The Effects on Rabbits of Immunization with Bovine Thyroid-Stimulating Hormone and its Subunits

GILDON N. BEALL, INDER J. CHOPRA, DAVID H. SOLOMON
JOHN G. PIERCE, and JAMES S. CORNELL

From the Department of Medicine, Harbor General Hospital, Torrance, California 90509 and the Departments of Medicine and Biochemistry, UCLA School of Medicine, Los Angeles, California 90024

ABSTRACT Rabbits were immunized with bovine thyroid-stimulating hormone (bTSH), bovine luteinizing hormone (bLH), and their subunits. In two immunization experiments, thyroid-stimulating activity was found in the serum of 6 out of 12 rabbits immunized with bTSHβ subunits. The thyroid-stimulating activity in the anti-bTSHβ sera was greater at 2 h than at 8, was eluted with the globulin fraction from Sephadex G-100, was completely neutralized by both anti-bTSH and anti-rabbit gamma globulin, and was completely suppressed by administration of triiodothyronine (T_3) to the immunized rabbit. These findings led to the conclusion that the thyroid-stimulating activity resided in soluble complexes of rabbit TSH bound to anti-bTSHβ. Two of nine rabbits immunized with bTSH developed thyroid-stimulating activity in their serum, but it was nonsuppressible by T_3. None of the animals immunized with bTSHα, bLH, bLHβ, or bLHα developed serum thyroid-stimulating activity.

Hypopituitary hypothyroidism, evidenced by decreased serum thyroxine (T_4) and thyroidal ^{131}I uptake and by the histologic appearance of large follicles with flat cells, was found in the bTSHβ- and bTSH-immunized animals, despite the presence of thyroid-stimulating activity in the serum of many. The reasons for this paradox are unclear; possibly the complexes block the effect of TSH on the rabbit thyroid.

INTRODUCTION

In the preceding paper we described the binding, neutralizing, and nonneutralizing antibodies that we found in the serum of rabbits after immunization with bovine thyroid-stimulating hormone (bTSH)[1] and its α and β subunits (1). Anti-bTSH and anti-bTSHβ bound [^{125}I] bTSH and [^{125}I]bTSHβ and neutralized, in vitro, the biological activity of bTSH. In addition, diluted, anti-bTSHβ sera, stored at $-20°C$ for many weeks, combined in vitro with bTSH to form an immune complex that was still biologically active as a thyroid stimulator when injected into mice for bioassay. In this paper we will describe a similar thyroid stimulator detected in the fresh, undiluted serum of several rabbits immunized with bTSHβ. Paradoxically, however, these rabbits were hypothyroid. The paper presents the results of our studies characterizing this thyroid stimulator.

METHODS

Animals, hormone preparations, and immunization procedures. These were described in the companion paper (1). Two immunization experiments were performed. Experiment I utilized bTSH subunits that were contaminated with bTSH. Experiment II used highly purified TSH subunits (1).

Thyroid stimulation. The McKenzie bioassay in mice was used (1). Thyroid-stimulating activity is presented as the response index (the percent of the zero-hour value). At places in the text we have, for the convenience of the reader, indicated the standard error of the mean computed from the individual response indices. Statistical comparisons, however, were made using the logarithm of the responses. Group means were compared with Student's *t* test.

Undiluted fresh rabbit serum killed many of the mice. Heating (to 56°C for 20 min), precipitation of globulins

Dr. Chopra is the recipient of U. S. Public Health Service NIH Research Career Development Award 1 KO 4 AM-70225.

Received for publication 21 May 1973 and in revised form 10 August 1973.

[1] *Abbreviations used in this paper:* α, alpha subunit; β, beta subunit (after TSH); b, bovine; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; GARGG, goat anti-rabbit gamma globulin; GPATSH, guinea pig anti-bTSH; LATS, the long-acting thyroid stimulator; LH, luteinizing hormone; PBS, phosphate buffered saline; T_3, triiodothyronine; T_4, thyroxine; TSH, thyroid-stimulating hormone.

with 50% ammonium sulfate, prolonged dialysis, and, when possible, storage for a few days at −20° appeared to be helpful in diminishing this serum toxicity. Consequently, these procedures were used for those portions of undiluted rabbit antisera intravenously injected into mice for bioassays.

