A QUANTITATIVE THEORY OF THE PRECIPITIN REACTION

VI. The Reaction between Mammalian Thyroglobulins and Antibodies to Homologous and Heterologous Preparations*

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Investigations on the mechanism of thyroid action have emphasized the importance of thyroxine, a crystalline degradation product of the protein, thyroglobulin, which appears to be the actual thyroid hormone. Only limited information is available on the chemical and physical properties of thyroglobulin, on the interrelationships of the thyroglobulins of various animal species, and on the immunological properties of the protein and antisera produced by its injection. Heketoen and his collaborators (1) have made a qualitative comparison and found a greater serological relationship between the mammalian thyroglobulins than between the thyroglobulins of mammals and chickens. On the other hand, absorption experiments revealed distinct differences between certain of the mammalian thyroglobulins. Since a deeper insight into these similarities and disparities might assist in an understanding of the nature of organ specificity it was felt that further study of the problem was warranted, especially since new and more sensitive quantitative methods have become available since the earlier work.

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Preparations of human, hog, beef, and sheep thyroglobulins were made according to (2). Evidence had already been obtained that hog and human thyroglobulins, at least, were of high molecular weight, essentially monodisperse (3), and contained but very small amounts of serum globulin when prepared in this way.

It has previously been shown that typical instances of the precipitin reaction could be quantitatively described by means of equations derived from the law of mass action (4–8). These equations and the significance of their constants, as well as empirical equations which in some instances fitted the data even more closely, were discussed in the preceding paper (8) and are therefore given only in their applied form in the tables in the present paper.

It was thought of interest to test this quantitative theory of the reaction mechanism in the additional case of a protein of far higher molecular weight than had previously been studied.

Taylor, Adair, and Adair (9) have doubted the validity of the assumption made in earlier papers of this series that all of the added antigen is precipitated in the presence of antibody excess. Opportunity for an experimental verification of this assumption was furnished by the use of thyroglobulin, a single antigenic substance containing iodine, an element which could be estimated quantitatively in the specific precipitates.

As a part of this study certain hapten reactions involving diiodotyrosine, thyroxine, and enzymic digests of thyroglobulin are presented, and their relation to thyroid action is discussed.

EXPERIMENTAL

The various thyroglobulins[1] were prepared essentially according to (2). N was taken as 15.8 per cent (2); the serum globulin in two preparations was determined by the quantitative precipitin method (10). Data on the Tg preparations are given in Table I.

Tg suspensions were made and injections carried out as described in (6). Rabbits were injected with 1 to 5 mg. of alum-precipitated Tg four times a week for 4 weeks with a rest period of 1 month between courses. All sera were collected sterilely and preserved with 1:10,000 merthiolate.[2] The quantitative precipitin

[1] Referred to throughout as Tg.

[2] Kindly presented by the manufacturers, Eli Lilly and Company, Indianapolis.

estimations were carried out as in previous communications by analysis of the washed specific precipitates for nitrogen by the micro Kjeldahl method. All analyses were made in duplicate unless otherwise indicated. To facilitate calculation N values are given to three decimal places although it is realized that the last figure is uncertain.

Owing to the presence of small amounts of serum globulin in the Tg preparations all anti-Tg sera were absorbed with small amounts of serum globulin of the same species as the Tg used for injection, until no further precipitation occurred. Only after this had been done was the Tg-antibody reaction studied. With regard to the proportion of anti-serum globulin found, anti-hog $12E_1$ serum 3.75_3 contained 0.07 mg. of anti-serum globulin N per ml. (analysis on 30 ml.). After absorption 1.07 mg. anti-Tg remained, so that of the total antibody produced during three courses of injection of Tg 6 per cent was due to the serum globulin in

TABLE I

Thyroglobulin Preparations

Preparation No.	Source	Iodine	Serum globulin
		per cent	*per cent*
17 B	Human	0.75	
19 B	"	0.62(5)	
21 B	"	0.31	
13 B_2	Hog	0.58	1.1
13 B_3	"	0.58	2.4
12 E_1	"	0.58	
F B_5	Beef	0.21	
5 A	"	0.68	
F B_4	Sheep	0.34	

the original Tg. Presence of excess serum globulin did not influence the amount of N precipitated by Tg from its antisera. Serum CQ5, absorbed with an adequate amount of hog serum globulin, and at another time with a considerable excess, gave with Tg 0.456 and 0.466 mg. N per ml., respectively. In another instance the excess of globulin was many times that required to inhibit precipitation of its antibody, yet this exerted no effect on the N precipitable by Tg.

The pH of all antisera was ascertained with the glass electrode[3] and, within the limits used, 6.8 to 8.05, no effect of pH change was noted on the amount of N precipitated. Since this is in agreement with previous work on other immune systems (Marrack and Smith (11); (6)), details are omitted.

The solubilities of hog and human Tg specific precipitates at 0°C. have been determined by estimation of N in precipitates washed with varying amounts of

[3] Courtesy of Mr. F. Rosebury, Department of Biochemistry.

saline, and in precipitates formed after dilution with saline or with normal rabbit serum. The data are summarized in Table II. It will be noted that the solubility of Tg-anti-Tg in 0.9 per cent saline is very small, about 0.003 mg. N per ml. Solubility in normal rabbit serum appeared to be negligible. The solubility of the specific precipitate is thus less than in the other rabbit antibody systems studied (6–8).

