CHARACTERIZATION OF THE LONG-ACTING THYROID STIMULATOR OF GRAVES' DISEASE*

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Following the discovery by Adams and Purves in 1956[1] of an abnormal substance in the blood of patients with hyperthyroidism, renewed interest has developed concerning the pathogenesis of Graves' disease. The occurrence of this substance, now referred to as the long-acting thyroid stimulator (LATS), has been confirmed by others,[2, 3] and it has been found in the serum of the majority of patients with hyperthyroidism. It is almost always detectable in those patients with exophthalmos and localized pretibial myxedema.[4]

Despite the suggestion that this factor is of etiologic importance in Graves' disease, little has been known of its nature or origin, and some doubt has been expressed whether it actually represents a distinct entity.[5] Various attempts to concentrate or isolate the active principle have been only partially successful. The percolation method for the purification of thyrotropin has failed.[6] Recently, a 5- to 10-fold concentration by means of filtration through Sephadex gel was described.[7]

This report describes a method of purification of the long-acting thyroid stimulator in which the activity was found to reside exclusively in the gamma globulin fraction of the serum proteins. Biologic activity was further identified in subunits of the gamma globulin; however, these fragments, in contrast to the intact material, produced an early peak response similar to that obtained with thyrotropin.

Materials and Methods.—Sources of plasma: The starting material used in the purification procedure was lyophilized citrated plasma or lyophilized serum from two patients with hyperthyroidism, exophthalmos, and consistently high titers of LATS-activity in the unaltered serum. One patient had pretibial myxedema; neither patient had an abnormal distribution of serum proteins as determined by paper electrophoresis.

Purification procedure: The lyophilized plasma, or serum, was dissolved in distilled water to make a 2.5% solution. Ammonium sulfate was added to a 40% concentration at 4°C. The precipitate was allowed to form overnight in the cold, collected by centrifugation, then dissolved and dialyzed against 0.1 *M* sodium chloride for 12 hr. The solution was then dialyzed 12 hr against 0.005 *M* phosphate buffer, pH 7.0. The euglobulin precipitate was removed, and the clear protein solution was passed through a 2.4-cm × 20-cm column packed with diethylaminoethylcellulose (DEAE-cellulose) which previously had been equilibrated with the phosphate buffer. The column load of the DEAE-cellulose (exchange capacity, 0.7 mEq/gm) was limited to 1.0 gm of protein. The unabsorbed protein fraction (gamma globulin) was dialyzed free of phosphate and was stored in the lyophilized state; the absorbed protein was removed by the passage of 0.2 *M* potassium phosphate through the column, dialyzed against 0.1 *M* sodium chloride, and lyophilized.

Neutralization studies: The active gamma globulin was dissolved in saline, mixed with an equal volume of rabbit antibody to human 7S gamma globulin (Hyland Laboratories, Lot. no RP 763F), and incubated at 37°C for 90 min. The solution was then held at 4°C overnight. Although no visible precipitate formed, the solution was centrifuged and the upper portion removed for assay. A control sample of active gamma globulin was treated in an identical fashion with the exception that saline was substituted for the rabbit antibody.

Reduction of the active gamma globulin: The method for the reduction and fractionation of gamma globulin described by Fleischman *et al.*[8] was used with minor modifications. A 2% solution of

gamma globulin in 0.55 *M* Tris (hydroxy-methyl) aminomethane-HCl, pH 8.2, was made 0.7 *M* with respect to 2-mercaptoethanol at room temperature. After 1 hr the solution was cooled in ice water, and an equal volume of 1.0 *M* iodoacetamide (recrystallized) was added. The pH of the solution was maintained near 8 by the addition of 25% trimethylamine in water. After 1 hr the solution was dialyzed for 16 hr at 4°C against 0.15 *M* sodium chloride.