Histologic procedures. At the completion of the immunization the animals were sacrificed, and the thyroid glands were removed. One lobe was fixed in Bouin's solution, and histologic sections were prepared and stained with hematoxylin and eosin. The other lobe was snap-frozen in isopentane cooled in a dry ice/acetone bath. The frozen block was stored at −70°C in the isopentane. Frozen sections 6 μm thick were subsequently prepared for fluorescent antibody studies.

Guinea pig anti-bTSH (GPATSH) was prepared by immunization with bTSH.[2] Globulins from this serum were conjugated with fluorescein isothiocyanate (FITC). A goat anti-rabbit gamma globulin (GARGG) conjugated with the FITC was obtained from Antibodies, Inc., Davis, Calif. Frozen sections of rabbit thyroid were stained directly with these conjugates. The conjugates were used at concentrations of both 1:4 and 1:8 in Coon's buffer both with and without absorption with rat liver powder. Three normal rabbit thyroids were used as controls.

Thyroid function studies. Total thyroxine (T_4) was assayed in rabbit serum by the method of Murphy and Pattee (2, 3). Thyroidal ^{131}I uptake at 24°C was measured as previously described (4).

Suppression of endogenous TSH in the immunized rabbits was accomplished by injecting 20 μg of triiodothyronine (T_3) subcutaneously each day for 8 days. On the eighth day the animals were bled, and thyroidal ^{131}I uptake was measured. The suppression with T_3 was performed in two stages. First, half the animals received T_3 while the rest received saline. Subsequently, the treatments were reversed. The animals were bled before and after each of the 8-day periods. The results were combined for analysis.

Effects of anti-TSH and anti-gamma G on thyroid-stimulating activity. 100 μl of GPATSH had no thyroid-stimulating activity and was capable of completely neutralizing the activity of 0.5 mU of bTSH. GARGG contained approximately 20 mg of antibody/ml. GPATSH, 0.9 ml, or GARGG, 9.0 ml, were incubated with 4.0 ml of rabbit serum containing the following thyroid stimulators: rabbit TSH (in the form of serum from a thyroidectomized rabbit); bTSH (Thytropar), and rabbit anti-bTSHβ that contained thyroid-stimulating activity. Incubation was for 1 h at room temperature and then overnight at 4°C. Precipitates were separated by centrifugation and discarded. Globulins were then precipitated from the supernate by the addition of an equal volume of saturated ammonium sulfate. The globulin precipitates were dissolved in and dialyzed against phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate buffer, pH 7.3) for the McKenzie bioassay.

Gel filtration of rabbit TSH and anti-bTSHβ. Rabbit serum containing rabbit TSH (serum from a thyroidectomized rabbit), serum containing thyroid-stimulating activity from a rabbit immunized with bTSHβ, and LATS and bTSH added to normal rabbit serum were all subjected to gel filtration on the same 2.8 × 36-cm column of Sephadex G-100. 4-ml portions were dialyzed vs. PBS and carefully layered onto the column. As PBS was pumped through the column at a flow rate of 50 ml/h, optical density

[2] Thytropar, Armour Pharmaceutical Co., Chicago, Ill.

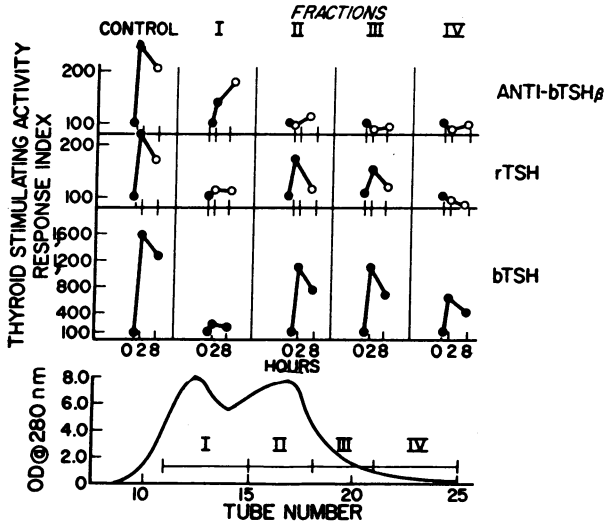

FIGURE 1 Thyroid-stimulating activity of the fractions eluted from Sephadex G-100 is shown for anti-bTSHβ, rabbit TSH, and bTSH. The ordinate is response index (percent of the zero-hour value) (log scale). Solid dots show statistically significant thyroid stimulation ($P < 0.05$). Response at 0, 2, and 8 h are shown. The pattern of protein elution from the column is shown at the bottom of the figure, optical density at 280 nm is on the ordinate. All the thyroid-stimulating activity of anti-bTSHβ emerges in fraction I. The activities of rabbit TSH (rTSH) and bTSH are found in fractions II, III, and IV.

