (tubes 29–47 and tubes 49–64) were pooled, concentrated against sucrose solution, dialysed against tris-HCl buffer and then further separated on Sephadex G-75 columns. The Sephadex G-75 elution patterns are presented in Figs. 5 and 6. From these gel filtration patterns it can be seen that essentially no protein was present in the void volume. Therefore the separation of undigested Tg from the peptic fragments of Tg was complete by this criterion. Analytical ultracentrifugation revealed components with sedimentation coefficients of 1·0 (pep I) and 0·6 (pep II).

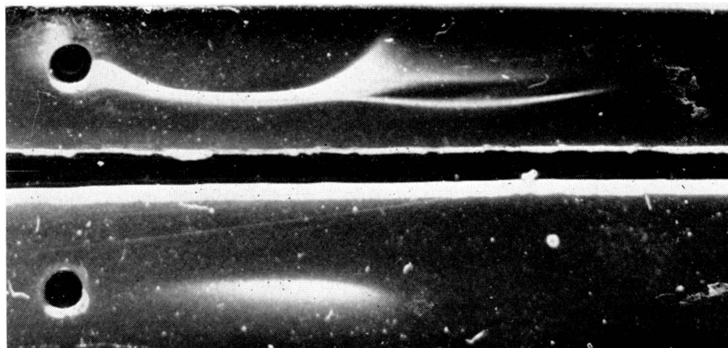
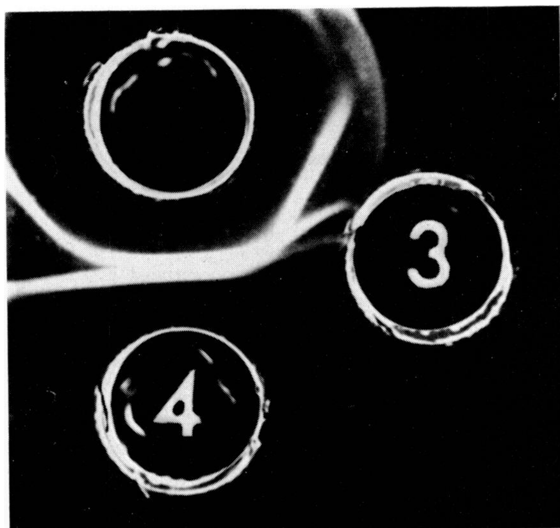


FIG. 1. Immunoelectrophoretic analysis of unseparated thyroglobulin–pepsin digest. Electrophoresis carried out in 2% agarose using barbital buffer, pH 8·2, ionic strength 0·025. Voltage maintained at approximately 5 V/cm for 90 min. Upper well, Tg digested with pepsin; lower well, intact Tg. Horizontal trough filled with heteroantiserum. The anode is to the right.



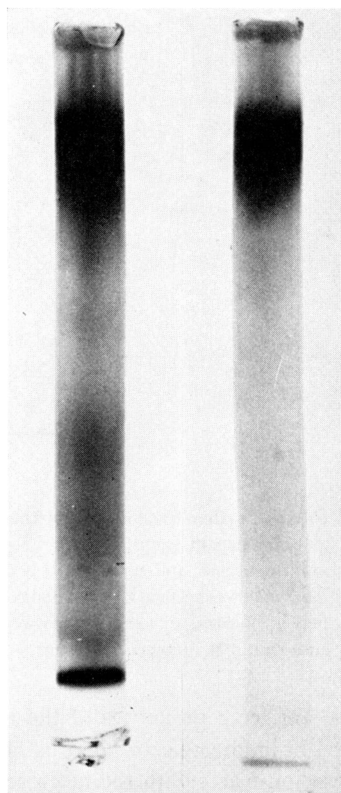


FIG. 2 FIG. 3

FIG. 2. Gel diffusion pattern of unseparated Tg–pepsin digest. Peripheral wells: 3, thyroglobulin-pepsin digest; 4, thyroid saline extract. Both antigens adjusted to a 1% protein concentration. Centre well, hetero-antiserum.

FIG. 3. Disc electrophoresis. On the right, intact thyroglobulin; on the left, thyroglobulin–pepsin digest. Both samples contained 250 μg of protein. Electrophoresis carried out at 5 mA/tube for 45 min. The anode is to the bottom.