TABLE II

Solubility of Thyroglobulin-Antithyroglobulin Precipitates

Volume saline washings	Total N precipitated	Solubility 0°C., N	Tests on supernatants	Volume	Total N precipitated	Solubility 0°C., N	Tests on supernatants
ml.	*mg.*	*mg. per ml.*		*ml.*	*mg.*	*mg. per ml.*	
Serum 3.75_3, anti-$12E_1$, hog 0.5 ml. serum, 0.198 mg. hog Tg N in 0.5 ml.				Serum 3.99_3 anti-19B, human 0.5 ml. serum, 0.158 mg. human Tg N			
4	0.620	0.003	Excess A, trace Tg	4	0.642	0.003 (5)	Trace A, trace Tg
8	0.608			8	0.628		
Volume							
Serum CQ 4, anti-$13B_2$, hog 1.0 ml. serum, 0.047 mg. hog Tg N				Serum 3.99_3 0.5 ml. serum, 0.158 mg. hog Tg N			
1.5	0.148	0.003	Excess A	1	0.252*	0.003 (5)	Excess A
8	0.128			5	0.238		
Dilution with normal rabbit serum: Serum 3.53_2, anti-13 B_3, hog 1.0 ml. serum, 0.079 mg. hog Tg N				Dilution with normal rabbit serum (compared with above)			
1.5	0.338	0.001	Excess A	4	0.254†	0.001	Excess A
8.5	0.332*			8	0.246		

* Single determination.

† Average of three determinations.

Since slightly greater amounts of total N were precipitated at 0°C. for 48 hours than in 2 hours at 37°C. and overnight in the ice box, the former conditions were used unless otherwise indicated; thus anti-hog Tg serum 2.61 gave 0.470 and 0.456 mg. N under the two sets of conditions. At 37°C. no difference in N precipitated was observed in ½ hour or 2 hours, and only additional traces of N were precipitated if the tubes were then allowed to stand overnight in the ice box.

Iodine Content of Specific Precipitates.—In order to ascertain whether or not

the iodine in the specific precipitates was derived entirely from the Tg, antibody-containing globulin was isolated from a typical serum, 3.75_2, anti-12 E_1, by precipitation with 0.85 volume of Na_2SO_4 solution, saturated at 35°C. After dialysis of the precipitate the resulting solution contained 1.56 mg. of N per ml. Duplicate 1.0 ml. samples of this solution were analyzed for I and were entirely negative, as was the saline used in the washings. Iodine determination reagents were I-free. The method used was that of Leipert (12) modified by Dr. G. L. Foster of this department to determine iodine quantities of about 10 γ in the presence of large amounts of protein (20,000 times). The writers wish to express their gratitude for Dr. Foster's aid with these determinations and for permission to use this as yet unpublished method. The above 3.75_2 antibody globulin contained 0.23 mg. of antibody N per ml.

In Table III are summarized the results of the micro iodine determinations on the specific precipitates from four antisera to both human and hog Tg of known N and I content. Points in the equivalence zone, and on both sides of the zone were selected so that the largest possible amounts of iodine could be measured. The same amounts of Tg as were used for precipitation were analyzed for I. After elimination of one determination as out of line, the iodine analyses given in Table III indicate that 96 to 101 per cent of the added Tg is recovered in the specific precipitates up to the first point at which a slight excess of Tg appears. Hooker and Boyd (13) similarly found 90 to 100 per cent of antigen precipitated in the case of hemocyanin. When an excess of Tg is added, a lower proportion of the iodine is, of course, recovered in the precipitate and the relative amount of I (antigen) recovered is that anticipated from the supernatant tests.

Addition of Increasing Amounts of Thyroglobulin to Homologous Antithyroglobulin Sera.—In Table IV are given the quantitative precipitin data obtained by the addition of increasing amounts of human, hog, beef, and sheep Tg to homologous antisera. The course of the reaction is recorded for sera of a single rabbit immunized with the Tg of a single species and given several courses of injections. Analogous data, omitted in the present report, were obtained on many sera, but a summary of data relating to the equivalence zone will be found in Table V for all sera on which a sufficient number of analyses were run. Equations [1] and [4] (*cf.* (8)), are given wherever possible for sera in Table IV and a comparison is made of the precipitated antibody N calculated from these equations with the experimentally

TABLE III

Estimation of Iodine in Thyroglobulin-Antithyroglobulin Precipitates

Tg added	I found	I calculated	I recovered	Total N precipitated	Tests on supernatants	A/Tg in precipitates, calculated from I and total N
mg.	*gammas*	*gammas*	*per cent*	*mg.*		
1.0 ml. anti-hog Tg 12E$_1$ serum 3.75$_3$, hog Tg, 0.58% I 2 washings, 2 ml. saline each						
1.5	8.8	8.7	101		Excess A	
2.0	11.1	11.6	96		" "	
2.5	14.1	14.5	97	1.26	Trace Tg, excess A	2.3
3.0	16.4	17.4	94		Trace Tg, trace A	
Anti-hog Tg 12E$_1$ serum 3.75$_2$, hog Tg, 0.58% I						
2.0*	10.9	11.6	94	1.15	Excess A, trace Tg	2.9
3.0†	17.1	17.4	98	1.72	" " " "	2.7

Tg added	I found	I calculated	I recovered	Total N precipitated	Tests on supernatants	A/Tg in precipitates, calculated from I and total N
mg.	*gammas*	*gammas*	*per cent*	*mg.*		
2.0 ml. anti-human Tg 19B serum 4.00$_1$, human Tg, 0.62(5)% I 2 washings, 2 ml. saline each						
1.25	7.6	7.8	97	0.63	Trace Tg, excess A	2.3
1.50	9.3	9.4	99		Trace Tg, trace A	
2.00	11.9	12.5	95		Excess Tg, trace A	
2.0‡	11.2	12.5	90		Excess Tg, no A	
2.0§ supn't.	1.0		98 (total)		Excess A	
1.0 ml. anti-human Tg 19B serum 3.99$_3$, human Tg, 0.62(5)% I						
2.0‖	12.3	12.5	98	1.36	Trace A, trace Tg	3.4
2.0‡‖	12.3	12.5	98		Excess A	
3 washings with 1.5 ml. saline 3rd washing: 0.6γ I						

* 2.0 ml. serum.

