# Neutralizing and Non-neutralizing Antibodies to Bovine Thyroid-Stimulating Hormone and its Subunits

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ABSTRACT To test the possibility that the long-acting thyroid stimulator (LATS) might represent an immune complex either of thyroid-stimulating hormone (TSH) with anti-TSH or of a subunit of TSH with an appropriate antibody, we immunized rabbits with bovine TSH (bTSH), bLH (luteinizing hormone), and their $\alpha$ and $\beta$ subunits (bTSH$\alpha$ and bTSH$\beta$). Binding, neutralizing, and nonneutralizing antibodies were demonstrated in the antisera obtained. First, antisera to TSH, TSH$\beta$, and TSH$\alpha$ all bound [$^{125}$I]TSH and [$^{125}$I]TSH$\beta$. Anti-bTSH$\beta$ antisera bound [$^{125}$I]bTSH$\beta$ better than did anti-TSH sera, while the binding of [$^{125}$I]bTSH was similar with both types of antiserum. Second, the thyroid-stimulating activity (McKenzie bioassay) of TSH could be neutralized by incubation with various dilutions of anti-TSH or anti-TSH$\beta$. Finally, when incubation mixtures containing TSH and dilutions of anti-TSH$\beta$ antisera that only partially neutralized TSH were treated with an antiserum against rabbit immunoglobulins to precipitate immune complexes, the bioassay response of the TSH was abolished. This phenomenon was not observed when antiserum to the intact hormone was substituted in the incubation mixture. The removal of TSH biological activity from a mixture of TSH and anti-bTSH$\beta$ by addition of an anti-immunoglobulin indicated that biologically active immune complexes were formed between TSH and anti-TSH$\beta$ but not between TSH and anti-TSH. The time-course of the bioactivity and several other characteristics of these complexes differentiate them from LATS.

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

*Received for publication 13 July 1973 and in revised form 10 August 1973.*

## INTRODUCTION

Bovine thyroid-stimulating hormone (bTSH) [1] has recently been shown to consist of two different subunits designated TSH$\alpha$ and TSH$\beta$. The subunits are separated by gel filtration in ammonium bicarbonate solution after treatment with 1.0 M propionic acid (1). Neither subunit is, by itself, a thyroid stimulator. Similar subunits have also been isolated from luteinizing hormone (LH) (2, 3). The $\alpha$-chains of LH and TSH have identical or nearly identical amino acid sequences and they will substitute for one another in reconstituting biologically active LH or TSH with the appropriate $\beta$-chain (1, 4, 5).

Antisera to human or bovine TSH incubated in vitro with appropriate amounts of either bTSH or hTSH can produce a thyroid-stimulating material that in some studies has been found to be more active at 8 h than at 2 h in the McKenzie bioassay, thus resembling the long-acting thyroid stimulator (LATS) rather than TSH (6, 7). We thought it important to investigate further the possibility that LATS might represent an immune complex of either TSH–anti-TSH or a subunit of TSH with an appropriate antibody. Consequently, we prepared rabbit antisera to purified bTSH and its subunits (8). In preliminary experiments, we found that sera from rabbits immunized with bTSH$\beta$ contaminated with bTSH or with purified bTSH alone caused thyroid stimulation in the mouse. This biological activity of the undiluted serum disappeared after several weeks storage at −20°C. We then studied the

[1] *Abbreviations used in this paper:* $\alpha$, alpha subunit; $\beta$, beta subunit (after TSH or LH); b, bovine; BSA, bovine serum albumin; GARGG, goat anti-rabbit gamma globulin; h, human; LATS, long-acting thyroid stimulator; LH, luteinizing hormone; PBS, phosphate buffered saline; TSH, thyroid-stimulating hormone.

ability of these antisera to combine with and neutralize bTSH. Similar studies were also done with antisera to highly purified preparations of bTSH subunits.

## METHODS

*Animals.* Healthy New Zealand white rabbits were immunized. Equal numbers of both sexes were used. Their weights at the beginning of the study ranged from 1.4 to 3.0 kg.