The A and B polypeptide chains of the reduced, alkylated globulin were separated by means of a Sephadex G-75 column (2.8 cm × 60 cm) at 4°C. Both the reduced protein and the Sephadex gel were equilibrated with 1 *N* acetic acid. The separated A and B chains were dialyzed against distilled water and lyophilized.

Papain digestion of the active gamma globulin: The technique for the enzymatic digestion of gamma globulin was similar to that described by Porter;[9] crystalline mercuripapain (Worthington Biochemical Corp., Lot no. 6111) was used. The chromatographic separation of the papain-digest using DEAE-cellulose was carried out as described by Edelman *et al.*[10] The conditions for gradient elution were: mixing chamber size, 800 ml; column size, 2.4 cm × 30 cm; buffer, potassium phosphate, pH 7.8; ionic strength, 0.005 *M* to 0.5 *M*. The fractions were dialyzed against distilled water and lyophilized.

Protein analysis: Protein concentrations were determined either by a modified Folin-Ciocalteau method[11] or by absorbance at 280 mμ.[12]

Electrophoresis on polyacrylamide: The method of disk electrophoresis as described by Ornstein and Davis[13] was used for the analysis of proteins at pH 9.5. Disk electrophoresis at an acidic pH was carried out as described by Reisfeld *et al.*[14] with the exception that a concentration of 8 *M* urea was added to the large pore and lower gels. The columns were prepared with 7.5% acrylamide for the lower gel.

Bioassay for thyrotropin and LATS: The method of bioassay developed by McKenzie[15] was used with minor modifications, and the data are presented in the form employed by him. A significant response was considered to be a 2- or 8-hr response greater than 150. Controls for each assay included saline or normal serum, USP thyrotropin reference standard at four dilutions, and a known LATS-active serum.

Results.—The results of the purification of active sera are shown in Figure 1. When serum was subjected to salt fractionation, 33 per cent of the initial protein was recovered in the ammonium sulfate precipitate. The LATS activity was associated

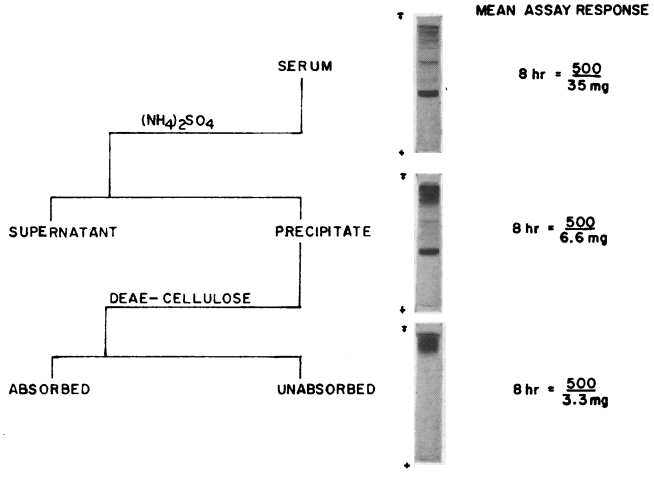

FIG. 1.—LATS purification scheme. The polyacrylamide gel illustrations indicate the protein electrophoretic pattern at pH 8.4 of the three LATS-active fractions: unaltered serum, ammonium sulfate precipitate, and the unabsorbed protein from DEAE-cellulose chromatography. Protein applied to the upper gel, 150 μg. The assay of these fractions for LATS is listed on the right; the results are expressed as the amount of intravenously injected protein required to produce equivalent 8-hr responses.