† 3.0 ml. serum used, single determination in 4 ml. vol.

‡ 1.5 ml. serum.

§ Added to excess antiserum and specific precipitate analyzed.

‖ Single determination.

found values. Agreement between the observed and calculated values was in some cases better and in others less satisfactory than in the examples given.

Several analyses of the specific precipitate were carried out in the region of excess Tg by the method given in (6). Antibody N:Tg N ratios as low as 0.6 and 0.4 were found in the hog Tg system.

Serial Additions of Thyroglobulin to Homologous Antiserum.—Table VI shows typical results of serial additions of small amounts of human and hog Tg to homologous antisera and contains a comparison with the total antibody N which would have been precipitated by single addition at the same Tg N value, calculated to the original volume from Equation [1] as given for the same serum in Table IV. The course of each serial reaction was described with considerable accuracy by the equations given in Table VI.

Heterologous Reactions.—A summary of the proportion of antibody precipitable by heterologous Tg from the various sera is given in Table VII with the age of the sera used. Data on several of the numerous possible cross reactions involving the four Tg's and their antibodies are given in Table VIII. Table IX shows the proportion of antibody precipitable by heterologous Tg remaining after partial precipitation of antiserum with homologous Tg.

Action of Various Related Haptens.—In view of the results reported by Adant and Spehl (14) and Snapper (15) *l*-3,5-diiodotyrosine[4] and *dl*-thyroxine were tested for hapten action in homologous and heterologous human and hog Tg-anti-Tg systems. *l*-3,5-Diiodotyrosine was used in 1:10,000 concentration in 0.9 per cent saline and also as its sodium salt at pH 7.32. *dl*-Thyroxine, due to its extreme insolubility in saline, was used as its disodium salt at pH 10.6.

Addition of these solutions to human and hog anti-Tg sera failed to produce precipitation, and did not inhibit precipitation on subsequent addition of homologous or heterologous Tg. In addition, the alcohol-precipitable material from successive peptic and tryptic[5] digestion of denatured hog Tg, with 41 per cent NH_2 N, gave only negligible precipitates with anti-Tg sera and showed no inhibiting effect.

[4] Kindly furnished by Dr. G. L. Foster.

[5] Merck's preparation containing polypeptidases; however, digest gave positive biuret test.

The polysaccharide isolated from Tg by alkaline hydrolysis[6] also failed to show hapten action.

DISCUSSION

The immunological property of thyroglobulin which has hitherto been most emphasized is its organ specificity (1). The question of antihormones has, however, recently become one of importance. If, as appears probable, thyroglobulin is the actual hormone of the thyroid gland instead of the crystalline degradation product, thyroxine, thyroglobulin stimulates the production of an antihormone as it readily gives rise to precipitins. The present quantitative study of precipitin reactions involving mammalian thyroglobulins was carried out in the hope of adding to the knowledge of thyroglobulin in both of the above respects and also in order to include in the series of precipitin reactions studied one in which the antigen was of high molecular weight (3).

While the thyroglobulin of no species has yet been obtained in crystalline form, those which have been studied (3) are essentially monodisperse. The thyroglobulins included in the present work behaved as single antigens, in that supernatants in the equivalence zone failed to show the presence of antigen or antibody when tested with fresh antiserum or antigen. Positive reactions in the supernatants from the equivalence zone may be taken as an indication of several components (*cf.* (8) for a more complete discussion). The preparations used (2) contained small amounts of serum globulin but any disturbing effects of this impurity were eliminated by absorption of all antisera with serum globulin of the same species as the thyroglobulin injected. In agreement with Hektoen and collaborators (1), no immunological relation between serum globulin and thyroglobulin could be detected, a result all the more striking since the thyroglobulins, although so closely related as to show a definite organ specificity, also exhibit marked species differences just as do the serum globulins. In the thyroglobulins these species differences appear to be related to an entirely different molecular configuration than in the case of the serum globulins, since addition of far more serum globulin than re-

[6] To be described in a subsequent communication.

quired to inhibit precipitation of the antiglobulin in the anti-Tg sera failed entirely to reduce the amount of antibody thrown down by Tg.

The solubility data given in Table II indicate a somewhat lower solubility for the Tg-anti-Tg precipitate than was found in this laboratory for other protein-antiprotein precipitates; that is, about 0.003 mg. of N per ml. as against 0.005 mg. in the egg albumin system (6) and 0.006 mg. in the serum albumin system (8). The solubility of the thyroglobulin specific precipitate in normal rabbit serum was only about 0.001 mg. of N per ml. These data also show that the composition of the specific precipitate does not depend on the concentration of antibody at equilibrium, but rather on the proportions in which the reactants are mixed. This would indicate that the considerations which led to the adoption of equations [1] to [4] (see introduction, Paper V (8)) were equally applicable to the Tg system.

Before the analytical data could be fully relied upon it seemed desirable to test the assumption made in previous papers of the series that a single antigen (or hapten) was quantitatively precipitated in the region of excess antibody and the equivalence zone if a test of the supernatant with more antiserum showed no trace of the antigen (or hapten). The reasons for this assumption were fully given in (6) but its validity was questioned by Taylor, Adair, and Adair (9). Since the iodine content of the Tg used was accurately known and anti-Tg had been found to be free from iodine, it was possible to precipitate a given amount of Tg iodine with an excess of antiserum and determine the proportion of the iodine in the washed specific precipitate. As will be seen from Table III, a recovery of 96 to 101 per cent was usually obtained. The above assumption is therefore shown to be justified in the one instance in which a direct test was possible, and this adds an additional reason for its use to those given previously (6).