measurement of the effluent at 280 nm revealed two protein peaks. The effluent was divided into four fractions. The first fraction contained a peak that included the globulins. The albumin was collected as fraction II. The trailing portion of the second peak was collected as fraction III. The remaining effluent fraction IV, contained no significant protein peak (Fig. 1). The fractions were concentrated by negative pressure dialysis to 4 ml for McKenzie bioassay.

Fluorescent tracing of immune complexes. Serum from rabbit 3 immunized with bTSHβ (experiment 1) was incubated with bTSH under conditions appropriate to form bioactive TSH–anti-bTSHβ immune complexes in vitro (1). Sufficient antiserum was used to make the final concentration 1:100 (vol/vol) in 4.0 ml of normal rabbit serum. Sufficient bTSH was used to make the final concentration 10 mU/ml. These mixtures were incubated 2 h at room temperature and overnight at 4°C. After centrifugation in the cold, the supernates were rewarmed, and 0.5-ml aliquots were injected into mice. Similarly prepared bTSH without antiserum and antiserum without bTSH were injected into control mice. Mice were sacrificed at 2, 8, and 24 h. Thyroid and other organs were frozen in isopentane/dry ice, sectioned at 6 μm and stained with FITC-GPATSH and FITC-GARGG.

Interaction of thyroid-stimulating materials. In an effort to explain the paradox of hypothyroidism in the presence of serum thyroid-stimulating activity, we examined the possible interactions and interference of thyroid stimulators and antisera. In these studies, mice appropriately prepared for the McKenzie assay were injected intravenously with 0.25 or 0.5 ml of a test substance after a 0-h bleed. Mate-

rials used for the first injection included normal rabbit serum, anti-bTSHβ serum from which previously present thyroid-stimulating activity was absent after T_3 administration, anti-LHα to which bTSHβ subunits were added, and bTSHβ. At various subsequent times, a second intravenous injection of normal rabbit serum, rabbit TSH, bTSH, or LATS was given.

In one type of experiment, the second injection was given 1 min after the first. The mice were then bled at 2 h and 8 h, and the response indices of thyroid stimulation were calculated. In other experiments, the 2-h response index after a first injection was obtained. 24 h later the procedure was repeated with a new test substance in the same mice. 2-hour response index on day 1 could thus be compared to 2-h response index on day 2; each mouse underwent four bleedings in this design. Yet another variation, involving no more than three bleedings per mouse, consisted of 0-h bleeding, followed by the first injection. Then some of the mice were bled at 2 h, while others injected with the same materials were bled not at 2 h but at 8 h, before a second injection and a 10-h bleeding. The 2-h response index to the second injection could thus be compared to the first injection, but not in the same mice.

RESULTS

Thyroid-stimulating activity. In experiment I, four out of six rabbits immunized with bTSHβ developed significant amounts of thyroid-stimulating activity in their serum. The group mean±SEM 2-h response index for all six anti-bTSHβ rabbit sera obtained at day 83 was 315±106% compared to a group mean of 93 ±14% for the preimmunization serum of the same six rabbits ($P < 0.02$). Of the animals immunized with bTSH, two of five developed significant thyroid-stimulating activity. The group mean 2-h response index for these bTSH-immunized rabbits rose from 78±7.9% before immunization to 195±28% at 83 days after immunization ($P < 0.01$). Significant thyroid-stimulating activity was not found in the serum of animals immunized with TSHα, LHα, or LHβ. These results are summarized in Fig. 2, which depicts the means for the 2-h response indices of the various groups. In those sera containing thyroid-stimulating activity, the response at 2 h in the bioassay was consistently greater than that at 8 h.

