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Research Article

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Inhibition of Long-acting Thyroid Stimulator by Thyroid Particulate Fractions *

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The long-acting thyroid stimulator (LATS), found uniquely in the serum of patients with Graves' disease, is closely associated with the immunoglobulin G (IgG) proteins of serum and is removed from serum by antibody to human IgG (2, 3). Since some IgG molecules function as antibodies, it seems possible that LATS is also an antibody. This possibility would be strengthened if LATS could be demonstrated to react specifically with an antigen. In an effort to demonstrate such a reaction we incubated serum containing LATS with insoluble fractions of thyroid tissue and evaluated the effects of this incubation by measuring LATS in the serum freed of suspended material by centrifugation. These experiments represent a confirmation and extension of studies reported by Kriss, Pleshakov, and Chien, in which they showed inhibition of LATS activity by slices and homogenates of dog and human thyroid (3). We also explored the specificity, stability, sensitivity, and, to some extent, the nature of the interaction between thyroid tissue and LATS.

Methods

Sera. Sera containing LATS were donated by five patients with Graves' disease. The great majority of the experiments used large donations of sera from two patients who had infiltrative ophthalmopathy, localized pretibial myxedema, and high serum concentrations of LATS (McKenzie assay response indexes, 1,400 and 1,000). These two patients were without medication when the serum was obtained. Previous hyperthyroidism had been treated with a subtotal thyroidectomy in one patient and radioactive iodine in the other.

Serum from the other three patients was used for only one experiment each. These patients all had infiltrative ophthalmopathy but did not have localized pretibial myxedema, and serum concentrations of LATS were relatively low (response indexes, 270, 215, and 179). At the time of venesection two of these patients were euthyroid without medication after treatment of hyperthyroidism with radioactive iodine. The third patient had never experienced hyperthyroidism but had developed spontaneous hypothyroidism. She was euthyroid while receiving desiccated thyroid medication at the time of study.

LATS assay. LATS was measured in mice by the McKenzie procedure (4) modified slightly in our laboratory (5). The volume of all injections was 0.5 ml. The variance of the response found in the assay procedure was highly correlated with the magnitude of that response, as has been reported by others (6). Therefore, statistical comparisons employed the logarithm of the response as the response metameter. The relationship between log dose and log response was determined for each batch of serum used in this study and for a standard serum provided by Dr. Joseph Kriss and identical to that used by him (3). Regression lines for the sera were parallel to that of the standard. Therefore, the LATS content of all samples was expressed in "Kriss units," by comparison of the mean log response for the assayed sample with the regression line previously obtained for the standard serum. Statistical comparisons were made with Student's t test, and significance was attributed to differences where p was less than 0.05.

Thyroid tissue. Portions of over 50 different thyroid glands were used during the study. Four of these had been surgically removed, two for hyperthyroidism and two for nodular goiter. The remainder had been removed at autopsy from patients succumbing to a variety of diseases.[1] Some glands were stored at 4° C for as long as 57 hours after death, but most were processed within 24 hours. Fat and excess connective tissue were removed with sharp scissors. The tissue was then blotted, weighed, cut up with scissors, and homogenized in a Virtis homogenizer in a convenient volume of 0.25 M sucrose (usually a 10% wt/vol suspension). Subcellular

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fractions were obtained from the homogenate with the techniques described by Hogeboom (7). A Spinco model L preparative ultracentrifuge with a no. 40 rotor was used. Centrifugal forces are given for the average radius of the centrifuge tube.

The light particle or microsomal fraction was that portion of the homogenate which did not sediment at a centrifugal force of 8,000 *g* in 20 minutes but did sediment in 1 hour at 100,000 *g*. The amount of a microsomal preparation used in an experiment was expressed in terms of the equivalent wet weight in grams (g-Eq) of the thyroid tissue from which it had been obtained.

Incubations. Substances that were studied for their ability to inhibit LATS activity were incubated with serum containing LATS. Subcellular particles were suspended in the serum by brief homogenization. Soluble tissue extracts were lyophilized and then suspended in the serum. After appropriate incubation with stirring, the sedimentable particles were removed by centrifugation at 100,000 *g* for 1 hour, and LATS was assayed in the supernatant. Serum treated identically but without the addition of tissue fractions was simultaneously assayed for LATS as a control.