It appears, therefore, that the ratios given in column 4 of Table IV actually represent the ratio of antibody N to Tg N in the Tg-homologous anti-Tg specific precipitate in the region of excess antibody and in the equivalence zone. Calculations of the composition of the precipitate from the iodine content gave similar ratios of the components, as shown in Table III. When these ratios were plotted against the Tg N precipitated it was usually possible to draw a straight line fairly satisfactorily through the points and thus obtain the constants

for equation [1] for the serum. Usually the points farthest from the line were at the extreme antibody excess end and near the antigen excess end of the equivalence zone. There was thus often a fraction of antibody present in the sera which formed compounds with Tg in which the antibody N:Tg N ratio was greater than 2R, and in such instances the empirical equation [3], in which the ratios were plotted against the square root of the Tg N in the precipitate, often showed better agreement in the region of large antibody excess since this relation covers the formation of compounds of three times the antibody N:Tg N ratio at the maximum (see introduction, Paper V (8)). In Table IV equations [1] and [4], derived from [2] and [3] by multiplying through by Tg N, are given in the instances in which sufficient data were available, and it will be noted that there is fair agreement of the experimentally determined antibody N values with those calculated from one or the other of these relations. It was evident in most cases, however, that connection of the actual experimental ratio:Tg points led to a curve and not a straight line.

From the above data the reaction between Tg and antibody may also be considered, as a first approximation, to take place in a series of bimolecular competing reactions between multivalent antigen and antibody before precipitation begins. The first step in the reaction would be

$$A + Tg \rightleftharpoons A \cdot Tg$$

followed, for example, in the region of antibody excess, by

$$A \cdot Tg + A \rightleftharpoons ATg \cdot A, \text{ and}$$

$$A \cdot Tg + A \cdot Tg \rightleftharpoons ATg \cdot ATg$$

This process would continue, leading to the formation of larger and larger aggregates until these finally precipitated from solution, possibly on account of their great size or because of the interaction of polar groups brought into juxtaposition leading to a diminution of affinity for water (*cf.* also Marrack (16)).

As nearly as could be estimated, R in equations [1] and [4] corresponded fairly closely with the antibody N:Tg N ratio at the antibody excess end of the equivalence zone (Table V). Usually only 80 to 90 per cent of the anti-Tg was precipitated at this point, so that estima-

TABLE IV

Addition of Increasing Amounts of Thyroglobulin to Homologous Antithyroglobulin Rabbit Serum

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Antibody N calculated from equation [4]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	*mg.*	
Anti-human Tg 17B serum 3.99						
Course 1. 1.0 ml. serum, human Tg 17B used						
0.079	0.350	0.271	3.4			Excess A
0.158	0.472	(0.314)	(2.0)			Trace A, trace Tg
Course 2. 1.0 ml. serum used						
Equation [1]: mg. antibody N pptd. = 6.5 Tg N − 15.0 (Tg N)^2						
Max. Tg N, A N = 0.217, 0.704 mg., respectively						
Equation [4]: mg. antibody N pptd. = 8.0 Tg N − 10.4 (Tg N)$^{3/2}$						
Max. Tg N, A N = 0.263, 0.700 mg., respectively						
0.144	0.784	0.640	4.4	0.625	0.582	Excess A
0.216	0.914	0.698	3.2	0.704	0.682	" "
0.288	1.018	0.730	2.5			No A or Tg
0.316	1.043*	0.727	2.3			" " " "
0.364	1.113†	0.749	2.1			" " " "
0.474	1.220†					Excess Tg
Course 3. 1.0 ml. serum used						
Equation [1]: mg. antibody N pptd. = 7.7 Tg N − 13.8 (Tg N)^2						
Max. Tg N, A N = 0.279, 1.073 mg., respectively						
Equation [4]: mg. antibody N pptd. = 11.2 Tg N − 14.2 (Tg N)$^{3/2}$						
Max. Tg N, A N = 0.277, 1.029 mg., respectively						
0.079	0.686‡	0.607	7.7	0.523	0.570	Excess A
0.158	1.024‡	0.866	5.5	0.872	0.864	" "
0.316	1.358‡	(1.042)	(3.3)			Trace A, trace Tg
0.395	1.408†					Excess Tg
Anti-hog Tg $12E_1$ serum 3.75						
Course 1. 2.0 ml. serum, hog Tg, $13B_2B_3$ used						
Equation [1]: mg. antibody N pptd. = 4.9 Tg N − 15.2 (Tg N)^2						
Max. Tg N, A N = 0.161, 0.395 mg. N, respectively						
0.015 (8)	0.098	0.082	5.2	0.074		Excess A
0.031 (6)	0.170	0.138	4.4	0.140		" "
0.079	0.352	0.273	3.5	0.292		" "
0.158	0.562	(0.404)	(2.6)	(0.394)		Trace A, trace Tg?
0.316	0.804					Excess Tg

* 1.5 ml. 1:1 serum dilution used, calculated to 1.0 ml. whole serum.

† 1.0 ml. 1:1 serum dilution used, calculated to 1.0 ml. whole serum.

‡ One determination only.