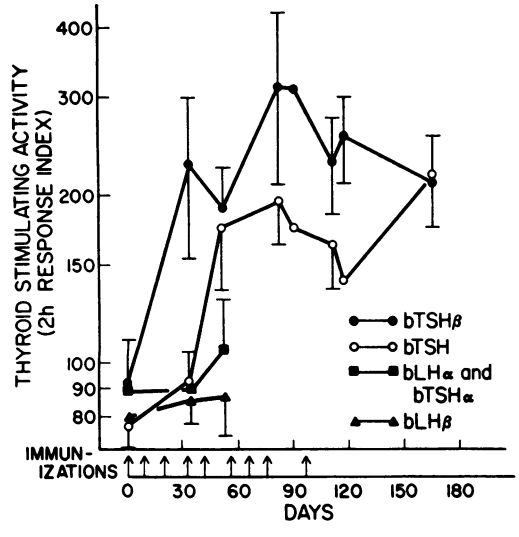

FIGURE 2 The thyroid-stimulating activity in the McKenzie assay is shown on the ordinate as the 2-h response index (percent of the zero-hour value) (note the log scale). For each group the mean±SEM is plotted for each bleeding. The dates of immunization are indicated by the arrows.

TABLE I
Effect of T_3 on Thyroid-Stimulating Activity

Immunization with:	2-h Response Index				
	Before	After saline	P	After T_3	P
	%, mean±SEM			%, mean±SEM	
bTSHβ	275±56	276±70	NS*	104±9	<0.02
bTSH	178±19	156±15	NS	188±23	NS

* $P > 0.05$.

Administration of T_3 was associated with a decrease in the thyroid-stimulating activity of the serum ($P < 0.02$) in all five of the bTSHβ-immunized rabbits studied (Table I). (Two of these animals did not have a significant increase in thyroid-stimulating activity before T_3 was given). Of the animals immunized with bTSH who were studied, only one of four had a decrease in thyroid-stimulating activity while receiving T_3.

In experiment II, using more highly purified subunits, immunization was less effective in producing serum thyroid-stimulating activity. It is possible that during the gel filtration step needed to remove the contaminating α-subunit, partial aggregation or other changes occurred in the β-preparation. However, two out of six rabbits immunized with purified bTSHβ did develop significant thyroid-stimulating activity, in contrast to none out of four animals immunized with intact bTSH. As in experiment I, the thyroid-stimulating activity, when present, disappeared temporarily when the animals were given T_3 for 8 days.

Thyroid function studies. In experiment I, serum T_4 fell significantly by day 52 in the animals immunized with bTSHβ (Table II), while uptake of radioiodine by the thyroid gland was not much affected until the study done on day 174. The mean 24-h thyroidal ^{131}I-uptake of the bTSHβ-immunized group was significantly less than the preimmunization mean value at day 174 (Table III). Some decline in T_4 values was also noted in rabbits immunized with bTSH, although the change was not statistically significant. Administration of T_3 suppressed the thyroidal ^{131}I uptake in all the animals.

TABLE II

Total Serum T_4

Immunization	Day 0	32	52	104	175
	μg/100 ml, mean ± SEM				
Experiment I					
bTSHβ	2.3 ±0.2	2.3 ±0.1	1.3* ±0.2	1.3* ±0.1	1.7 ±0.3
bTSHα	2.1 ±0.2	2.2 ±0.1	1.4 ±0.2		
bLHα	3.5 ±0.4	2.8 ±0.2	2.3 ±0.3		
bTSH	2.3 ±0.4	2.4 ±0.3	1.6 ±0.3	1.4 ±0.2	2.1 ±0.2
bLHβ	2.2 ±0.1	2.4 ±0.1	2.0 ±0.2		
Experiment II: day	0		77		
bTSHβ	2.4 ±0.4		2.0 ±0.2		
bTSHα	2.1 ±0.3		2.3 ±0.1		
bTSH	2.0 ±0.4		1.5 ±0.6		
BSA	1.3 ±0.1		2.2 ±0.4		

* Significantly different ($P < 0.05$) from preimmunization value.

In experiment II, total serum T_4 did not change significantly when assayed at day 77 (Table II). The thyroidal ^{131}I-uptakes declined in all the rabbits, including the BSA-immunized controls, but the change was significant only in the rabbits immunized with bTSH and its subunits (Table III).

The effects of anti-TSH and anti-gamma globulin on thyroid-stimulating activity. The thyroid-stimulating activity found in the serum of rabbits immunized with bTSHβ was strikingly inhibited after incubation of the serum in vitro with either GPATSH or GARGG.