Stability of the thyroid microsomes to heat was studied by preincubation of a microsomal suspension in phosphate-buffered physiological saline (pH 7.2). The microsomes were then resedimented by centrifugation and incubated with serum containing LATS. The effect of ribonuclease T_1 [2] was studied in a similar fashion after incubating from 100 to 800 U of the enzyme with microsomal suspensions at pH 7.5 and 37° C for 1 to 4 hours.

Enzymatic inhibitors. Several substances capable of inhibiting various enzymatic systems were studied for their effects on the LATS-thyroid microsomal interaction. The potential inhibitors were added to the serum in appropriate concentrations before incubation with the microsomes. After incubation and centrifugation the serum was dialyzed against phosphate-buffered physiological saline (pH 7.2) overnight before LATS assay.

Extractions. Various procedures were tested for their ability to solubilize the microsomal LATS inhibitor. Freezing and thawing were accomplished by immersion of a vessel containing the microsomal suspension into acetone in dry ice and warm tap water, respectively. Ultrasonic vibration was performed with an M.S.E. Mullard ultrasonic disintegrator [3] operated at 20,000 cycles per second for 20 minutes with the probe barely in contact with the microsomal suspension, which was cooled in an ice bath. Washed thyroid microsomes were incubated for 20 hours at 4° C with 0.26% sodium deoxycholate, 8 M or 4 M urea, 5.2 M acetic acid, or 0.5 N NaOH. The supernatants and sediments obtained after centrifugation at 100,000 *g* for 1 hour were dialyzed against phosphate-buffered physiological saline and incubated with serum containing LATS.

Thyroactive materials. Sodium-1-thyroxine was dissolved in 0.1 ml of 1 N NaOH. This solution was added to 5 ml of serum containing LATS, and incubation and bioassay were performed exactly as in the experiments with tissue fractions. The control was 5 ml of serum containing LATS to which 0.1 ml of 1 N NaOH had been added.

Human thyroglobulin [4] was dissolved directly in serum at a concentration of 5 mg per ml. Incubation and bioassay were performed in the usual manner. To investigate the effect of thyroactive materials on thyroid-stimulating hormones (TSH), we dissolved appropriate amounts of TSH in 5 ml isotonic NaCl and added thyroxine as described above.

Nitrogen. Nitrogen was measured by the Kjeldahl procedure (8).

Enzyme assays. Cathepsin (acid protease) in tissue fractions was assayed by incubation at 37° C for 30 minutes with a hemoglobin substrate in 0.2 N acetic acid (9). Acid phosphatase was measured with CalSuls; [2] β-glucuronidase activity was measured with a phenolphthalein glucuronide substrate [5] (10).

Iodine analysis. Two g-Eq of thyroid microsomes was incubated in normal serum and centrifuged at 100,000 *g*, and the supernatant was analyzed for iodinated compounds by the chromatographic method of Hellman, Tschudy, Robbins, and Rall (11).[6] In this method, serum is applied to the column at alkaline pH, and then a predominantly iodoprotein fraction is eluted at pH 7.0, iodotyrosines at pH 4.0 to 2.2, and iodothyronines at pH 1.4.

Results

Twenty-three of 26 experiments in which microsomes isolated from various thyroid glands were incubated with LATS resulted in significant inhibition of LATS (Figure 1). The mean reduction in LATS activity was 74% of the concentration present in the serum initially. Each of the four subcellular fractions prepared (nuclei, mitochondria, microsomes, and cell sap) reduced LATS activity after incubation, but the microsomal fraction was much the most active. Microsomal fractions inhibited the LATS activity of each of the five sera used in the study.

The source of the thyroid tissue used seemed to be of little consequence. The microsomal fraction obtained from tissue stored at 4° C for as long as 57 hours after death was still effective as an inhibitor of LATS. Glands removed at autopsy from patients dying of a variety of diseases were as active as the normal portions of thyroid glands removed surgically for adenoma. Fractions prepared from two thyrotoxic glands were neither

[2] Calbiochem, Los Angeles, Calif.

[3] Measuring and Scientific Equipment, Ltd., London.

[4] Provided by Dr. John G. Pierce.