TABLE IV—*Concluded*

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Antibody N calculated from equation [4]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	*mg.*	
Anti-hog Tg $12E_1$ serum 3.75						
Course 2. 1.0 ml. serum, hog Tg $12E_1$ used						
0.158	0.556‡	0.398	2.5			Excess A
0.174	0.590‡	0.416	2.4			" "
0.248	0.720‡	(0.472)	(1.9)			No A, trace Tg?
Course 3. 1.0 ml. serum, hog Tg $12E_1$ used.						
Equation [1]: mg. antibody N pptd. = 5.5 Tg N − 9.1 $(\text{Tg N})^2$						
Max. Tg N, A N = 0.302, 0.830 mg. N, respectively						
Equation [4]: mg. antibody N pptd. = 8.2 Tg N − 10.4 $(\text{Tg N})^{3/2}$						
Max. Tg N, A N = 0.277, 0.756 mg. N, respectively						
0.032	0.230	0.198	6.2	0.167	0.202	Excess A
0.079	0.508	0.429	5.4	0.378	0.417	" "
0.158	0.784	0.626	4.0	0.642	0.641	" "
0.237	1.018	0.781	3.3	0.793	0.740	" "
0.316	1.168	0.852	2.7			" "
0.395	1.256	(0.861)	(2.2)			Trace A, trace Tg
Anti-sheep Tg FB_4 serum 4.93						
Course 1. 1.0 ml. serum, sheep Tg FB_4 used						
0.040	0.168	0.128	3.2			Excess A
0.079	0.268	0.189	2.4			Trace A, no Tg
0.249	0.408					Excess Tg
Course 2. 1.0 ml. serum, sheep Tg FB_4 used						
Equation [1]: mg. antibody N pptd. = 6.3 Tg N − 29 $(\text{Tg N})^2$						
Max. Tg N, A N = 0.109, 0.342 mg. N, respectively						
0.040	0.262	0.222	5.6	0.206		Excess A
0.079	0.394	0.315	4.0	0.317		Trace A, trace Tg
0.111	0.456‡	0.345	3.1	0.342		" " " "
0.158	0.508	(0.350)	(2.2)			No A, trace Tg
0.332	0.480					Excess Tg
Anti-beef Tg 5A serum 4.83						
Course 1. 1.0 ml. serum, beef Tg 5A used						
Equation [1]: mg. antibody N pptd. = 4.5 Tg N − 14 $(\text{Tg N})^2$						
Max. Tg N, A N = 0.161, 0.362 mg., respectively						
0.040§	0.213	0.173	4.3	0.158		Excess A
0.079	0.342	0.263	3.3	0.269		" "
0.158	0.498	0.340	2.2	0.361		" "
0.190	0.542‡	0.352	1.9			Trace A?, no Tg
0.237	0.610‡	0.373	1.6			" " " "

§ 2.0 ml. serum used, calculated to 1.0 ml

tion of the total antibody N content of an anti-Tg serum would involve precipitation in the usual way with a slight excess of Tg, estimation of the excess Tg in an aliquot portion of the supernatant by addition to an antiserum calibrated against Tg according to (10), subtraction of the Tg in the total supernatant from the total amount of Tg originally added, and subtraction of the difference from the total N found.

While few analyses of the composition of the Tg-anti-Tg precipitate have been carried out in the region of excess Tg, ratios as low as 0.6 and 0.4 were found for antibody N:Tg N in these analyses. These

TABLE V

Equivalence Zone Ratios of Thyroglobulin Antisera

Serum No.	Antibody N:Tg N ratio at antibody excess end of equivalence zone	Antibody N:Tg N calculated from equation [1]	Antibody N:Tg N ratio at antigen excess end of equivalence zone	Serum No.	Antibody N:Tg N ratio at antibody excess end of equivalence zone	Antibody N:Tg N calculated from equation [1]	Antibody N:Tg N ratio at antigen excess end of equivalence zone
Human-anti-human				Hog-anti-hog			
3.56_1	> 3.6	4.0		CQ 4_1	< 2.2	2.1	
3.99_1	(3.0)		(2.0)	CQ 5_1	> 2.0	2.1	
3.99_2	(3.0)	3.3	(2.2)	2.40_1	< 1.8	1.9	
3.99_3	> 3.3	3.9	< 3.3	2.61_2	< 2.5	2.7	
				2.61_3	2.1		
Sheep-anti-sheep				3.75_1	< 3.5	2.5	< 2.6
4.93_1	2.4			3.75_2	< 2.4		< 1.9
4.93_2	(4.0)	3.2	(2.2)	3.75_3	< 2.7	2.8	(2.2)
				3.97_1	2.1	2.1	
Beef-anti-beef							
4.83_1	< 2.2						

Values in parentheses deduced from nearest actual determination.

instances are an exception to the usual finding that most of the specific precipitate is derived from the antiserum. Ratios smaller than unity were also encountered by Hooker and Boyd (13) in the hemocyanin system and recognized as characteristic of specific precipitates containing antigens of high molecular weight.

It is apparent from Table V that R at the antibody excess end of the equivalence zone was higher in the human Tg-antibody system than in the hog Tg sera. Unfortunately, insufficient data were obtained to fix the other end of the equivalence zone with any certainty, but the

ratios appeared to vary around 2 in both systems. Since it has been shown that the molecular weights of human Tg and hog Tg are the same (3), the apparently broader equivalence zone in the former in-

TABLE VI

Serial Additions of Thyroglobulin to Homologous Antisera, Calculated to Original Volume