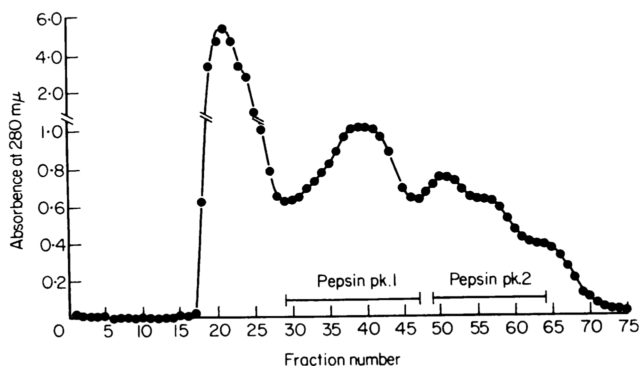


FIG. 4. Separation of undigested Tg from pepsin-produced fragments on Sephadex G-200. The thyroglobulin–pepsin sample was concentrated by dialysis against sucrose, and equilibrated with 0·1 M tris-HCl buffer, pH 8·0. A 3 ml sample (425 mg) was applied to the column (2·5 cm i.d. × 75 cm) equilibrated with the same tris-HCl buffer. Samples of 5·0 ml were collected. The protein fraction eluted first from the column containing undigested Tg. The subsequent fractions, indicated by vertical lines, were pooled and concentrated for further fractionation on Sephadex G-75 columns.

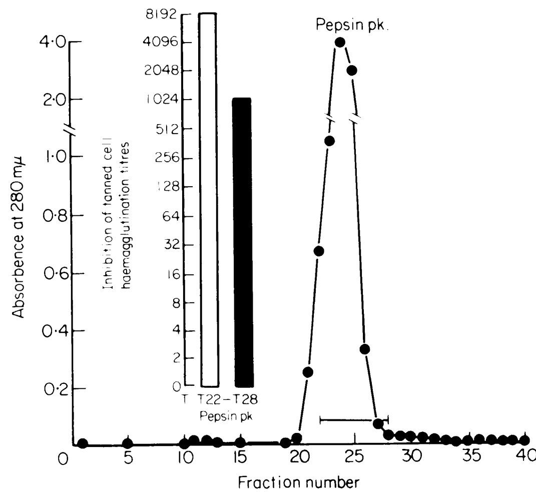


FIG. 5. Further fractionation of the G-200 isolated pepsin fragment of Tg on Sephadex G-75. The thyroglobulin–pepsin sample (tubes 29–47 from Sephadex G-200 fractionation) was concentrated by dialysis against sucrose, and equilibrated with 0·1 M tris-HCl buffer, pH 8·0. A 5·1 ml sample of this protein solution (86·2 mg) was applied to a Sephadex G-75 column (2·5 cm i.d. × 75 cm) equilibrated with the tris-HCl buffer. Pep I, indicated by vertical lines, represents the tubes which were pooled for antigenic analysis. □, Rabbit antiserum; ■, human antiserum.

The antigenic properties of the two pepsin-derived fragments of Tg, viz. pep I and pep II, were studied by immunoelectrophoresis and gel diffusion. In immunoelectrophoresis (Fig. 7), pep I produced two precipitin arcs with the heteroantiserum not seen with intact Tg. Pep II did not show any precipitin reaction. With the autoantiserum (Fig. 8) pep I resulted in a major precipitin arc (similar in electrophoretic mobility to intact Tg) and a minor, more rapidly migrating arc, not seen with intact Tg. The pep II fragment did not show any precipitin reaction. Gel diffusion analysis with the autoantiserum showed a reaction of complete identity between Tg and pep I (Fig. 9). Again, the pep II fragment did not precipitate.

When pep I and pep II were separated by disc electrophoresis (Fig. 10), rapidly migrating protein components are seen that are not detectable (at equivalent protein concentration) with the intact Tg.

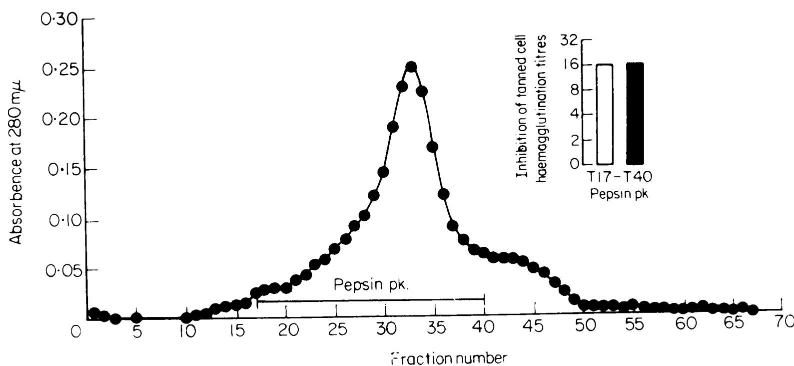


FIG. 6. Further fractionation of the G-200 isolated pepsin fragment of Tg on Sephadex G-75. The thyroglobulin–pepsin sample (tubes 49–64 from Sephadex G-200 fractionation) was concentrated by dialysis against sucrose, and equilibrated with 0·1 M tris-HCl buffer, pH 8·0. A 4·9 ml sample of this protein solution (32·3 mg) was applied to a Sephadex G-75 column (2·5 cm i.d. × 75 cm) equilibrated with the tris-HCl buffer. Pep II indicated by vertical lines, represents the tubes which were pooled for antigenic analysis. □, Rabbit antiserum; ■, human antiserum.