[5] Sigma Chemical Co., St. Louis, Mo.

[6] The analyses were performed by Dr. Vincent J. Pileggi of Bio-Science Laboratories, Los Angeles, Calif.

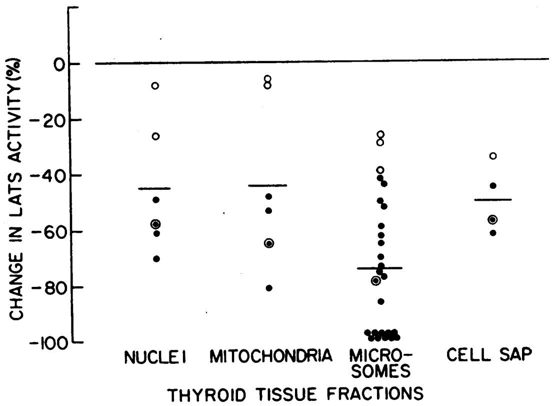

FIG. 1. INHIBITION OF LONG-ACTING THYROID STIMULATOR (LATS) ACTIVITY WHEN INCUBATED WITH FRACTIONS OF THYROID TISSUE. Sedimentable material was removed by centrifugation for 1 hour at 100,000 *g*, and LATS was assayed in the supernatant. The ordinate expresses change in LATS activity calculated as per cent of the control value. In this and in Figures 3 and 4 closed circles represent values of LATS activity significantly different from control values ($p < 0.05$ by *t* test), and open circles represent statistically insignificant results. The horizontal bars indicate mean values. The circled dots represent the results from incubation of serum containing LATS with fractions of a portion of thyroid gland surgically removed from a patient with Graves' disease.

more nor less active than tissue from normal glands. Both patients had the clinical features of Graves' disease, but neither had LATS activity in the serum.

Lyophilization of microsomes reduced LATS-inhibiting activity slightly. Heating the thyroid microsomal fraction to 55° C or above for 20 minutes destroyed its capacity to inhibit LATS. The results of two experiments illustrating this point are shown in Table I. Microsomal suspensions incubated with ribonuclease T_1 retained their inhibitory activity.

Inhibition of LATS activity was roughly proportional to the amount of the microsomal fraction used. Two g-Eq of fresh microsomes usually produced significant and often complete inhibition. When varying amounts of a large pool of lyophilized microsomes were tested for LATS-inhibiting activity, a roughly linear relationship was demonstrated when the logarithm of the quantity of microsomes used was plotted against the per cent decrease in LATS activity (Figure 2).

Although there were obvious differences in the amounts of microsomes isolated from different glands, neither the volume nor the nitrogen content of the microsomes correlated well with their inhibitory activity. Six washed microsomal preparations had a mean nitrogen content of 0.70 mg per g-Eq ± 0.17 (SD).

The inhibition of LATS activity by thyroid microsomal fractions was demonstrated over a range of times of incubation from 1 to 4 hours at 37° C and from 4 to 20 hours at 4° C. There was no demonstrable trend toward increased or decreased effect with time.

Inhibition of LATS appeared to be specific for

TABLE I

*Inhibition of LATS activity when incubated with thyroid microsomes preheated to various temperatures**

	% change in LATS activity	p
1. Thyroid microsomes		
Kept at 4° C	−44	<0.02
Heated for 20 minutes		
at 37° C	−52	<0.01
at 55° C	− 9	NS
at 70° C	+30	NS
at 100° C	+52	NS
2. Thyroid microsomes		
Kept at 4° C	−62, −100	<0.001
Heated for 20 minutes		
at 100° C	−25, −4	NS

* LATS = long-acting thyroid stimulator.

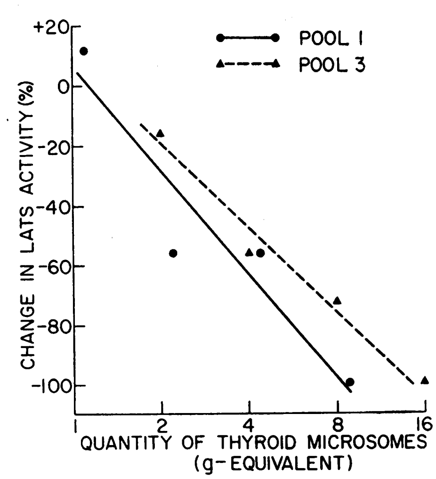

FIG. 2. EFFECT OF QUANTITY OF MICROSOMES ON THE DEGREE OF INHIBITION OF LATS ACTIVITY DURING INCUBATION. A logarithmic scale is employed for grams-equivalent of thyroid microsomes. For two pools of microsomes a roughly linear relationship obtains between per cent change in LATS activity and the logarithm of the quantity of microsomes used.