Total of successive Tg N additions	Total N precipitated	Total antibody N by difference	Ratio antibody N:Tg N in total precipitate	Ratio antibody N:Tg N in each precipitate	Antibody N calculated from equation [1], Table IV
mg.	*mg.*	*mg.*			*mg.*
7.0 ml. anti-human Tg 17B serum 3.99, total A N 5.11 mg.					
mg. antibody N pptd. = 13.3 Tg N − 8.0 (Tg N)²					
Max. Tg N, A N = 0.832, 5.53 mg., respectively					
0.031(6)	0.440	0.408	12.9	12.9	
0.066	0.899	0.833	12.6	12.5	
0.102	1.345	1.243	12.2	11.3	
0.141	1.837	1.696	12.0	11.6	
0.183	2.336	2.153	11.8	11.0	
0.228	2.848	2.620	11.5	10.4	
0.276	3.342	3.066	11.1	9.3	
0.327	3.843	3.516	10.8	8.7	1.90
0.382	4.257	3.875	10.2	6.5	2.17
0.441*	4.611	4.170	9.5	5.0	2.45
Another bleeding, 3.99_3, gave 95% antibody N pptn.					
5.0 ml. 3:1 anti-hog Tg $13B_2$ serum CQ 5, total A N 1.55 mg.					
mg. antibody N pptd. = 5.9 Tg N − 10.5 (Tg N)²					
Max. Tg N, A N = 0.281, 0.826 mg., respectively					
0.023(7)	0.180	0.156	6.6	6.6	
0.050	0.316	0.266	5.3	4.2	
0.079	0.464	0.385	4.9	4.1	
0.111	0.621	0.510	4.6	4.0	0.420
0.146	0.785	0.639	4.4	3.7	0.540
0.184	0.943†	0.759	4.1	(3.1)	0.665
Another antibody solution, 3.75_2, gave 75% A N pptn.					

The above runs were not made in duplicate.

* Next determination lost, showed slight excess Tg in supernatant.

† Allowed to stand 3 days at 0°C. Trace Tg in supernatant.

stance would seem due to the reactivity of the anti-human Tg with more groupings on the antigen molecule than react with the anti-hog Tg (*cf.* 6).

Perhaps for this reason, which would in some measure determine the range of combining proportions between antigen and antibody, serial additions of human Tg to anti-human Tg serum 3.99_2 showed an unusually large Danysz effect (Table VI). All except the final precipitates were characterized in this instance by very high antibody N:Tg N ratios and the equation for the reaction was very different from that obtained with the same serum by addition of increasing amounts of Tg (Table IV). The appearance of Tg in the final supernatants from the serial experiment before all of the antibody N had been precipitated, indicates, as in other systems, the presence in the antiserum of antibodies of varying reactivity toward the antigen. It is probable, also, that the great size of the Tg molecule increases the tendency toward large Danysz effects, owing to the relatively large number of immunologically reactive groupings available. A Danysz effect of similar proportions was obtained with a later bleeding from the same rabbit. On the other hand, two hog Tg-anti-hog Tg serial experiments with much weaker sera gave smaller Danysz effects resembling those observed in other systems (5, 6, 8).

The remaining tables, VII, VIII, and IX, give data relating to the organ specificity of the thyroglobulins. Hektoen and collaborators (1) showed that mammalian thyroglobulins were closely enough related to be termed organ specific, but that they were not necessarily identical. This conclusion is confirmed by the present quantitative studies and extended in a number of respects. In Table VII are given the percentages of cross reacting antibody in many of the sera for which data with homologous Tg are recorded in Tables IV and V. It will be noted that the proportion of cross reacting antibody increases, in general, with prolonged immunization, as would be expected. Moreover, the reciprocal cross reactions appeared to occur to about the same extent, as shown by the human Tg-anti-hog Tg and hog Tg-anti-human Tg data in Tables VII and VIII.

It is shown in Table VII that the cross reactions, also, may be quantitatively expressed by equations [1] to [4], and in this respect the Tg reactions differ radically from two of those previously subjected to quantitative study (17, 18). The antigens in a third cross reaction, crystalline horse serum albumin and R-salt-azo-biphenyl-azo-crystalline horse serum albumin (8) resembled each other even more closely

than do the thyroglobulins, since they behaved identically in antisera to serum albumin. In the present study 75 to 80 per cent crossing was shown in both directions between sheep and bovine Tg and their antisera, the relationship being the closest of those studied. According to Adant and Spehl (14) sheep and bovine Tg show little crossing, but it is difficult to say whether their conclusion is due, as seems possible

TABLE VII

Approximate Percentage of Cross Reacting Antibody and Age of Antithyroglobulin Sera

Serum No.	Amount of cross reacting antibody	Age of serum	Serum No.	Amount of cross reacting antibody	Age of serum
	per cent	*mos.*		*per cent*	*mos.*
	Hog-anti-human			Human-anti-sheep	
3.56_1	20	1.5	4.93_2	14	3
3.99_1	20	3			
3.99_2	36	10		Hog-anti-sheep	
3.99_3	44	6	4.93_2	40	2
	Human-anti-hog			Sheep-anti-beef	
CQ 4_1	15	2	4.84_1	(75)	2
CQ 5_1	20	2.5			
2.61_3	30	6		Hog-anti-beef	
2.64_1	18	9	4.84_1	(50)	2
3.75_1	12	0.5			
3.75_2	21	6		Human-anti-beef	
			4.84_1	15	2
	Beef-anti-sheep		4.83_1	15	2
4.93_1	80	6			
4.93_2	80	2		Beef-anti-hog	
			4.94_1	45	3

Values in parentheses are approximate.

from their data, to inhibition of the cross reaction by the use of excessive amounts of Tg, to reliance on antigen dilution as a measure of antibody reactivity, or to other factors. Human Tg differs markedly from the others in the series, for in most of the sera obtained after short courses of injections crossing was less than 20 per cent in either direction. The quantitative precipitin method therefore gives results corresponding in general to the biological relationship of the animals

TABLE VIII

Addition of Increasing Amounts of Heterologous Thyroglobulin to Antithyroglobulin Rabbit Serum