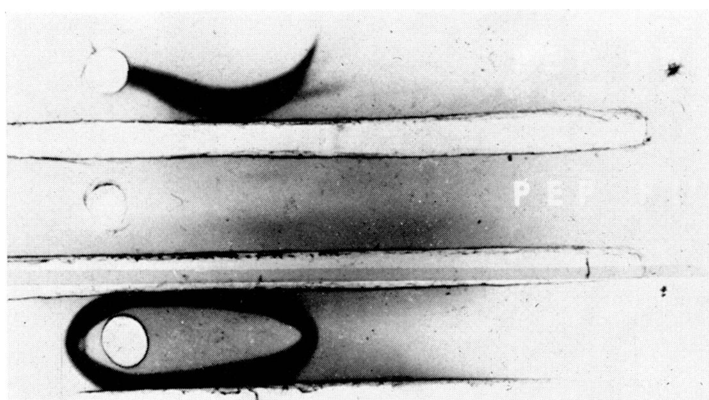


FIG. 7. Immunoelectrophoresis analysis of pep I and pep II isolated by Sephadex G-75 gel filtration. Electrophoretic conditions as described in the legend of Fig. 1. Upper antigen well, pep I, 3·4 mg/ml; middle antigen well, pep II 1·1 mg/ml; lower antigen well, intact Tg, 7·7 mg/ml. Horizontal troughs filled with the hetero-antiserum absorbed with normal human serum. The anode is to the right.

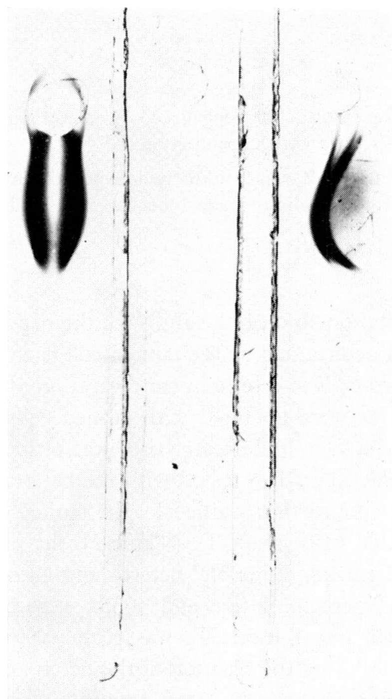


FIG. 8. Immunoelectrophoretic analysis of pep I and pep II isolated by Sephadex G-75 gel filtration. Electrophoretic conditions as described in the legend of Fig. 1. Left antigen well, pep I, 13·1 mg/ml; middle antigen well, pep II, 3·4 mg/ml; right antigen well, intact Tg, 7·7 mg/ml. Antiserum troughs filled with auto-antiserum. The anode is to the bottom.

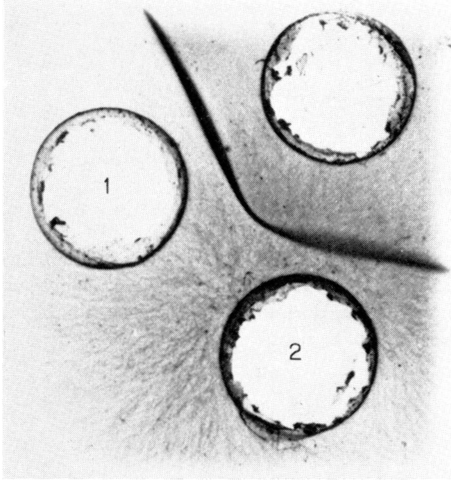


FIG. 9

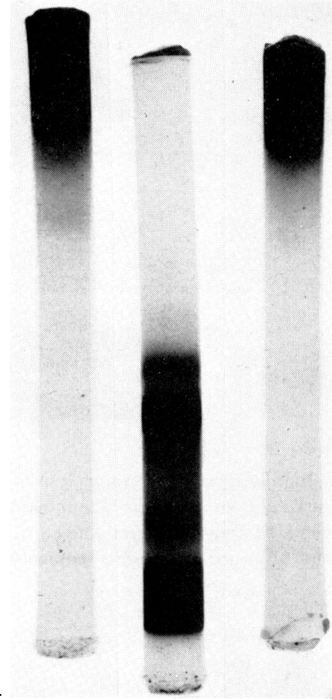


FIG. 10

FIG. 9. Gel diffusion pattern of pep I isolated by Sephadex G-75 gel filtration. Peripheral wells; 1, intact Tg, 7·7 mg/ml; 2, pep I, 13·1 mg/ml. Centre well, autoantiserum.