TABLE III

Percent of Thyroidal ^{131}I uptake (Mean ± SEM)

Experiment I: Day	0	34	84	174		
Immunization						
bTSHβ	14.0 ±3.5	9.8 ±2.1	8.6 ±1.3	4.5* ±1.2		
bLHα and bTSHα	16.6 ±1.9	13.1 ±1.4				
bTSH	9.6 ±4.4	7.9 ±1.1	9.2 ±1.5	5.0 ±1.0		
bLHβ	10.5 ±1.9	11.5 ±1.9				
Experiment II: day	0	28	40	47	61	77
Immunization				$\bar{p}T_3$		
bTSHβ	10.7 ±1.4	8.0 ±1.8	8.2 ±1.2	3.4* ±0.5	5.5* ±0.8	3.2* ±0.5
bTSHα	13.5 ±1.7	8.3 ±1.5	9.1 ±1.2	5.0* ±1.0	7.6* ±1.8	7.8* ±0.8
bTSH	12.0 ±2.6	6.4 ±1.7	8.3 ±3.0	4.2* ±1.7	6.9 ±2.9	4.2* ±2.1
BSA	10.0 ±2.4	4.5 ±0.5	7.4 ±0.9	3.9* ±1.0	8.5 ±1.3	8.3 ±3.0

* Significantly different ($P < 0.05$) from preimmunization value.

TABLE IV

Incubation of Anti-bTSHβ with GPATSH and GARGG

	2-h Response Index		
	Control	GPATSH	GARGG
	%, Mean ± SEM		
anti-bTSHβ	266 ±26	121 ±13*	137 ±19*
rabbit TSH	399 ±62	92 ±4*	301 ±52
bTSH	704 ±64	107 ±15*	425 ±57
LATS	531 ±64	602 ±60	485 ±122

* $P < 0.001$ (Student's t test vs. control)

Neither of these antisera effected the thyroid-stimulating activity of LATS. GPATSH fully neutralized both rabbit TSH and bTSH, whereas GARGG had no significant effect on either (Table IV).

The inhibition by GPATSH of the thyroid-stimulating activity of rabbit anti-bTSHβ serum was not accompanied by a detectable decrease in the binding of either [^{125}I]bTSH or [^{125}]bTSHβ by the serum. In contrast, the inhibition of thyroid-stimulating activity of the rabbit anti-bTSHβ by GARGG was associated with loss from the supernate of nearly 90% of both [^{125}I]bTSH and [^{125}I]bTSHβ binding activity (Table V).

Histologic findings. Sections of the thyroid glands from animals immunized with bTSH and bTSHβ contained large but normal-appearing follicles with flat thyroid acinar cells. Only two–four follicles were seen per 40 × field. In normal rabbit thyroid, acinar cells were taller, and 6–10 follicles were found per 40 × field. There was no histologic evidence of thyroiditis in the immunized rabbits.

Incubation of frozen sections with fluorescence-labeled GPATSH or GARGG did not reveal any staining that suggested deposition of TSH or rabbit gamma globulin in the thyroid.

Gel filtration. The biologic activity of anti-bTSHβ antiserum emerged from G-100 in the first (globulin) peak. Both rabbit TSH and bTSH were retarded and eluted from the same column with later protein frac-

TABLE V

The Effect of GPATSH and GARGG on Thyroid-Stimulating (TS), [^{125}I]bTSHβ-Binding, and [^{125}I]bTSH-Binding Activities of Rabbit Antisera to bTSHβ

	TS activity 2-h Response Index	Binding activity	
		[^{125}I]bTSHβ	[^{125}I]bTSH
	%	*μl serum needed to bind 50%*	
anti-bTSHβ	266 ±26	0.020	0.40
anti-bTSHβ plus GPATSH	121 ±13	0.018	0.40
anti-bTSHβ plus GARGG	137 ±19	0.17	3.0

tions (Fig. 1). Interestingly, the thyroid-stimulating-activity of the gel-filtered anti-bTSHβ serum was greater at 8 h than at 2 h. A second experiment of this type gave similar results with elution of a "long-acting" thyroid stimulator in the first fraction from Sephadex G-100.