*Hormone preparations.* The preparations of bTSH used to make subunits had a potency of 30–40 U/mg. Two different groups of animals were immunized. The subunits of bTSH used for the first group (experiment I) contained significant thyroid-stimulating activity in both the McKenzie assay and by measurement of the uptake of $^{32}P$ into chick thyroids. (The activity of the bTSH$\beta$ subunit was 6% of the original bTSH activity in the McKenzie assay, and the bTSH$\alpha$ was found to contain 10–15% of the original TSH activity in the chick assay.) Contamination of the subunit preparations with bTSH is the most probable cause of the residual activity. This results from incomplete separation of the subunits with subsequent recombination to form intact bTSH (5). The subunits of LH were prepared by countercurrent distribution (1). Their potency was approximately 0.04 × N.I.H.-LH-S1 standard for the $\alpha$-subunit and 0.08 for the $\beta$-subunit.

In experiment II, highly purified subunits prepared by a second gel filtration were used for immunization. These bTSH$\beta$ and bTSH$\alpha$ subunits contained no detectable thyroid-stimulating activity when assayed at a concentration of 500 ng/mouse. They could be recombined to produce bTSH with approximately 25% of the original activity (5).

*Immunization.* In experiment I, 23 rabbits were used. Six were immunized with bTSH$\beta$, three with bTSH$\alpha$, three with bLH$\alpha$, five with bTSH (Thytropar, Armour Pharmaceutical Co., Chicago, Ill.), and six with bLH$\beta$. All of the rabbits were injected with antigen on days 1, 10, 20, 32, and 42. The protein was dissolved in 1.0 ml of phosphate-buffered saline (PBS) (0.14 M NaCl, 0.01 M sodium phosphate buffer, pH 7.3) or, in the case of bTSH$\beta$ subunits, 0.012 M glycine-NaOH, pH 9.5. The solutions were then homogenized in an equal volume of complete Freund's adjuvant, and a total of 2 ml of homogenate was injected into two subcutaneous sites on the back. The first injection of the subunits was 200 $\mu$g. Subsequently, 100-$\mu$g injections were alternated with 200 $\mu$g. The first injection of bTSH was 330 $\mu$g. Subsequently, 165-$\mu$g injections were alternated with 330 $\mu$g. After the fifth immunization, the experiment was continued only with the animals immunized with bTSH$\beta$ and bTSH, the former receiving 200 $\mu$g and the latter 6 mg/injection on days 56, 66, 76, and 97. The animals were bled on days 0, 32, 52, 66, 76, 83, 90, 104, 111, 118, and 175. The binding of [$^{125}$I]bTSH and [$^{125}$I]bTSH$\beta$ was assessed with the day 52 serum.

As will be described in the following paper, some of these sera contained thyroid-stimulating activity. That activity disappeared after storage at −20° C. Such sera from bleedings on days 32, 52, and 66 were used for the neutralization studies.

For experiment II, 21 rabbits were immunized, 6 with bTSH$\alpha$, 6 with bTSH$\beta$, 4 with bTSH (the purified preparation used to make the subunits), and 5 with bovine serum albumin (BSA). Immunizations were performed on days 1, 10, 20, 30, 40, 50, and 65 with the same technique described for experiment I. The animals received either 100 $\mu$g of the subunits, 200 $\mu$g of bTSH, or 500 $\mu$g BSA at each immunization. Bleedings were obtained on days 0, 28, 40, 47, 61, and 77. The studies on neutralization and binding of bTSH were all performed with serum obtained on day 77.

*Thyroid-stimulating activity.* The McKenzie bioassay was performed as previously described (9). Groups of six appropriately prepared mice were used for each bioassay. Each mouse received 0.5 ml of test material intravenously, and bleedings were obtained at 0, 2, and 8 h. Thyroid-stimulating activity of TSH is presented as the response index at 2 h (the percent of the zero hour value). Statistical comparisons were made using the logarithm of the responses. Group means were compared by Student's *t* test.