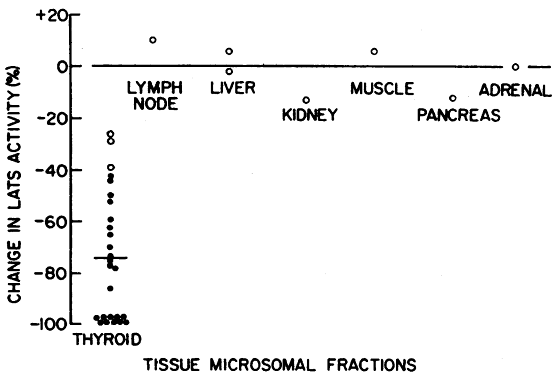

FIG. 3. INHIBITION OF LATS ACTIVITY WHEN INCUBATED WITH TISSUE FRACTIONS. None of the microsomal fractions obtained from tissues other than thyroid-inhibited LATS activity.

thyroid tissue fractions. Microsomes isolated from liver, kidney, muscle, pancreas, adrenal, and lymph node did not inhibit LATS activity (Figure 3).

Inhibition of LATS by thyroid microsomes was not affected by a number of inhibitors of enzymatic activity (Table II).

Extraction of LATS-inhibiting material from microsomes. The cell sap prepared from thyroid tissue homogenized in 0.25 M sucrose was partially effective in inhibiting LATS. The sedimented microsomes were, however, much more active. Resuspension of the microsomes in 0.25 M sucrose or 0.15 M saline followed by sedimentation at 100,000 *g* was not effective in liberating any inhibitory material into the supernatant liquid. Freezing and thawing the suspended microsomes ten times in saline or ultrasonic vibration of such a saline suspension (20,000 cycles per second for 20 minutes) did not release any inhibitory material into the supernatant at 100,000 *g* unless a large amount, such as 10 g-Eq, was used (Table III). The sedimented microsomes usually retained their activity after these procedures; measurements of nitrogen in the pellets indicated that little material had been solubilized.

TABLE II

The lack of effect of various agents on inhibition of LATS by thyroid microsomes

Agent added	Concentration	% change in LATS activity
None		−75
Soy bean trypsin inhibitor	0.2 %	−74
Sodium azide	10^{-2} M	−70
Potassium cyanide	10^{-3} M	−79
Sodium fluoride	10^{-3} M	−61
Diisopropyl fluorophosphate	10^{-2} M	−58
Ethylenediaminetetra-acetic acid	10^{-2} M	−69
Dinitrofluorobenzene	10^{-3} M	−74

TABLE III

Extraction of LATS inhibitor from thyroid microsomes

Extracting solution	% change in LATS activity: After incubation with supernatant	After incubation with sediment
0.15 M saline		
37° C 1 hour	−16, − 3	
Frozen and thawed ten times	−35, −42, −42, +20	−70,* −100*
Ultrasonically vibrated	− 8, −42*	0
Serum proteins		
Normal human serum, 37° C 1 hour	−100,* −38	
Normal human serum, frozen and thawed ten times	−48,* −68*	−76*
4% human serum albumin, frozen and thawed ten times	+20	−100*
1% bovine serum albumin, 37° C 1 hour	−61*	−69*
Others		
Sodium deoxycholate 0.26%	−26, −23	−21, 0
Urea 4 M	+ 8	0
Urea 8 M	−15, 0	+48
Acetic acid 5.2 M	+ 5	
NaOH 0.5 N	0	

* Statistically significant change.