FIG. 10. Disc electrophoresis. On the left, pep I, in the middle pep II, on the right intact Tg. All samples contained 800 μg of protein. Electrophoresis carried out at 5 MA/tube for 45 min. The cathode is uppermost.

In order to augment the precipitation studies, the ability of the pepsin-derived fragment to react with the hetero- and auto-antisera was determined by the tanned cell haemagglutination reaction. Intact Tg and pep I were used to coat tanned cells at a protein concentration of 100 μg/ml (Table 1). Equivalent tanned cell haemagglutination titres were obtained with tanned cells coated with intact Tg or pep I with the autoantiserum. However, a four-fold greater titre was observed with cells coated with pep I compared to those coated with intact Tg when tested with the heteroantiserum. The relative antigenic potencies of pep I and intact Tg were further studied by the tanned cell haemagglutination-inhibition test. When tanned cells were coated with intact Tg (Table 2), the pep I fragment was as effective in inhibiting the reaction as intact Tg using either the hetero- and autoantiserum. When pep I fragment was used as coating antigen, equivalent inhibition end points were obtained with the autoantiserum; however, slightly less (2·5-fold) pep I than intact Tg was required to produce total inhibition. Pep II was significantly less capable of inhibiting the reaction of the heteroantiserum with either intact Tg or pep I. In the case of the autoantiserum, very large amounts of pep II were required to produce complete inhibition.

The immunogenicity of the pepsin-derived fragments was determined by intradermal injection of the isolated fragments emulsified with complete Freund's adjuvant. Circulating antibody to the peptic fragments was measured by the tanned cell haemagglutination test. It was found that pep I was more immunogenic than pep II (Table 3). Very little antibody activity was obtained when anti-pep I and anti-pep II were tested with tanned cells coated with normal human serum.

TABLE 1. Tanned cell haemagglutination test: direct haemagglutination by hetero- and autoantisera of intact thyroglobulin and pep I

Antisera	Tanned cells coated with: Intact Tg (100 μg/ml)	Pep I (100 μg/ml)
Heteroantiserum	260,000*	1,024,000
Autoantiserum	260,000	260,000

* Figures represent the greatest dilution of antiserum producing a positive haemagglutination reaction.

TABLE 2. Tanned cell haemagglutination inhibition: inhibition of hetero- and autoantisera produced by intact Tg and pep I

Antiserum*	Haemagglutination antigen†	Inhibition produced by: Tg	Pep I	Pep II
Heteroantiserum	Intact Tg	0·03‡	0·03	0·49
	Pep I	0·02	0·02	0·49
Autoantiserum	Intact Tg	0·03	0·03	1·95
	Pep I	0·004	0·01	31·25

* Antiserum dilution: heteroantiserum, 1:25,000; autoantiserum, 1:25,000.
† Antigens used to coat tanned cells at a protein concentration of 100 μg/ml.
‡ Represents the amount of each fraction (μg) needed to inhibit completely the tanned cell haemagglutination reaction.

TABLE 3. Tanned cell haemagglutination test: titres of rabbit antisera produced by the peptic fragments of human Tg

	Tanned cells coated with: Human thyroid extract	Normal human serum
Antiserum	100 μg/ml	200 μg/ml
Anti-pep I rabbit serum	120,000*	2
Anti-pep II rabbit serum	2,000	16

* Figures represent the greatest dilution of antiserum producing a positive haemagglutination reaction.

Rabbits injected with the pepsin-derived isolated fragments were skin tested in order to determine if these fragments were capable of eliciting a delayed hypersensitivity reaction. It was found (Table 4) that the rabbits immunized with pep I and challenged with the homologous antigen (pep I) produced marked induration and erythema within 2 hr and reached maximum reactivity in 24 hr. The induration and erythema remained the same throughout the observation period which lasted 72 hr.

The thyroids of other rabbits immunized with pep II were examined and found to be normal in appearance. Those immunized with Tg and with pep I were infiltrated with mononuclear cells, giving the appearance of focal lymphocytic thyroiditis.

