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Thyrotropin Receptors in Thyroid Plasma Membranes

CHARACTERISTICS OF THYROTROPIN BINDING AND SOLUBILIZATION OF THYROTROPIN RECEPTOR ACTIVITY BY TRYPTIC DIGESTION

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Biologically active bovine ^{125}I-thyrotropin preparations have been prepared, characterized, and used to evaluate the optimal conditions for thryotropin binding to bovine thyroid plasma membranes *in vitro*. Binding of ^{125}I-TSH has a pH optimum around 6.0 and is sensitive to the choice and concentration of buffer. Binding is inhibited by salts, especially those containing magnesium and calcium ions; magnesium concentrations optimal for adenylate cyclase assays (2 to 5 mM) result in 85 to 98% inhibition of binding. Binding is temperature sensitive. At 37° binding has its highest initial level; however, instability of the membrane at this temperature causes a rapid loss of binding activity. Binding at 0° is optimal in 30 min and at the same level as initial binding at 37°; since there is no decrease in binding activity, it has been chosen as the optimal temperature. Thyrotropin, luteinizing hormone, the β subunit of thyrotropin, and the α subunit of thyrotropin have relative binding affinities for the thyrotropin receptors of 100, 10, 2, and <0.5, respectively. In all of these characteristics, ^{125}I-thyrotropin at 1.5×10^{-5} M concentrations has the same properties of binding to bovine plasma membranes as do [^{3}H]thyrotropin preparations which have been previously characterized (Amir, S. M., Carraway, T. F., Jr., Kohn, L. D., and Winand, R. V. (1973) *J. Biol. Chem.* **248,** 4092-4100) and used to study binding at 5×10^{-6} M concentrations.

^{125}I-TSH binding as a function of hormone concentration results in curved Scatchard plots; however, Hill plots of these same binding data are linear and have a slope of 0.65. Taken together, these data suggest that the heterogeneity in thyrotropin binding constants which is evident in the Scatchard plot reflects a negatively cooperative relationship among the thyrotropin receptor sites, *i.e.* decreased hormonal affinity as hormone concentrations increase. Adenylate cyclase studies yield kinetic plots which also exhibit negative cooperativity; corrections for thyrotropin bound under the adverse binding conditions of the adenylate cyclase assays suggest that K_m values for thyrotropin in this enzymatic assay are compatible with binding constants measured by the ^{125}I-thyrotropin preparations.

Tryptic digestion destroys binding activity on the thyroid plasma membrane but releases specific thyrotropin receptor activity into the supernatant phase. Chromatography on Sephadex G-100 indicates that this solubilized receptor fragment has a molecular weight between 15,000 and 30,000.

In a previous report (1) we described the binding of bovine [^{3}H]thyrotropin to receptors on isolated bovine thyroid membranes. The specificity of binding and its relationship to biologic function were indicated by the following observations: unlabeled TSH[1] was able to compete with or chase the [^{3}H]TSH binding to the receptors whereas other hormones and albumin could not; there was a near absence of specific binding to muscle and liver plasma membranes; and [^{3}H]TSH binding correlated with adenylate cyclase activation. In a subsequent study (2) we showed that luteinizing hormone, a structural analog of TSH, could at high concentrations compete with TSH for the thyrotropin receptor despite a negligible or significantly lower effect on thyroid biologic activity and that the absence of significant effects on thyroid function by the α and β subunits of TSH could be correlated with their very low levels of binding to the TSH receptor. These studies of TSH

[1] The abbreviations used are: TSH, thyroid-stimulating hormone or thyrotropin; LH, luteinizing hormone.

binding to bovine thyroid plasma membranes were initiated as a consequence of our work on experimental exophthalmos. In these studies (3–10), we had observed (*a*) that specific TSH receptors could be detected on plasma membranes derived from the retro-orbital tissue of the guinea pig, a mammalian model of exophthalmos (9, 10); (*b*) that the sera of exophthalmic patients contained a γ-globulin which could induce experimental exophthalmos in fish (7, 8); and (*c*) that this γ-globulin significantly increased the *in vitro* binding of TSH to TSH receptors on plasma membranes of retro-orbital tissue but not to TSH receptors on thyroid plasma membranes (1, 9, 10). These observations suggested that specific TSH receptors not only existed in retro-orbital as well as thyroid tissue but that there was a functional or structural difference between these receptors which could account for the increased TSH binding to the retro-orbital tissue receptors in the presence of this "autoimmune" γ-globulin.

In the present report, we have further characterized the properties of the TSH receptor in bovine thyroid plasma membranes, and in an accompanying report (10) we have further characterized the properties of the TSH receptor on guinea pig retro-orbital tissue plasma membranes in an effort to uncover functional or structural differences which might exist. In both studies we have used both a tritiated TSH preparation which has been previously described (1–5) and an iodinated TSH preparation which is characterized in this report. In contrast to the [^{3}H]TSH which is used at much higher concentrations, the ^{125}I-TSH has been used at hormone concentrations *in vitro* which could be expected *in vivo*. Although we show that the binding properties of TSH receptors on thyroid and retro-orbital tissue plasma membranes are effectively the same except for the γ-globulin effect, we show that a fragment of the TSH receptor released by tryptic digestion of thyroid plasma membranes is structurally different from a component of the TSH receptor which can be released by tryptic digestion of retro-orbital tissue plasma membranes (10).