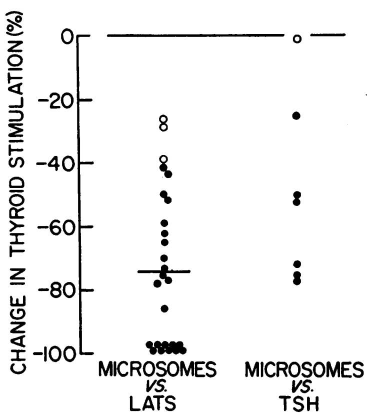

FIG. 4. EFFECT OF INCUBATION WITH THYROID MICROSOMAL FRACTION ON ACTIVITY OF BOVINE THYROID-STIMULATING HORMONE (TSH) AND HUMAN LATS.

TABLE IV

*Effect of thyroactive materials on response to LATS and TSH**

Experiment no.	Material	Dose per mouse	% change in activity	p
		μg		
8-hour response to LATS				
22	Thyroxine	0.18	−21	NS
22	Thyroxine	0.55	−21	NS
22	Thyroxine	1.67	− 3	NS
22	Thyroxine	5.0	−24	NS
5	Thyroxine	10.0	−18	NS
13	Thyroxine	100.0	−35	NS
21	Thyroxine	100.0	−60	<0.01
15	Human thyroglobulin	2,500.0	−18	NS
2-hour response to TSH				
22	Thyroxine	1.67	−38	<0.05
21	Thyroxine	100.0	−66	<0.01

* TSH = thyroid-stimulating hormone.

On the other hand, simple incubation or freezing and thawing of microsomes in a medium containing protein, such as normal human serum or bovine serum albumin, was successful in transferring some inhibitory activity to the supernatant of the 100,000 *g* centrifugation, but the sedimented material still retained most of its original activity.

Sodium deoxycholate (0.26%), 8 M and 4 M urea, 5.2 M acetic acid, and 0.5 N sodium hydroxide all solubilized some portion of a microsomal suspension. Unfortunately, these procedures apparently destroyed the LATS-inhibitory capacity of the microsomes, since neither supernatants nor sediments at 100,000 *g* were effective inhibitors of LATS (Table III).

Effect of microsomal fractions on thyrotropin (TSH). Microsomes were incubated with solutions containing known amounts of bovine TSH in order to ascertain the effects of the thyroid microsomes on another type of thyroid stimulator. After centrifugation the supernatant was assayed for TSH activity. TSH was inhibited by the microsomes with reasonable consistency (Figure 4). However, the microsomes were more effective in inhibiting LATS than TSH, if the 2-hour response to TSH can be compared to the 8-hour response to LATS.

Thyroactive materials and thyroid stimulation. Thyroactive materials might have been released from the thyroid microsomes into the serum during incubation to act directly to inhibit the response of the mouse thyroid gland to thyroid-stimulating agents in the serum. This possibility was examined by studying the effect of thyroactive materials on thyroid stimulation. As much as 2.5 mg of thyroglobulin per mouse had no effect on LATS, whereas as little as 8.75 mg of microsomal protein completely inhibited an equivalent amount of LATS activity. Huge amounts of thyroxine (100 μg per mouse) did inhibit the mouse ^{131}I-release response to LATS or TSH. Smaller amounts were without effect on LATS but did inhibit TSH (Table IV).

When 2 g-Eq of thyroid microsomes was incubated in serum and then centrifuged at 100,000 *g*,

TABLE V

Iodinated materials in serum after incubation with various substances

Preparation	Expected iodine	Iodoprotein (H_2O wash)	Iodotyrosines (pH 4.0–2.2)	Iodothyronines (pH 1.4)	Total iodine recovered	Total iodine by direct analysis
				μg/0.5 ml		
Thyroid microsomes in serum		0.42	0.12	0.06	0.60	0.80
Thyroxine, 100 μg per 0.5 ml serum	65	2.5	0.13	41.0	44.0	48.0
Human thyroglobulin, 5 mg per 0.5 ml serum		21.0	0.19	0.38	21.0	26.0