exclusively with the precipitated protein; repeated assay failed to detect activity in the supernatant fraction. Disk electrophoresis (pH 9.5) of the active precipitate indicated that there had been a concentration of the globulin proteins. When the ammonium sulfate precipitate was dissolved and dialyzed against phosphate buffer, a euglobulin precipitate formed which represented 8 per cent of the protein. This material was inactive when assayed. After passage of the soluble protein through DEAE-cellulose, the activity was recovered in the unabsorbed fraction. This material was homogeneous on disk electrophoresis, and the pattern was identical to normal 7S gamma globulin. The identification of this protein fraction as primarily 7S gamma globulin was further suggested by immunoelectrophoretic studies.[16] This active fraction accounted for 8–10 per cent of the initial serum protein or 60 per cent of the gamma globulin of the unaltered serum. The mean assay response of the three active protein fractions is indicated in the right column of Figure 1. When 35 mg of protein as unaltered serum was injected, an 8-hr response of 500 was obtained. An equivalent response followed the intravenous injection of 6.6 mg of the ammonium sulfate precipitate or 3.3 mg of the unabsorbed gamma globulin; thus the final purification step represented a 10-fold purification of the LATS activity.

Since it was possible that the LATS activity of the 7S gamma globulin fraction was not truly associated with these proteins but had merely been recovered in the same fraction, neutralization studies with antiserum to human 7S gamma globulin were performed. As shown in Table 1, the addition of rabbit antiserum to human

TABLE 1

NEUTRALIZATION OF LATS ACTIVITY BY ANTI-7S GAMMA GLOBULIN

LATS active 7S gamma globulin (mg)	Rabbit antibody to human 7S gamma globulin	Assay response ± SE*	
1.2	5.7 mg	2 hr 81 ± 13	
		8 hr 104 ± 12	①
1.2	...	2 hr 213 ± 30.0	
	(Saline control)	8 hr 260 ± 25.9	①
1.5	5.7 mg	2 hr 163 ± 30.6	
		8 hr 180 ± 30.9	②
1.5	...	2 hr 373 ± 26.8	
	(Saline control)	8 hr 459 ± 28.8	②

* SE, standard error of mean of 5 mice.
① and ②, paired values significantly different [$p > 0.001$ (2-tail test)].

7S gamma globulin caused a significant fall in LATS activity. In two separate experiments, the mean 8-hr response to the injection of 1.2 and 1.5 mg of active gamma globulin per animal was reduced from 260 to 104 and from 459 to 180, respectively, by the addition of the rabbit antibody. These results indicated that the isolated 7S gamma globulin was directly responsible for the LATS response.

The reduction of the active gamma globulin with mercaptoethanol in a neutral aqueous solution and subsequent alkylation with iodoacetamide led to a product soluble in water or saline, and LATS activity was retained (Table 2). Separation of the reduced material using Sephadex G-75 yielded two peaks (Fig. 2) identified as polypeptide chains A and B. Recovery of the protein following reduction and separation approached 90 per cent, and 24 per cent of the protein was found in the second peak. Figure 3 illustrates the electrophoretic pattern of the various protein preparations in polyacrylamide-8 *M* urea gel at pH 4.5. The separation of poly-