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	
		Anti-human Tg serum 3.99			
		Course 2. 1.0 ml. serum, hog Tg $12E_1$ used			
		Equation [1]: mg. antibody N pptd. = 2.6 Tg N − 6.4 (Tg N)^2			
		Max. Tg N, A N = 0.203, 0.264 mg., respectively			
0.040	0.130	0.090	2.3	0.094	Excess A, no Tg
0.079	0.242	0.163	2.1	0.165	" " " "
0.158	0.406	0.248	1.6	0.251	" " " "
0.211	0.472	(0.261)	(1.2)		Trace A, trace Tg
		Course 3. 1.0 ml. serum, hog Tg $12E_1$ used			
		Equation [1]: mg. antibody N pptd. = 3.3 Tg N − 6.1 (Tg N)^2			
		Max. Tg N, A N = 0.270, 0.446 mg., respectively			
0.079	0.336	0.257	3.3	0.223	Excess A, no Tg
0.158	0.516*	0.358	2.3	0.370	" " " "
0.237	0.624†	0.387	1.6	0.439	No Tg
0.316	0.780†	(0.464)	(1.5)		Trace Tg
0.632	1.016†				Excess Tg
		Anti-sheep Tg serum 4.93			
		Course 1. 1.0 ml. serum, beef Tg 5A used			
		Equation [1]: mg. antibody N pptd. = 2.8 Tg N − 10.5 (Tg N)^2			
		Max. Tg N, A N = 0.133, 0.187 mg., respectively			
0.040	0.144	0.104	2.6	0.095	Excess A, no Tg
0.063	0.190	0.127	2.0	0.134	" " " "
0.079	0.224	0.145	1.8	0.155	" " " "
0.158	0.354	(0.196)	(1.2)		No A, trace Tg
		Course 2. 1.0 ml. serum, beef Tg 5A used			
		Equation [1]: mg. antibody N pptd. = 4.4 Tg N − 17 (Tg N)^2			
		Max. Tg N, A N = 0.129, 0.285 mg., respectively			
0.040	0.210	0.170	4.3	0.149	Excess A, no Tg
0.079	0.310	0.231	2.9	0.242	" " " "
0.102	0.380	0.278	2.7	0.272	No A, no Tg
0.158	0.440	(0.282)	(1.8)		No A, trace Tg

* Single determination.

† 0.5 ml. serum, calculated to 1.0 ml.

TABLE VIII—*Concluded*

Tg N added	Total N precipitated	Antibody N by difference	Ratio antibody N:Tg N	Antibody N calculated from equation [1]	Tests on supernatants
mg.	*mg.*	*mg.*		*mg.*	
		Anti-beef Tg serum 4.84			
		Course 1. 1.0 ml. serum, sheep Tg FB_4 used			
0.040	0.132	0.092	2.3		Trace A, no Tg
0.079*	0.178	(0.099)	(1.3)		Trace Tg
0.158	0.170	(inhibition)			
		1.0 ml. serum, hog Tg $12E_1$ used			
0.040	0.104	0.064	1.6		Excess A
0.079	0.118	(inhibition)			
		1.0 ml. serum, human Tg 21B used			
0.015(8)	0.041‡	(0.025)	(1.6)		Trace Tg
0.040*	0.042	(inhibition)			
		Anti-hog Tg serum 4.94			
		Course 1. 1.0 ml. serum, beef Tg 5A used			
0.020	0.058‡	0.038	1.9		Excess A, no Tg
0.031(6)	0.072	0.040	1.3		No A, no Tg

‡ 2.0 ml. samples calculated to 1.0 ml.

from which the Tg were derived, and should be a more useful tool in serological studies of such relationships than the inaccurate, qualitative dilution methods customarily employed. Rough measurements of relative precipitate volumes in the cross reactions of mammalian sera were made by Nuttall and Strangeways (19).

It is also evident from Table VIII that 2R in equation [1] is usually considerably lower than in the homologous reaction (Table IV). Since the molecular weights of human Tg and hog Tg, at least, have been shown to be the same (3) the ratio effect may be most simply explained by the assumption that the antibody reacts with fewer immunologically active groupings on the heterologous Tg molecule than on the homologous Tg.

Another aspect of the cross reactions is brought out in Table IX. Several sera were fractionally precipitated by the homologous Tg so that as much antibody N, or somewhat more, was removed than had previously been found to enter into the cross reaction. Analysis of

the remaining antibody for cross reacting antibody N showed that nearly one-half remained, so that the heterologous anti-Tg was by no means confined to the portion of the antibody most reactive with homologous Tg. The figures in column 3 show, however, that the initial precipitate was relatively high in the heterologous anti-Tg.

Since data are available on the molecular weights of thyroglobulin (3) and at least a limited number of antibodies formed in the rabbit (20) it is possible to calculate the empirical composition of the specific precipitate at certain limiting points or regions of the reaction range. In the region of extreme antibody excess, as in the serial experiments

TABLE IX

Partial Precipitation of Antisera with Homologous Thyroglobulin, Followed by Precipitation with Heterologous Thyroglobulin

Homologous Tg N added	Homologous antibody N precipitated	Per cent of total antibody N precipitated	Total cross reacting antibody N in serum	Antibody N precipitated by heterologous Tg from supernatant of homologous precipitation	Per cent of heterologous antibody N precipitated
mg.	*mg.*		*mg.*	*mg.*	
Anti-human Tg 17B serum 3.56_1, 2.0 ml.					
Hog Tg used in cross reaction					
0.015(8)	0.126	28	0.097	0.043	44
Anti-hog Tg $12E_1$ serum 3.75_1, 2.0 ml.					
Human Tg used in cross reaction					
0.015(8)	0.082	17	0.084	0.035	42
Anti-hog Tg $13B_2$ serum 2.61_3, 2.0 ml.					
Human Tg used in cross reaction					
0.015(8)	0.106	27	0.093	0.041	44