Injection of TSH–anti-TSH complexes into mice. After injection into mice of appropriate mixtures of anti-bTSHβ and bTSH, granular deposits of rabbit gamma globulin were detected by immunofluorescence with FITC-GARGG in the small blood vessels of all the organs studied, including the thyroid. These deposits were not found after control injections of normal rabbit serum containing either TSH or anti-bTSHβ. The deposits of rabbit gamma globulin were consistently present at 8 h and less frequently demonstrated 2 h and 24 h after injection. No fluorescence was detected with FITC-GPATSH.

Effects of antisera on thyroid-stimulating activities. Several experiments were undertaken in an effort to discern the cause of the hypothyroidism in the immunized rabbits. The possibility that bTSHβ might competitively interfere with the action of endogenous rabbit TSH was investigated by preliminary injection of bTSHβ or control material, followed 1 min later by injection of bTSH into the test mice. The preliminary injection of 200 ng bTSHβ did not interfere with the response to 0.15 or 0.6 mU of subsequently injected bTSH.

When antisera obtained from bTSHβ-immunized rabbits were administered to mice 5 min, 8 h, or 24 h before administration of TSH, the thyroid-stimulating activity of the TSH (either bovine or rabbit) could be significantly lessened or completely eliminated (Table VI). This inhibition of TSH activity was not dependent on thyroid-stimulating activity in the anti-bTSHβ antisera, because antisera of this type obtained from animals in whom thyroid-stimulating activity had been suppressed by T_3 administration were still effective in blocking the mouse response to TSH. Antisera that had lost their thyroid-stimulating activity during storage were also inhibitory to subsequently administered TSH. Anti-bLHα had no such effect. The thyroid-stimulating response at 2 h to LATS was not inhibited by anti-bTSHβ (Table VI).

TABLE VI

Effects on Thyroid Stimulators of the Injection of Anti-bTSHβ 24 h Earlier (Mean±SEM)

Day 1		Day 2	
Preliminary injection	2-h response index	Thyroid Stimulator	2-h response index
	%		%
Normal rabbit serum	123±13	LATS	911±142
"	100±4	bTSH, 0.5 mU	664±199
"	159±20	rabbit TSH	499±51
Anti-bTSHβ	186±15	LATS	850±40
"	149±5	bTSH, 0.5 mU	87±11*
"	152±29	rabbit TSH	128±40*

* $P < 0.001$ compared to control after normal rabbit serum.

DISCUSSION

Immunization of rabbits with bTSHβ and to a lesser extent with bTSH resulted in the appearance of serum thyroid-stimulating activity together with suppression of thyroid function. It seems reasonable to believe that the hypothyroidism was instrumental in causing the appearance of thyroid-stimulating activity, and the suppression of thyroid-stimulating activity by administration of T_3 supports that belief. Despite this, there was no obvious relationship between the values for thyroid stimulation and the thyroid function measurements. Serum T_4 values and ^{131}I uptakes were as low in the rabbits without as in those with detectable thyroid-stimulating activity in their sera.

Thyroid-stimulating activity. The thyroid-stimulating activity of unfractionated serum resembled TSH in its time-course. 2 h activity was greater than 8 h. Administration of T_3 to the rabbits caused a temporary suppression of the thyroid-stimulating activity in the serum of the bTSHβ-immunized animals. This demonstrated not only that the activity was due to TSH but that the thyroid still was responsive to TSH and that the TSH was being secreted by the rabbit pituitary, which was capable of a normal negative feedback response to T_3 administration.

There is good evidence that most of this rabbit TSH activity was present in the serum of the rabbits as a TSH–anti-bTSHβ immune complex. The gel filtration experiments indicated that the thyroid-stimulating activity was globulin-bound, in contrast to the behavior of both rabbit TSH and bTSH, which were eluted from Sephadex G-100 at the same position as serum albumin. In addition, both guinea pig anti-bTSH and GARGG neutralized almost all the thyroid-stimulating activity of the anti-bTSHβ serum whereas anti-gamma globulin sera did not inactivate bTSH or rabbit TSH mixed with normal rabbit serum. Hoffmann, Mason, Good, Hetzel, and Ferguson, and Meek have previously demonstrated that some in vitro mixtures of TSH and anti-TSH have thyroid-stimulating activity when injected into the mouse (5, 6). Our experience with these immunized rabbits is the first demonstration that such biologically active complexes may be formed in vivo. It is particularly interesting that this phenomenon appeared to be related especially to the immunization with bTSHβ.