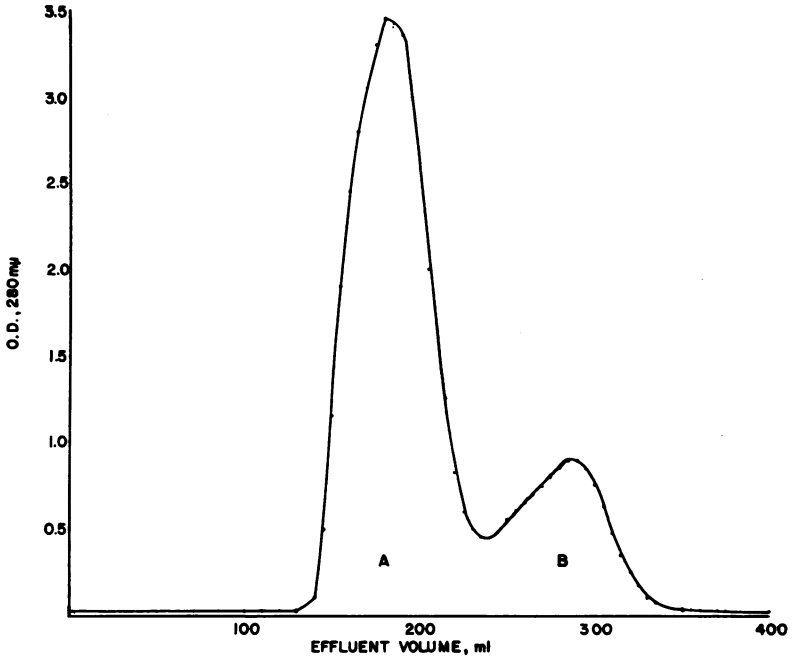

FIG. 2.—Fractionation of reduced LATS-active gamma globulin with Sephadex G-75 in 1.0 *N* acetic acid. Column load, 200 mg; fraction size, 4 ml; recovery, 87%.

peptide chains A and B appears complete; the slower moving bands in the sample of chain A probably represent aggregates.[8]

When the separated A and B polypeptide chains were assayed, the activity was found exclusively in chain A (Table 2); and, based on protein concentration, a 25-fold increase in activity had been achieved. This finding was noted on two separate fractionations when 1 *N* acetic acid was used as the dissociating medium; however, less satisfactory results were obtained when propionic acid was employed.

Hydrolysis of the active gamma globulin with papain yielded three fractions which were separated using DEAE-cellulose (Fig. 4). Assay (Table 3) showed that activity was associated entirely with piece I. Some loss of activity was sustained following digestion with papain and isolation of the fragments; larger quantities of piece I were required to produce a significant response in comparison to the active 7S gamma globulin.

An important change in the character of the assay response was noted with the active gamma globulin subunits. Throughout the early purification steps (to and

TABLE 2

MERCAPTOETHANOL REDUCTION OF LATS ACTIVE GAMMA GLOBULIN

Material	Assay concentration (mg protein/mouse)	Assay response at 8 hr ± SE*
Whole serum	12	432 ± 42.1
7S gamma globulin	1.5	459 ± 28.8
Reduced gamma globulin	2.5	462 ± 41.6
A chain	0.45	393 ± 32.9
B chain	2.8	117 ± 16.8

* SE, standard error of mean of 5 mice.

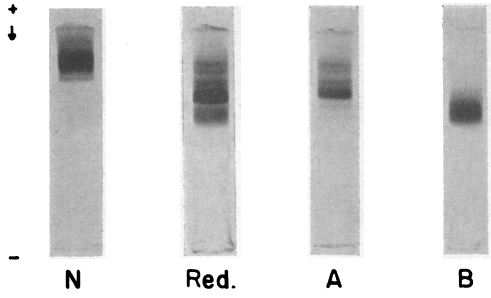

FIG. 3.—Electrophoresis in polyacrylamide-8 *M* urea at pH 4.5 of LATS-active gamma globulin (*N*), whole reduced LATS-active gamma globulin (*Red.*), and *A* and *B* fractions. Protein applied to the upper gel, 100–150 μg.

including the intact 7S gamma globulin), the bioassay response of the active extracts was always "long-acting," i.e., the 8-hr response was significantly greater than the 2-hr response (Fig. 5). However, once the gamma globulin protein had been broken into its various active fragments, either by cleavage of the disulfide bonds or by enzymatic digestion, the maximum stimulation occurred at 2 hr. In this respect the response curve for the polypeptide chain A and the papain-digest piece I resembled that produced by thyrotropin.

Discussion.—The described procedures for the concentration of the long-acting thyroid stimulator of Graves' disease indicate that the active constituent of the plasma is a 7S gamma globulin. This finding is in contrast to previous fractionation studies of unaltered serum by McKenzie[17] and Munro[18] who found the activity to be distributed in various protein fractions, although the former investigator noted that a higher proportion of the activity resided in the gamma globulin fraction. Furthermore, their studies were made without initial concentration of the globulins by salt fractionation, and the DEAE-cellulose separation step varied from the one described in this report. Using similar methods of separation, Kriss *et al.*[19] have recently reported the association of LATS activity with the 7S gamma globulin fraction of a patient's serum. It therefore appears that by using these specific measures of concentration of the gamma globulin proteins, a reliable means has been achieved of obtaining a 10-fold purification of LATS activity from serum. This should prove advantageous in the study of patients when LATS is not conclusively detected in unaltered serum.