*Binding of [$^{125}$I]bTSH or [$^{125}$I]bTSH$\beta$ by rabbit antisera.* Appropriate dilutions of rabbit sera were incubated 24 h with trace quantities of the polypeptides labeled with $^{125}$I by the method of Greenwood, Hunter, and Glover (10). Specific activities always exceeded 200 $\mu$Ci/$\mu$g. The labeled polypeptide was obtained as a single peak from Sephadex G-75 gel filtration. To separate globulin-bound from free hormone or subunit, goat anti-rabbit gammaglobulin (GARGG) was added in antibody excess (11, 12). Control tubes without rabbit antisera were routinely included. Five concentrations of each antiserum were assayed. The resultant binding (percent of maximum) was plotted against amount of antiserum, and the amount needed for 50% binding was read from the curve.

These studies were performed with serum from the 52nd-day bleeding of six rabbits immunized with bTSH$\beta$, four with bTSH$\alpha$, four with bTSH, and one with LH$\beta$ in experiment I, and the 77th-day bleeding of six rabbits immunized with bTSH$\beta$, six with bTSH$\alpha$, four with bTSH, and five with BSA in experiment II.

*Effects of antisera on the thyroid-stimulating activity of bTSH.* Antisera were combined with bTSH in a rigidly prescribed manner so that final antiserum concentrations of 1:33 to 1:2,700 (vol/vol) and final bTSH concentrations of 5 mU/ml were used. To duplicate tubes, sufficient rabbit antiserum to give a desired final concentration in 5.0 ml was brought to 0.05 ml with normal rabbit serum and added to 25 mU bTSH dissolved in 0.1 ml 2% BSA. This antiserum-TSH mixture was then brought to a volume of 1.0 ml with 2% BSA and incubated for 2 h at room temperature. GARGG, 0.2 ml, was then added to one of the tubes, and the volume of both tubes was brought to 5.0 ml by the addition of more 2% BSA. After another incubation for 30 min at room temperature, the samples were stored overnight at 4°C and centrifuged in the cold, and the supernates were decanted. The supernates, rewarmed to room temperature, were then bioassayed in mice. A sufficient number of concentrations of each antiserum was incubated with TSH to permit construction of a dose-response curve varying from $<2$ to $>98$% neutralization. The residual TSH was quantitated by comparison with a standard TSH bioassay curve. Neutralization of 50% of the 5 mU/ml bTSH was read from the curve.

*Effects of dilution of the TSH-anti-TSH complexes.* In the experiments described in the previous paragraph, the antiserum-TSH mixtures were prepared and incubated in a 1.0 ml volume for subsequent dilution to 5.0 ml. Thus, ratios of antiserum and TSH were varied but the final dilution (from 1.0 ml to 5.0 ml) was constant. To assess the possible effect of dilution of the complexes in the mouse, complexes formed at a constant TSH to anti-

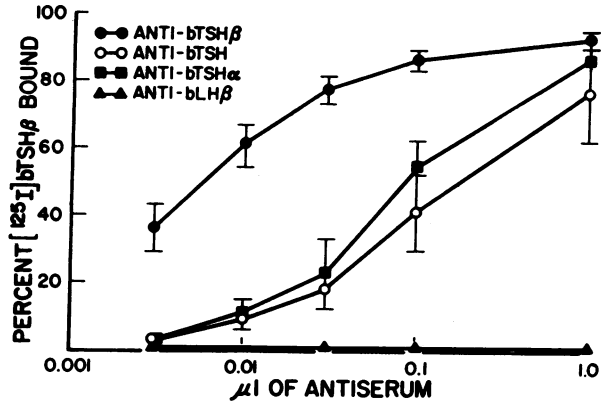


FIGURE 1 Binding of [$^{125}$I]bTSHβ by the rabbit antisera of experiment I obtained at day 52. The group mean and SEM are shown for each point. The anti-bTSHβ binds [$^{125}$I]bTSHβ much more avidly than does the anti-bTSH.

serum ratio were diluted variably. These diluted complexes were then subjected to McKenzie bioassay.

## RESULTS

*Binding of iodinated TSH and its subunits by the antisera.* The bTSHβ antisera of experiment I showed considerable specificity for [$^{125}$I]bTSHβ. Such antisera bound this subunit much more avidly than did antisera raised to bTSHα or bTSH (Fig. 1). In contrast, the binding of intact [$^{125}$I]bTSH was similar with anti-bTSHα, anti-bTSH, and anti-bTSHβ. 50% of the tracer was bound by similar quantities of all three types of antisera although the anti-bTSHβ antiserum bound slightly more TSH when limited amounts of antibody were present (Fig. 2).