T_3 administration did not usually suppress the thyroid-stimulating activity found in the serum of rabbits immunized with intact bTSH, suggesting either that the thyroid-stimulating activity of anti-bTSH sera was not due to rabbit TSH–anti-bTSH complexes or that the pituitary had escaped from normal control. Although it is possible that the injected bTSH combined in vivo with anti-bTSH, forming immune complexes of bTSH–anti-bTSH, such bTSH–anti-bTSH complexes were not formed in vitro under the conditions we used (1). The thyroid-stimulating activity of these anti-bTSH antisera, although statistically significant, was so weak that nonspecificity could not be excluded (7).

Thyroid stimulation by both anti-bTSHβ and anti-bTSH sera was confirmed by the use of the double isotopic modification of the McKenzie assay (8). No shift of [^{125}I]T_4 from tissues to serum was found in the bioassay mice.

The bTSHβ preparation used for immunization in experiment I did contain intact bTSH equivalent in thyroid-stimulating activity to 6% of the starting bTSH. It needs to be considered whether that degree of contamination puts into question our conclusion that the unique results of immunization were due to the use of bTSHβ as an antigen. Certainly immunization with bTSH (Thytropar), albeit a preparation of lesser potency, produced much less thyroid-stimulating activity. A more telling point, however, was the difference between the complexes formed: definitely rabbit TSH–anti-bTSHβ in the first instance and questionably bTSH–anti-bTSH in the second. Furthermore, the binding studies demonstrated that immunization with bTSHβ produced antisera that as a group bound bTSHβ more avidly than bTSH (1). In contrast, anti-bTSH sera bound both proteins equally well. When highly purified subunits (9) were used for immunization in Experiment II, similar, though less impressive, results were obtained. Finally, the anti-bTSHβ and anti-bTSH sera were found to differ markedly in their ability to form antigen-antibody complexes possessing thyroid-stimulating activity when bTSH was added in vitro (1).

Is there any possibility that these complexes we have described are analogous to LATS? It seems unlikely. The rabbit TSH–anti-bTSHβ complexes found in the unfractionated anti-TSHβ sera were not long-acting. Also in contrast to LATS, the activity was suppressed by T_3 administration and by in vitro incubation with GPATSH. Long-acting, globulin-bound thyroid-stimulating activity was obtained by Sephadex G-100 gel filtration. We did not study this further since the response indices for this eluted material were small and nonspecificity of the bioassay could not be excluded.

Since our evidence suggests that the thyroid stimulator in the bTSHβ-immunized rabbits is rabbit TSH–anti-bTSHβ and the pituitary seems capable of normal suppression by T_3, then increased production of rabbit TSH must be due to a deficiency of T_4 and T_3 in the serum. Such hypothyroidism was demonstrated conclusively by the presence of lowered serum T_4 levels and decreased thyroidal ^{131}I uptake in the bTSHβ-immunized group. Similar studies with the bTSH-immunized group were also suggestive of hypothyroidism. The histological appearance of the thyroid glands in both groups of animals suggested a lack of stimulation, i.e., TSH deficiency, rather than a primary thyroid lesion of any kind. Similar findings have been reported previously after immunization with bTSH (10).

We have considered several possible causes of this hypothyroidism. First, bTSHβ might be a competitive inhibitor of rabbit TSH. This possibility was investigated in the mouse. It was clearly shown that preliminary injection of bTSHβ did not inhibit subsequent response of the mouse thyroid to bTSH. Although it is possible that the rabbit thyroid and rabbit TSH might behave differently, this seems unlikely. A second possibility, that the hypothyroidism might be caused by thyroiditis, was not supported by the histological appearance of the glands. No thyroiditis was found. Third, rabbit TSH might be neutralized by circulating antibodies so that it was unable to stimulate the thyroid. Almost all of the animals immunized with bTSH and its subunits had serum antibodies that neutralized TSH. In addition, preliminary injection of such sera inhibited subsequent thyroid stimulatory response to TSH in the mouse. At least some of the hypothyroidism found, particularly in the animals immunized with bTSH, was undoubtedly related to these neutralizing anti-TSH antibodies. This explanation, however, is incomplete, since it cannot account for the demonstration in some hypothyroid animals of both gamma globulin-bound thyroid-stimulating activity due to rabbit TSH and the in vitro demonstration of biologically active nonneutralizing antibodies (1). We have proposed another hypothesis, that the nonneutralized complex becomes bound to the TSH receptor, preventing further thyroid stimulation by either rabbit TSH or rabbit TSH-anti-bTSHβ complexes. This explanation is attractive, but we have not yet been able to support it by any unequivocal experiment.