the supernatant serum contained only small amounts of iodinated materials (Table V). Iodothyronine iodine was only 0.06 μg per 0.5 ml of serum, of which 0.02 μg was actually accounted for by the thyroxine iodine of the unincubated serum, separately measured. The least amount of thyroxine (100 μg per 0.5 ml) that even inconsistently inhibited LATS activity (Table IV) yielded an iodothyronine iodine concentration of 41 μg per 0.5 ml serum by this method. Thus, the amount of hormonal iodine eluted from thyroid microsomes by incubation in serum was one-thousandth that required to produce any significant inhibition of LATS activity and was less than that present in any of the amounts of thyroxine added, without effect, to sera containing LATS in the experiments of Table IV. Similarly, the iodoprotein eluted from thyroid microsomes, 0.42 μg per 0.5 ml serum, was only one-fiftieth that introduced into serum by the addition of 2.5 mg of human thyroglobulin, which was ineffective in inhibiting LATS activity. Thus, neither the iodothyronine nor iodoprotein released into serum from the microsomal fraction approached the amount of thyroactive material required to inhibit LATS activity in the McKenzie assay.

Enzymatic activity of microsomes. Another possible explanation of the inhibition of LATS by microsomes was that LATS might have been destroyed by an enzyme in thyroid microsomes. This hypothesis would be favored if the microsomal preparation contained lysosomes. Consequently, the activity of several acid hydrolases in the microsomes was studied before and after repeated freezing and thawing to disrupt any lysosomes that might have been present. Table VI shows the results of these experiments. Acid hydrolases typical of those found in lysosomes were present in the thyroid microsomes, but the amount of enzyme free and active was not increased substantially by repeated freezing and thawing, thus providing no evidence for intact lysosomal packets of bound, inactive enzymes in the sediment at 100,000 *g*. Both cathepsin (acid protease) and β-glucuronidase were present in the supernatant saline in substantial concentration. Such extracts did not inhibit LATS (Table III).

TABLE VI

Acid hydrolases in a thyroid microsomal preparation

Preparation	Acid phosphatase	β-Glucuronidase	Cathepsin
	Bessey-Lowry U/L	*U*	*U*
1. Suspended in 0.25 M sucrose	10.5	60	1.8
Supernatant*	1.5	24	1.3
Sediment	8.4	32	.9
2. a. Suspended in 0.15 M saline	19.6		5.5
Supernatant*	1.3		4.1
Sediment	15.3		1.9
b. Suspended in 2% human serum albumin			
Supernatant	1.4		3.7
Sediment	16.2		4.0

* After freezing and thawing suspension ten times, then centrifugation at 100,000 *g* for 1 hour.

Recovery of LATS from microsomes. Our original hypothesis was that the microsomal fraction contained a specific substance that combined with and removed LATS from the serum as an insoluble antigen would adsorb an antibody. To test this hypothesis, we attempted to recover

TABLE VII

Recovery of LATS by acid extraction of thyroid microsomal pellet

Experiment no.	LATS initially in serum	LATS remaining in serum after incubation	LATS removed during incubation	LATS in washes of pellet	LATS in pH 3.0 extract of pellet	Significance p*	% recovered in pH 3.0 extract†
16	5.8‡	1.4	4.4	0	1.0	<0.001	23
18					0.73	<0.01	
24	4.7	1.3	3.4	0	0.60	<0.02	18
32	27.0	11.0	16.0	0.73	2.2	<0.001	13

* Significance of difference between LATS response to pH 3.0 extract and response to saline or normal serum control.
† LATS in pH 3.0 extract/LATS removed during incubation × 100.
‡ LATS is expressed in Kriss units (see Methods for definition).

LATS from sedimented thyroid microsomes that had first been incubated with and had inhibited a serum containing LATS. Whereas simple washing of such a microsomal pellet with normal serum did not usually free LATS activity, incubation of the pellet in 5 to 10 ml of a 0.1 N glycine-HCl buffer at pH 3.0 for 1 hour at 37° C led to recovery of a significant amount of LATS in the buffer (Table VII). This result occurred in four consecutive experiments.

Discussion

We have described inhibition of LATS during incubation with thyroid microsomal suspensions. Several explanations of this phenomenon are tenable. The apparent specificity of thyroid tissue for the inhibition and the recovery of LATS activity from the microsomes in an acid eluate suggest that an antigen-antibody combination may have taken place. Other evidences of an immunologic reaction are thus far lacking, however.