The antisera from experiment II were less potent than the experiment I antisera in their ability to bind

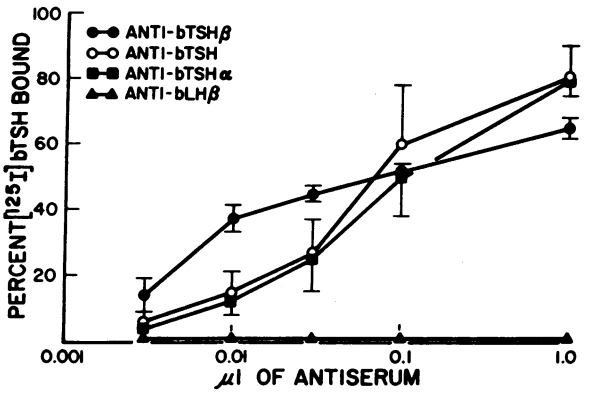


FIGURE 2 Binding of [$^{125}$I]bTSH by rabbit antisera of experiment I obtained at day 52. Percent bound peptide is plotted against the microliters of antiserum added. Mean±SEM of each group is shown. Antisera to either bTSH or its subunits bind bTSH equally well.

TABLE I

*Antiserum Required to Bind 50% of Tracer [$^{125}$I]bTSH (mean ±SEM)*

| Antiserum | Experiment I No. | I | Experiment II No. | II |
|---|---|---|---|---|
| | | μl | | μl |
| Anti-bTSH | 4 | 0.31±0.22 | 4 | 1.32±1.23 |
| Anti-bTSHβ | 5 | 0.08±0.02 | 5* | 2.78±0.89 |
| Anti-bTSHα | 3 | 0.16±0.09 | 4* | 3.14±1.73 |

* In addition, one anti-bTSHβ serum and two anti-bTSHα sera did not bind 50% of the tracer even when 100 μl of antiserum was added to the 1.0 ml incubation mixture.

[$^{125}$I]bTSH (Table I). Although the mean binding of the anti-bTSH sera exceeded mean binding by anti-subunit sera, the spread of values was large, and the differences were not significant. Antisera to LH and BSA did not bind [$^{125}$I]bTSH.

*Neutralization of TSH by the antisera.* Almost all of the antisera to bTSH and bTSHβ subunits were highly effective in neutralizing the biological activity of bovine TSH. 1 ml of the most potent anti-bTSHβ serum (#29) was capable of neutralizing over 10,000 mU of bTSH (Table II). The activity of individual antisera varied widely. The least active sera neutralized less than 25 mU bTSH/ml of antiserum.

TABLE II

*Antiserum Required to Neutralize 50% of 5 mU/ml bTSH*

| Rabbit | Experiment I | Rabbit | II |
|---|---|---|---|
| | μl/ml | | μl/ml |
| anti-bTSHβ | | | |
| 3 | 0.46 | 38 | 17.0 |
| 29 | 0.22 | 42 | 3.5 |
| 13 | 0.46 | 43 | 4.6 |
| 13 | 0.46 | 43 | 4.6 |
| 14 + 16 | 0.60 | 45 | * |
| | | 46 | 4.8 |
| | | 47 | 2.4 |
| mean±SEM | 0.44±0.08 | | 6.5±4.5 |
| anti-bTSH | | | |
| 12 | 0.55 | 39 | 3.8 |
| 27 | * | 48 | 1.6 |
| 19 | 2.00 | 49 | 12.0 |
| 26 | 4.40 | 50 | 3.9 |
| mean±SEM | 2.32±1.22 | | 5.4±2.3 |

* Concentrations up to 30 μl/ml (1:33) did not neutralize significant amounts of bTSH.

Anti-bTSH sera had greater neutralizing potency than did the anti-bTSHβ sera. The neutralizing activity, like the [$^{125}$I]bTSH binding activity, was greater in the sera from experiment I than the sera from experiment II.

Biologic neutralizing potency and binding of [$^{125}$I]-bTSH tracer may be related phenomena. The Spearman rank-order correlation coefficient ($r$) for correlation of 50% neutralization with 50% binding was $+0.73$ ($P < 0.05$) in experiment I but only $+0.47$ (NS) for experiment II.