Recent developments concerning the structural subunits of the gamma globulins have stimulated interest concerning the location of antibody activity. It has now been repeatedly demonstrated[20–22] that the gamma globulin protein is composed of two pairs of polypeptide chains (A and B) held together in part by five disulfide bonds. Papain digestion splits the molecule into three fragments: two identical pieces containing all of chain B and a part of chain A (piece I and II), and the third containing the remaining part of chain A (piece III).[23] Antibody activity has been associated with piece I and II and with polypeptide chain A[23, 24]; however, recently it has been suggested that chains A and B are necessary for the complete expression

TABLE 3

PAPAIN DIGESTION OF LATS ACTIVE GAMMA GLOBULIN

Material	Assay concentration (mg protein/mouse)	Assay response at 8 hr ± SE*
Serum	12	518 ± 45.1
7S gamma globulin	3.0	672 ± 36.6
Papain digest	5.0	297 ± 38.8
Piece I	9.0	266 ± 36.0
Piece II	5.0	109 ± 4.1
Piece III	7.5	95 ± 2.8

* SE, standard error of mean of 5 mice.

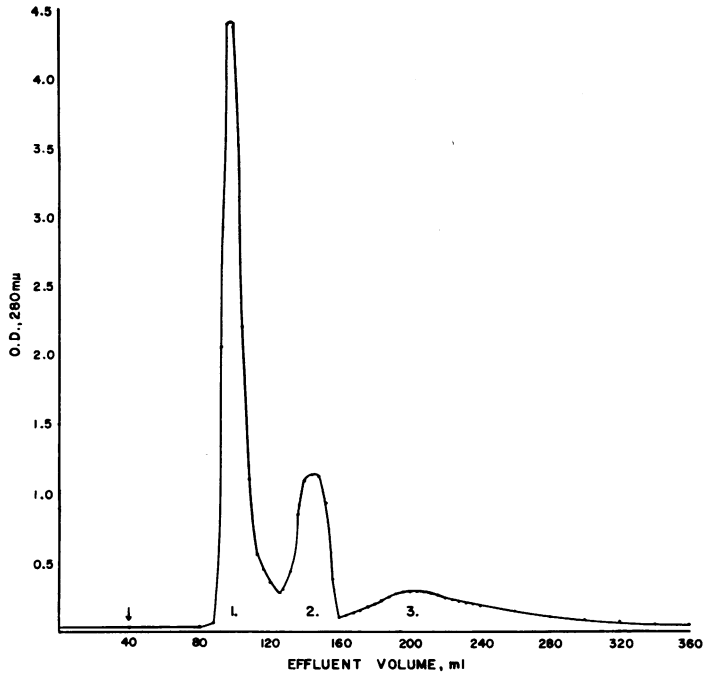

FIG. 4.—Chromatographic separation of papain-digest using DEAE-cellulose at pH 7.8. ↓: starting point of gradient elution (0.005 *M*–0.5 *M* phosphate buffer). Column load, 150 mg; fraction size, 4 ml; protein recovery, 80%.

of antibody activity.[25,26] The present study suggests that the active site for thyroid stimulation of the LATS-active gamma globulin is in that part of chain A included in the papain-digest piece I. This conclusion would agree with one proposed location of the antibody-combining site.[23, 27]