TABLE 4. Skin tests: reactions at 24 hr of rabbits immunized with pepsin-derived fragments of human Tg

	Rabbits immunized with:			
	Pep I		Pep II	
Skin test antigen	Induration*	Erythema†	Induration	Erythema
Pep I	+++	+++	++	++
Pep II	n.d.‡	n.d.	–	–
Intact Tg	+++	+++	±	+

* Induration: +++, 0·2–0·5 cm in diameter; ++, 0·1–0·2 cm in diameter; +, 0·5–0·1 cm in diameter; ±, slightly raised (0·05 cm); –, no reaction.

† Erythema: +++, 2·0–3·8 cm in diameter; ++, 1·5–1·9 cm in diameter; +, trace of erythema; –, no reaction.

‡ n.d.; Not done.

DISCUSSION

When human Tg is hydrolysed with pepsin, and intact Tg is separated from the digestate, the peptide fragments (termed pep I and pep II) remain. Elution patterns of these two components from Sephadex (Figs. 5 and 6) fail to reveal any intact thyroglobulin. From the retardation on Sephadex G-75 the molecular weight of both fragments can be estimated at less than 50,000, pep II being the smaller of the two fragments. The sedimentation constants are compatible with these estimates of molecular weight.

Studies of these peptic fragments by immunoelectrophoresis (Figs 7 and 8) and Ouchterlony gel diffusion analysis (Fig. 9) suggest that pep I has all of the antigenic determinants of intact Tg in its reactivity with both hetero- and auto-antisera. Electrophoretically, at least two novel components could be recognized in pep I. Two lines were also seen in gel diffusion precipitation with the rabbit antiserum, and they merged smoothly with the precipitation band produced by intact Tg. In haemagglutination inhibition, pep I was comparable to intact Tg in its ability to inhibit the reactions of the heteroantiserum with Tg, and somewhat more capable of inhibiting the autoantiserum in a weight basis. These findings indicate that pep I has all of the antigenic determinants of intact Tg and that they are readily available to complex with antibody.

The smaller isolated fragment, pep II, failed to show any precipitin reaction in immunoelectrophoresis or gel diffusion tests with either the hetero- or auto-antiserum. Further antigenic studies utilizing the inhibition of the tanned cell haemagglutination reaction (Table 2) revealed pep II to be a very weak inhibitor, needing 25 times more protein than pep I to produce total inhibition of the reaction of the hetero-antiserum. It required 65 times more pep II than pep I to produce total inhibition of the reaction of the autoantiserum with Tg and 3000 times more to totally inhibit the reaction with pep I.

Immunogenic studies of the peptic fragments reveal that pep I is highly immunogenic when emulsified with complete Freund's adjuvant and injected intradermally into the rabbit. That most of the antibody is directed toward thyroid components as evidenced by the lack of significant ractivity towards tanned cells coated with normal human sera (Table 3).

The ability of the peptic fragments to elicit a delayed hypersensitivity reaction was determined by rabbit skin tests. Pep I is as effective as intact Tg in producing induration and erythema. As with *in vitro* tests for antigenicity (lack of precipitin reaction in immunoelectrophoresis and gel diffusion) pep II was found not to stimulate induration and erythema in the rabbit injected with pep II and challenged with the homologous (pep II) antigen. Correspondingly, pep II was apparently unable to produce thyroiditis in rabbits.

Previous studies utilizing trypsin to fragment thyroglobulin from various species (Metzger *et al.*, 1962; Mates & Shulman, 1967, 1968; Stylos & Rose, 1969; Ghayasuddin & Shulman, 1970; Salable

et al., 1973) have revealed that trypsin-derived fragments of thyroglobulin bear all the major antigenic determinants reactive with hetero-antiserum, but lack some autoantigenic determinants. Essentially the same result (i.e. a substantial loss of autoantigenic determinants) has been reported by Rose & Stylos (1969), Salabe *et al.* (1973) and Smith & Shulman (1973) when thyroglobulin is reduced and alkylated.

The data presented in the present paper suggest that autoantigenic determinants (as tested by reaction with human thyroiditis serum) were reduced in the pep II fragment. Whether the depletion of these antigenic determinants is due to destruction by the enzyme used (pepsin), or production of small molecules which were subsequently lost during the isolation procedure are questions that can only be determined by additional studies. On the other hand, it is apparent that other small molecular weight fragments (pep I) of about 50,000 daltons can be produced from native thyroglobulin which retain full reactivity with autoantiserum and are capable of inducing delayed hypersensitivity and thyroiditis in the rabbit. In this regard, such enzyme-derived fragments may be important as initiators of the autoimmune response in the human.

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