*Nonneutralizing antibodies.* When incubation mixtures containing TSH and dilutions of anti-bTSHβ sera that only partially neutralized bTSH were treated with GARGG to precipitate immune complexes, the bioassay response of the supernate for TSH was further neutralized. Since free TSH was unaffected by GARGG, we ascribe this difference to nonneutralizing antibodies to bTSH which combined with TSH but did not neutralize its biological activity. These nonneutralizing antibodies were demonstrated in almost all of the animals immunized with bTSHβ and none of those immunized with bTSH. Figs. 3 and 4 present examples of this increased neutralization by GARGG added to an anti-bTSHβ serum and the absence of this effect

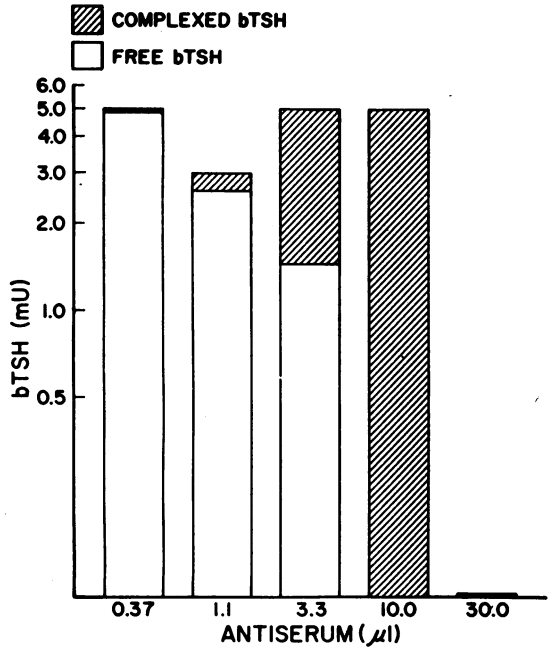


FIGURE 3 Anti-bTSHβ serum (#38) plus 5 mU/ml bTSH. The height of the bars indicates amount of TSH biological activity remaining after incubation with the amounts of antiserum indicated. Open bars indicate free bTSH. Cross-hatched bars indicate amounts of bTSH complexed to rabbit IgG and neutralized only by the addition of GARGG.

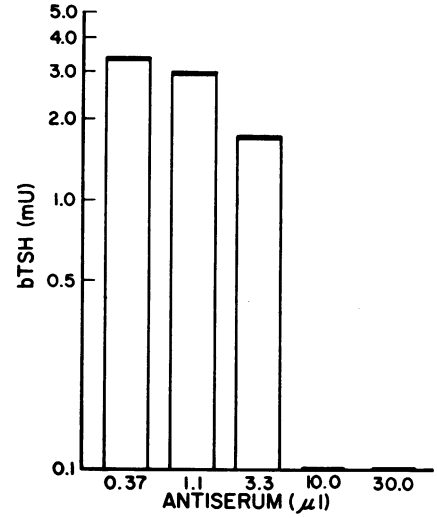


FIGURE 4 Anti-bTSH serum (#19) plus 5 mU/ml bTSH. Open bars indicate free bTSH (bioassay result after addition of GARGG). Increasing amounts of anti-bTSH neutralize increasing amounts of TSH. GARGG does not further reduce the biological activity.

with an anti-TSH serum. The amounts of bTSH–anti-bTSHβ complex neutralized and thus demonstrated by the addition of GARGG varied widely. Addition of GARGG neutralized from 0 to >98% of the activity of the 5 mU/ml TSH present.

This nonneutralizing activity of anti-bTSHβ was most marked in the sera with the least neutralizing potency. In fact, the two activities were significantly negatively correlated ($r = -0.74$). This relationship did not explain the lack of nonneutralizing antibodies in the anti-bTSH sera. These antisera had neutralizing activity in the same range as the anti-bTSHβ sera, so the striking difference between antisera to bTSH and bTSHβ was not related to differences in neutralizing potency. These observations are summarized in Table III.