It is possible that the thyroid tissue enzymatically destroys LATS. Maloof and Soodak have reviewed previous studies of proteases and peptidases in thyroid tissue (12). Both types of activity have been found. Unfortunately, the susceptibility of these enzymes to inhibitors has not been clearly delineated. These enzymes were extractable into saline, as was the acid protease we found in thyroid microsomal fractions, whereas LATS-inhibitory material was not. The LATS-inhibitory activity was not altered by including in the incubation a number of substances that are frequently capable of blocking enzymatic reactions (Table II). Additional evidence against destruction of LATS during the incubation was its recovery from acid washings of microsomes that had been incubated with serum containing LATS.

Also to be considered is the possibility that thyroactive materials released from the microsomes inhibited the response of the mouse thyroid gland to thyroid-stimulating materials, since huge amounts of thyroxine were capable of doing just that. The extractability of the inhibitor into serum but not into saline would be consistent with the behavior of thyroxine. However, the amount of iodine-containing material found in the supernatant serum after incubation with microsomes could not have produced such inhibitory activity, even if all the iodine were in the form of thyroxine and triiodothyronine. Actually, only a small proportion of the iodine extractable from the microsomes was in the form of iodothyronine.

One hypothesis of the mechanism by which LATS might induce hyperthyroidism is that it is an antibody to a genetic repressor (13) or to some other inhibitor of thyroxine manufacture or release. If such were the case, these "antigenic" sites in the thyroid gland in Graves' disease ought to be saturated and therefore fail to combine with and inactivate the "antibody" LATS *in vitro*. The presence of inhibitory activity in preparations from the thyroid glands of our two patients with Graves' disease appears superficially to be in conflict with this hypothesis. However, neither of these patients had LATS detectable in serum. In contrast, Pinchera, Pinchera, and Stanbury reported failure of thyroid slices and cell membranes to inhibit LATS activity; the donor of thyroid tissue in their study had a high concentration of LATS in the serum (14). The difference between their findings and ours may perhaps be explained on the basis of saturation of the thyroidal inhibitor as a result of prior exposure *in vivo* to high levels of LATS.

Separation of subcellular components by homogenization and differential centrifugation is acknowledged to be associated with admixture of the various fractions. This may account for the presence of inhibitory activity in all the subcellular fractions in addition to its apparent concentration in the microsomal fraction. The weak but significant inhibitory activity of lyophilized cell sap indicates that some of the inhibitor was soluble, but efforts to solubilize larger amounts of inhibitory activity from microsomes were unsuccessful. The microsomal fraction of thyroid tissue should contain fragments of cell membranes, including both the external plasma membrane and the endoplasmic reticulum. Studies to further characterize the site of the microsomal inhibitory activity are in progress.

The inhibition of TSH by microsomes, which confirms the inhibition demonstrated for whole thyroid tissue by Rawson, Graham, and Riddell (15), demonstrates that at least one other thyroid-stimulating substance can be inhibited under these conditions. In our hands as little as 1.67 μg of thyroxine added to 0.4 mU of TSH inhibited the thyroidal response of the assay mouse to the

TSH. Major and Munro noted no alteration in the response to 0.5 mU of TSH when 1.0 μg of thyroxine was added (16). The effect of larger amounts of thyroxine has not previously been reported. We did not further examine the effect of small amounts of thyroxine on the bioassay; therefore the interpretation of the reduction in TSH activity after incubation with thyroid microsomes remains in doubt. The data thus far do not establish that any kind of combination or interaction necessarily occurred between TSH and the microsomes. The same doubt does not cloud the LATS-microsome interaction, since LATS is not inhibited by any amount of thyroxine less than 100 μg per assay mouse.

Although much remains to be discovered, these experiments can be used to provide comfort and support for the hypothesis that LATS is an antibody. An antibody has been defined as a "humoral globulin produced by the body in response to an antigen and capable of reacting with the antigen in some observable way" (17). The evidence appears strong that LATS is intimately associated with and perhaps identical to IgG, since it travels with IgG in all the usual electrophoretic and chromatographic procedures (2, 3, 18, 19), is removed from serum by incubation with antihuman IgG (3, 19), and can apparently be found in the heavy chain fraction formed by partial degradation of IgG (19).