If these sera from rabbits are capable of stimulating the mouse thyroid, why can't they stimulate the rabbit thyroid? There are a number of possible causes for the difference between mice and rabbits. It might be

proposed that the rabbit TSH–anti-TSHβ complexes are more easily dissociated in the mouse than in the rabbit. In that way neutralized TSH might be released to assert its activity. This idea was not supported by the dilution of preformed complexes (Reference 1, Table V). Such diluted complexes did not release TSH activity. We cannot exclude the possibility that mouse thyroid has a greater binding affinity for rabbit TSH than does rabbit thyroid, although this seems unlikely. Whatever the cause, species differences to thyroid stimulators do seem to exist, as illustrated by the recent report of Onaya, Kotoni, and Yamada that the serum of hyperthyroid patients with Graves' disease contains a potent human thyroid-stimulating substance that is inactive in the mouse (11).

ACKNOWLEDGMENTS

We wish to express thanks to Mr. Raymond Ajugwo, Mrs. Natalie Limberg, Mrs. Ruey S. Ho, and Mr. Jeffrey Keene who provided the laboratory assistance; to Frank Hirose, M.D., who helped us to evaluate the thyroid histology; and to Mrs. Hilda Dornfest who typed the manuscript.

This work was supported in part by grants from the U. S. Public Health Service (AM-13126, C-2290, and GM-364) and The John A. Hartford Foundation, Inc.

REFERENCES

1. Beall, G. N., I. J. Chopra, D. H. Solomon, J. G. Pierce, and J. S. Cornell. 1973. Neutralizing and nonneutralizing antibodies to bTSH and its subunits. *J. Clin. Invest.* **52**: 2979.
2. Murphy, B. E. P., and C. J. Pattee. 1964. Determination of thyroxine utilizing the property of protein-binding. *J. Clin. Endocrinol.* **24**: 187.
3. Murphy, B. E. P. 1965. The determination of thyroxine by competitive protein-binding analysis employing an anion-exchange resin and radiothyroxine. *J. Lab. Clin. Med.* **66**: 161.
4. Beall, G. N., and D. H. Solomon. 1968. Thyroid-stimulating activity in the serum of rabbits immunized with thyroid microsomes. *J. Clin. Endocrinol.* **28**: 503.
5. Hoffmann, M. J., E. K. Mason, B. F. Good, B. S. Hetzel, and K. Ferguson. 1967. Studies of the nature of plasma thyroid stimulating activity in hyperthyroidism. *Australas. Ann. Med.* **16**: 98.
6. Meek, J. C. 1970. Formation of LATS by human thyrotropin-antibody complexes. *J. Clin. Endocrinol.* **31**: 48.
7. Chopra, I. J., and D. H. Solomon, and N. P. Limberg. 1970. Specific and nonspecific responses in the bioassay of long acting thyroid stimulator (LATS). *J. Clin. Endocrinol.* **31**: 382.
8. Solomon, D. H., G. N. Beall, and I. J. Chopra. 1970. Stimulation of release of thyroid iodine by serum of thyroid-immunized rabbits. *J. Clin. Endocrinol.* **31**: 603.
9. Pierce, J. G. 1971. Eli Lilly lecture: the subunits of pituitary thyrotropin—their relationship to other glycoprotein hormones. *Endocrinology.* **89**: 1331.
10. Lachiver, F., Y.-A. Fontaine, E. Lopez, and A. Martin. 1969. Hypoactivité de la glande thyroide chez les lapins immunisés contre l'hormone thyréotrope de boeuf. *Ann. Endocrinol.* **30**: 267.
11. Onaya, T., M. Kotoni, and T. Yamada. 1972. Evidence for the existence of a human thyroid-stimulating substance in the serum of hyperthyroid patients with Graves' disease. *Excerpta Med. Int. Cong. Ser.* **256**: 193.