The biological activity detected in the mixtures of antiserum with bTSH was greater at 2 h than at 8. This was true of the nonneutralized bTSH bound to rabbit gamma globulin as well as the free bTSH. Table IV documents these observations. For comparison, the 2 h/8 h ratio of the response indices for LATS is approximately 0.7, and that for bTSH is 1.4 (13).

*Effects of dilution of the TSH-anti-TSH complexes.* Dilution of preformed complexes did not result in the release of biologically active TSH (Table V).

## DISCUSSION

The heterogeneity of antibodies has been repeatedly documented and requires no special emphasis. To some extent, such heterogeneity may be a reflection of the

TABLE III

*Frequency of Neutralizing and Binding Activity in Rabbit Antisera to bTSH and bTSHβ*

| Antisera | Antibody activity | | | |
|---|---|---|---|---|
| | Total number | Number binding TSH | Number neutralizing TSH | Number with non-neutralizing antibodies* |
| Anti-bTSHβ (impure) | 4 | 4 | 4 | 3 |
| Anti-bTSH (Thytropar) | 4 | 4 | 3 | 0 |
| Anti-bTSHβ (free of bTSH) | 6 | 5 | 5 | 5 |
| Anti-bTSH (purified) | 4 | 4 | 4 | 0 |

*$P < 0.01$ for $X^2$ comparing bTSH with bTSHβ.

methods used to demonstrate the antibody. Thus the antibodies we have described as binding [$^{125}$I]bTSH or [$^{125}$I]bTSHβ undoubtedly encompass some or all of the antibodies we have designated as neutralizing or nonneutralizing, based on their biological activity in the presence of TSH.

The nonneutralizing antibodies combine with bTSH but do not completely inhibit its biological activity. These soluble antigen-antibody complexes can be precipitated or neutralized by the addition of GARGG. Since we have been able to describe this activity only in terms of the bioassay results, quantitation of the antibody is difficult and not easily comparable with the binding activity.

There are several possibilities as to the nature of neutralizing and nonneutralizing antibodies. The most obvious possibility is that the neutralizing antibody combines with a hormonally active portion of bTSH while nonneutralizing antibody combines elsewhere. Neutralization could also be caused by precipitation or by high affinity antibodies that produce conformational changes in the TSH. The former seems unlikely since workers interested in radioimmunoassay of TSH have found that it is difficult to achieve complete precipitation. Another possibility is that the apparent differences in antibodies, neutralizing vs. nonneutralizing, may represent varied rates of clearance of immune complexes in the bioassay mice.

More bTSHβ is bound by anti-bTSHβ than by anti-bTSH. These same antisera bind bTSH similarly. This difference supports the idea that the differing incidence of nonneutralizing antibodies in bTSHβ- vs. bTSH-immunized groups is due to the antigen used. The results of experiment II, using highly purified β-subunits, were similar in this respect to those of experiment I, using β-subunits contaminated with bTSH. In this context, the contamination seems insufficient to have affected the results. The increased binding and neutralizing activity of experiment I antisera as compared to experiment II antisera might be due to the subunit contamination with bTSH in experiment I, but a more likely explanation is that the purification procedure used in experiment II partly destroyed the antigenicity of the subunits.

Nonneutralizing antibodies to TSH form antigen-antibody complexes in vitro that are biologically active thyroid stimulators in the mouse. The biological ac-

TABLE IV

*Time-Course of Biological Activity of bTSH-Antiserum Mixtures*

| | Assays | Response index 2 h/8 h |
|---|---|---|
| | *n* | *mean (range)* |
| anti-bTSHβ plus bTSH | 30 | 1.5 (0.8–1.8) |
| anti-bTSHβ plus bTSH plus GARGG | 25 | 1.6 (1.1–1.9) |
| anti-bTSH plus bTSH | 13 | 1.4 (1.2–2.0) |
| anti-bTSH plus bTSH plus GARGG | 13 | 1.4 (1.1–1.6) |