(Table VI) ratios of antibody N to Tg N (or antibody to Tg) as high as 12.9 were encountered. If it be admitted from the work quoted above that the ratio of the molecular weights of antibody to thyroglobulin is approximately 150,000:700,000, or 0.21, the antibody:Tg ratio 12.9 would correspond roughly to the empirical composition Tg A_{60}. Ordinarily, however, the values for 2R, as shown in Table IV, do not exceed 8, so that most of the antibody present in a relatively smaller quantity of serum could not form compounds of higher A content than approximately TgA_{40}. The equivalence zone ratios of about 3 and 2 would then correspond roughly to TgA_{14} and TgA_{10}

while the ratio 0.4 in the region of excess antigen would indicate an empirical composition of about TgA_2. The soluble compound or compounds in the inhibition zone in the region of large Tg excess would then have an equivalent composition between this and TgA. It is, of course, not intended to propose these as the chemical formulas of definite, isolable compounds, but they at least indicate the molecular composition of the precipitate at definite points or regions in the reaction range. The extraordinarily great range of combining proportions indicates, it is believed, a very large number of reactive groupings in or on the large thyroglobulin molecule.

Adant and Spehl (14) and Snapper and Grünbaum (15) found no cross reactions between Tg and antisera to artificial iodoproteins or between iodoproteins and Tg antisera. The latter workers also failed to get inhibition of Tg-anti-Tg precipitation by diiodotyrosine or thyroxine and concluded that these two substances do not exist as such in thyroglobulin. While we have also failed to observe inhibition by these amino acids[7] we do not think Snapper and Grünbaum's conclusion justified. A simple calculation shows that even the large Tg molecule contains at most two or three thyroxine groups, not more than eight to twelve diiodotyrosine units, and much unsubstituted tyrosine as well (21). It has been shown above that Tg contains possibly 40 to 60 immunologically reactive groupings and while the iodinated amino acids are chemically the most distinctive and physiologically the most important, there is no reason for or against their being of any significance in the serological reactions of the protein. Moreover, the failure to effect a change in the reactivity of serum albumin in anti-serum albumin sera by introduction of large arylazo groups into at least a high proportion of the tyrosine molecules present in the antigen (8) indicates that chemical changes in the tyrosine groupings are not necessarily accompanied by pronounced changes in specificity.

SUMMARY

1. Quantitative data for both homologous and heterologous precipitin reactions of human, hog, beef, and sheep thyroglobulins show that

[7] The alcohol-insoluble portion of peptic and tryptic digests of Tg and the polysaccharide isolated from Tg after alkaline hydrolysis failed either to precipitate anti-Tg sera or to inhibit precipitation by Tg.

these reactions have the same mechanism as other instances of the precipitin reaction and may be expressed quantitatively by the same equations derived from the law of mass action.

2. It is shown that all of the added antigen is precipitated in the region of antibody excess and in the equivalence zone, so that in these portions of the reaction range the composition of the specific precipitate may be calculated from the nitrogen precipitated and the amount of antigen nitrogen added.

3. The thyroglobulin-antibody reaction is characterized by low antibody N to antigen N ratios, as would be expected with an antigen of high molecular weight. Molecular ratios varying from 60:1 to 1:1 were calculated for the extremes of the reaction range, indicating a very large number of immunologically reactive groupings on the thyroglobulin molecule.

4. Failure of thyroxine or diiodotyrosine to inhibit specific precipitation was confirmed, but it is shown that this need not mean that these substances do not occur in thyroglobulin, as has been claimed.

BIBLIOGRAPHY

1. Hektoen, L., Fox, H., and Schulhof, K., *J. Infect. Dis.*, 1927, **40**, 647, and earlier papers.
2. Heidelberger, M., and Palmer, W. W., *Jour. Biol. Chem.*, 1933, **101**, 433.
3. Heidelberger, M., and Pederson, K. O., *J. Gen. Physiol.*, 1934, **17**, 341.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 559.
5. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 467.
6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 697.
7. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1937, **65**, 647.
8. Kabat, E. A., and Heidelberger, M., *J. Exp. Med.*, 1937, **66**, 229.
9. Taylor, G. L., Adair, G. S., and Adair, M. E., *J. Hyg.*, 1934, **34**, 118.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55**, 555.
11. Marrack, J. R., and Smith, F. C., *Brit. J. Exp. Path.*, 1931, **12**, 30.
12. Leipert, T., *Biochem. Z.*, 1934, **270**, 448.
13. Hooker, S. B., and Boyd, W. C., *J. Immunol.*, 1936, **30**, 33.
14. Adant, M., and Spehl, P., *Compt. rend. Soc. biol.*, 1934, **117**, 230.
15. Snapper, I., and Grünbaum, A., *Wien. klin. Woch.*, 1935, **48**, 1199.
16. Marrack, J. R., Chemistry of antigens and antibodies, *Great Britain Med. Research Council, Special Rep. Series, No. 194*, 1934.
17. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1934, **59**, 519.

18. Heidelberger, M., Kabat, E. A., and Shrivastava, D. L., *J. Exp. Med.*, 1937, **65**, 487.
19. Nuttall, G. H. F., and Strangeways, T. S. P., Blood immunity and blood relationships, Cambridge University Press, 1904.
20. Heidelberger, M., and Pederson, K. O., *J. Exp. Med.*, 1937, **65**, 393.
21. Leland, J. P., and Foster, G. L., *J. Biol. Chem.*, 1932, **95**, 165. Cavett, J. W., *J. Biol. Chem.*, 1936, **114**, 65.