At this time there is no good evidence that LATS arises from antigenic stimulation, but McKenzie and Gordon recently reported production of LATS by lymphocytes in tissue cultures, affording some evidence for manufacture of LATS by body cells (20). Finally, the experiments reported here provide data perhaps best explained by an antigen-antibody reaction, although they cannot be construed as firm evidence for such reaction.

It may be useful at this time to examine the hypothesis that Graves' disease is caused by autoallergy. Milgrom and Witebsky's postulates are generally accepted as reasonable albeit strenuous requirements to establish an autoallergic etiology for a disease (21). These requirements can be summarized as follows: *1*) the recognition of an antibody or cellular immune reaction active at body temperature in humans with the disease, *2*) the recognition of a specific antigen in the human tissue involved in the disease, *3*) production of antibody in experimental animals stimulated by the antigen, *4*) the appearance of pathologic changes similar to the human disease in a corresponding tissue in a sensitized experimental animal, and *5*) passive transfer of the disease with serum or immunologically competent cells.

If LATS is an antibody, the first requirement has been met. Adams has reviewed the evidence strongly suggesting that LATS is the cause of the hyperthyroidism of Graves' disease (22). Our data suggest that the thyroid gland contains a substance which interacts with LATS as an antigen, but this is hardly clear enough as yet to satisfy the second requirement for the demonstration of an antigen. Search for such an antigen should probably not be confined to the thyroid gland because Graves' disease involves other tissues, including retro-orbital connective tissue and muscle as well as skin and subcutaneous tissue of the pretibial region and occasionally elsewhere (23). Normal skin does not interact with LATS in our system; no studies have as yet been done with the involved tissues of patients with Graves' ophthalmopathy or localized myxedema. The third and fourth requirements cannot be met until an antigenic substance has been isolated. As yet we have been unable to detect LATS activity in serum of rabbits receiving injections of human thyroid microsomal material. Graves' disease is associated with functional changes and little in the way of specific pathology, except for hyperplasia and lymphocytic infiltration of the thyroid gland and edema, metachromasia, and lymphocytic infiltration of retro-orbital tissues (24). It would seem reasonable to accept functional changes due to immunization in an experimental animal instead of pathologic changes to satisfy Witebsky's fourth requirement. So far neither has been observed. The fifth requirement, passive transfer, has been clearly demonstrated in human neonates who, if exposed *in utero* to mothers with Graves' disease and LATS in the serum, develop transient hyperthyroidism. In this situation LATS is found in the serum of the neonate and disappears at approximately the half-life of IgG (25, 26). One can conclude that the hypothesis that Graves' disease is caused by autoallergy is not an improbable one, although it is far from established at the present time.

Summary

Fractions of thyroid tissue were incubated with serum containing the long-acting thyroid stimulator to discover whether any interaction occurred. All subcellular fractions of thyroid tissue reduced long-acting thyroid stimulator activity, but the microsomal fraction contained the most potent and consistent inhibitor.

The microsomal inhibitor was consistently present, potent, effective in proportion to quantity, stable at 4° C, heat labile, and resistant to ribonuclease and several inhibitors of enzymatic activity. Microsomal fractions isolated from six nonthyroidal tissues did not inhibit the long-acting thyroid stimulator.

The inhibitory activity could not be satisfactorily extracted from the microsomes by any means used. Small amounts of this activity were extracted into protein-containing solutions but not into physiological saline.

Thyrotropin was inhibited by incubation with thyroid microsomes, but small amounts of thyroxine also decreased thyrotropin effect in the bioassay system; a direct interaction of thyroid microsomes and thyrotropin was therefore not established. On the other hand, the long-acting thyroid stimulator was not inhibited by any amount of thyroxine below 100 μg per mouse.

Although the thyroid microsomal fractions contained acid hydrolases, like those found in lysosomes, the presence of such enzymes did not correlate with inhibition of the long-acting thyroid stimulator.

Long-acting thyroid stimulator was recovered repeatedly from acidic extracts of microsomes that had previously been incubated with serum containing the long-acting thyroid stimulator, sedimented, and washed in normal serum. We believe these observations support the hypothesis that the long-acting thyroid stimulator is an antibody reacting with an antigen in the thyroid microsomal fraction.

The associated hypothesis that Graves' disease is a disorder of autoallergy has been discussed.

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