TABLE V

*Failure to Produce Thyroid-Stimulating Activity by Diluting Neutralized Complexes*

| | 2-h Response Index | |
|---|---|---|
| | Without GARGG | With GARGG |
| | *mean ± SEM* | |
| bTSH, 5. 0 mU/ml | 1,315±222 | |
| 1.67 | 1,005±110 | |
| 0.56 | 548±43 | |
| anti-bTSHβ, 1.1 μl/ml plus bTSH, 5 mU/ml | 370±35 | 190±7 |
| anti-bTSHβ, 3.3 μl/ml plus bTSH, 5 mU/ml | 149±20 | 190±21 |
| same, diluted 1:3 | 114±16 | 157±26 |
| same, diluted 1:9 | 78±7 | 103±6 |

tivity, however, is short-acting (2-h activity greater than 8-h). One of the reasons for immunizing rabbits with TSH and its subunits was to investigate further the possibility that LATS might represent some form of an immune complex of IgG antibody with a hormonally active antigen. Several groups have previously studied TSH antisera in various combinations with TSH. McKenzie and Fishman noted that an anti-bTSH they used had a thyrotropic effect on the mouse thyroid (14). Hoffmann, Mason, Good, Hetzel, and Ferguson found long-acting thyroid stimulating activity (8-h greater than 3-h activity) when bTSH or myxedematous human serum was mixed with diluted anti-bTSH (6). They proposed that immune complexes had been formed during the incubation period. After combining hTSH and diluted anti-hTSH, Meek also detected long-acting thyroid-stimulating activity which he termed "LATS" (7). In contrast we have found when gamma globulin-bound TSH was injected into bioassay mice the thyroid-stimulating activity was greater at 2 h than at 8 h. We are unable to reconcile the differences in these observations. Hoffmann et al. did not attempt and Meek was unsuccessful in precipitating the alleged complex with GARGG (probably because insufficient GARGG was added). All three groups have raised antisera in a similar fashion with roughly equivalent amounts of antigen and duration of immunization. Incubations were also similar although we were always careful to keep the total amount of rabbit serum constant by adding normal rabbit serum. Such a step seems critical only if GARGG is to be added. Hoffmann et al. used intraperitoneal injections of uncentrifuged material in the bioassay mice, whereas we and Meek centrifuged and injected the supernate intravenously. Despite these differences it seems clear that each group has produced TSH–anti-TSH complexes in vitro capable of thyroid-stimulating activity. Should we infer that LATS is a similar complex? We do not feel that the fact that Hoffmann et al. and Meek have demonstrated a long-acting thyroid stimulating activity with TSH–anti-TSH complexes argues very strongly for the concept. The time of peak thyroid stimulation is probably not a completely reliable basis for the differentiation of thyroid stimulators. LATS can be converted from long-acting to short-acting by cleaving the gamma globulin with papain (15). It is likely that other procedures that affect such things as the size, persistence in the circulation, immune elimination, and binding characteristics of thyroid stimulators also alter their time-course of action. It is notable that the TSH–anti-TSH or TSH–anti-TSH$\beta$ complexes formed in vitro are still inhibited by anti-TSH. Numerous investigators have noticed that anti-TSH antisera do not neutralize LATS (6, 7, 14, 16–18); this has also been our experience. On the other hand TSH is always neutralized if enough antiserum is added. In addition, ethanol extraction of serum destroys LATS but does not extract TSH, LATS persists in the serum of hypophysectomized patients (19), and we have been unable to liberate any thyroactive material from LATS by dialysis or affinity chromatography (20). Dissociation of LATS with low pH and NaSCN has been attempted by ourselves and others. The results have been difficult to interpret since LATS activity is generally decreased by any such treatment but no TSH-like material has been recovered from column-eluates or dialysates. Finally, in recent, also unpublished, experiments we have not found any evidence for binding of hTSH by LATS-IgG nor of binding of bTSH or its subunits by LATS-IgG or its fragments produced by cleavage with papain.

Present evidence continues to indicate that LATS is an ordinary IgG and not an immune complex, despite the interesting knowledge that anti-bTSH$\beta$ sera can form nonneutralized complexes with TSH.

## ACKNOWLEDGMENTS

Our thanks go to Mr. Raymond Ajugwo, Mrs. Ruey S. Ho, Mr. Jeffrey Keene, Mrs. Natalie Limberg, and Miss Tiiu Reino for laboratory assistance and to Mrs. Hilda Dornfest for typing the manuscript.

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

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