If the long-acting thyroid stimulator is indeed an antibody, as the present work indicates, it becomes necessary to consider the presence of LATS as representing a disorder of the body's immune mechanism. In contrast to the usual protective function of antibody, LATS has the biologic effect of an abnormal stimulation of the thyroid gland. The concept of an altered immune response is supported by the clinical observations of an association of thymic enlargement (noted as early as 1914 by Halsted[28]) lymphocytosis, splenomegaly, and other immune disorders[29] with thyrotoxicosis. Moreover, the difference in biologic activity between the intact gamma globulin ("long-acting") and its active fragments ("short-acting") suggests the possibility that LATS represents a thyrotropinlike substance bound to antibody. Definition of these active fragments should clarify this suggestion and may offer clues to the origin of this material. Finally, the identification of LATS with the 7S gamma globulins serves as a tentative basis for the immunosuppressive therapy which has been used in the treatment of the severe complications of Graves' disease. The recent report of the disappearance of LATS by corticosteroid therapy supports this contention.[30]

Summary.—A method for the isolation of the long-acting thyroid stimulator has been described which identifies the activity with the 7S gamma globulin proteins.

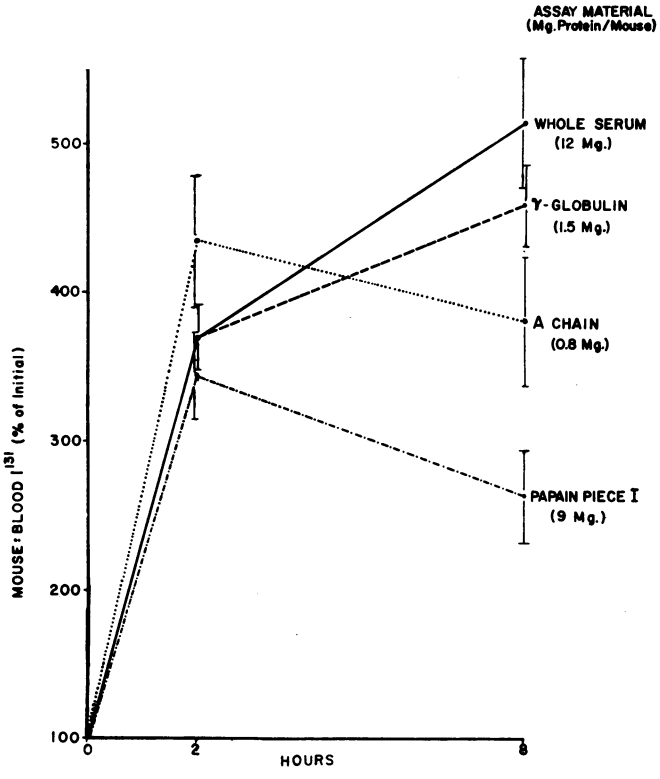

FIG. 5.—Bioassay response at 2 and 8 hr after injection of LATS-active serum, 7S gamma globulin, polypeptide chain A, and papain-digest piece I. Results are plotted as mean response ± standard error of the mean of 5 mice. The downward slopes of chain A and piece I are significant ($p < 0.05$).

Activity is retained in a single fraction when the protein is subjected to chemical reduction or to enzymatic digestion; however, the bioassay response shows the polypeptide subunits to be short-acting and to resemble thyrotropin. Thus the prolonged thyroid stimulation which is characteristic of LATS is dependent upon the intact gamma globulin protein. Because of the well-recognized function of the 7S gamma globulins to serve as antibody, it appears that the long-acting thyroid stimulator may in some manner be associated with a specific antigen-antibody response.

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*AN IMPROVED MAGNETIC DENSITOMETER: THE PARTIAL SPECIFIC VOLUME OF RIBONUCLEASE**

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This communication describes an increase in precision of the magnetic densitometer for small volumes[1] and presents results of partial specific volume determinations on ribonuclease.

Because of the importance of the partial specific volume in characterizing a macromolecule, a need exists for a method of making rapid, accurate density measurements on small volumes. Conventional pycnometric techniques and the moving, magnetically controlled buoy of Lamb and Lee[2] and MacInnes *et al.*[3] are sufficiently accurate when large amounts of material are available; for most biological macromolecules, however, it is difficult and often impossible to obtain adequate amounts in high purity. Density gradient columns of immiscible liquids,