MATERIALS AND METHODS

Hormone and γ-Globulin Preparations—Purified bovine TSH was prepared as described in an accompanying report (10) and purified [^{3}H]TSH was prepared and characterized as previously described (1). Purified TSH was nonenzymatically iodinated with ^{125}I using stoichiometric concentrations of hormone, chloramine T, and sodium [^{125}I]iodide as has been described by Lesniak *et al.* (11) for human growth hormone. Alternatively, labeling was performed by adapting an enzymatic method (12), *i.e.* the lactoperoxidase coupling procedure. In both cases the ^{125}I-labeled hormone was purified by Sephadex G-100 chromatography as described by Liao and Pierce (13) in order to remove free iodine, hormone aggregates, and iodinated subunits of the hormone.

Unlabeled TSH preparations initially had a mean thyrotropic (14) activity of 24 ± 4 i.u./mg, and [^{3}H]TSH preparations initially had a mean activity of 21 ± 4 i.u./mg. ^{125}I-TSH preparations had a mean thyrotropic activity of 16 to 19 i.u./mg (Table I) although it must be emphasized that McKenzie (14) assays of ^{125}I preparations were performed on preparations analogously reacted with cold iodine rather than ^{125}I for obvious considerations. All preparations similarly stimulated adenylate cyclase activity of thyroid membranes (Table I and Fig. 1) and induced the formation of follicle-like structures in thyroid tissue cultures (18–23). The specific radioactivity of different [^{3}H]TSH preparations ranged between 0.2 and 0.5 Ci/mM; the ^{125}I-TSH preparations had specific radioactivities of between 50 and 500 Ci/mM.

γ-Globulin from the sera of patients with Graves' disease and no exophthalmos, from patients with Graves' disease and malignant exophthalmos, or from normal controls were purified by chromatography on DEAE-cellulose (24). They were exhaustively dialyzed against 0.01 M ammonium bicarbonate and were lyophilized before being evaluated for long-acting thyroid stimulator activity (25), exophthalmogenic activity (26, 27), or activity in binding assays.

Preparation of Plasma Membranes and Measurement of TSH Binding to Plasma Membranes—Plasma membranes from bovine thyroid tissue were prepared and had the same characteristics as previously described (1). Assays of TSH binding used a standard incubation medium containing 25 mM Tris-acetate, pH 6.0, 0.6% bovine serum albumin, labeled TSH (10,000 to 250,000 cpm), and 5 to 50 μg of membrane protein in a final volume of 100 μl. The standard incubation was at 0° for a period of 1 hour. The technique of the assay involved vacuum filtration through an oxoid filter (Amersham/Searle Corporation) and used a modification of the procedure previously

TABLE I

Properties of labeled TSH preparations

Property	Unlabeled TSH	[^{3}H]TSH	^{125}I-TSH	
			Nonenzymatically labeled[a]	Enzymatically labeled[b]
Thyroid stimulating activity (i.u./mg)[c]	24 ± 4	21 ± 4	16 ± 3	19 ± 4
Adenylate cyclase stimulation[d]	D. R.[e]	D. R.	D. R.	D. R.
Thyroid cell differentiation in tissue culture[f]	+	+	+	+
Specific radioactivity (Ci/mM)[g]	–	0.2–0.5	50–500	50–500

[a] Prepared using the procedure of Lesniak *et al.* (11).

[b] Prepared using a lactoperoxidase procedure (12).

[c] Measured in the McKenzie bioassay (14).

[d] Measured as described (15–17); see Fig. 1.

[e] Dose response same between 10^{-8} and 10^{-5} M.

[f] Thyroid cells in primary culture form pseudo follicles when exposed to TSH after trypsinization (18–23). Each of the labeled preparations was included in the culture medium in place of unlabeled TSH and at approximately the same concentrations, *i.e.* 10 milliunits/ml of media or approximately 0.5 μg/ml of media. Differentiation was monitored microscopically; a plus (+) indicates pseudo follicle formation as demonstrated in an accompanying report (23).

[g] Measured on 4 to 10 different preparations of each type using a Beckman model 255 liquid scintillation spectrometer with a tritium counting efficiency of 54% and a ^{125}I counting efficiency of 86%.

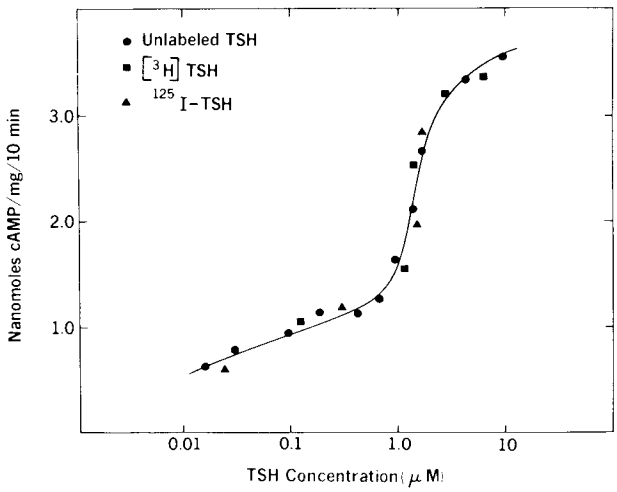

FIG. 1. The effect of [^{3}H]TSH, ^{125}I-TSH, and unlabeled TSH on the adenylate cyclase activity of the bovine thyroid plasma membranes used for binding studies in the present report. The reaction was performed as described under "Materials and Methods." It was initiated by membrane protein and allowed to proceed for 10 min at 37°. All points are means of triplicates. An optimal magnesium concentration, 4 mM, is present, as well as an optimal buffer pH and concentration, 0.01 M Tris-chloride at pH 7.6 (16, 17).

detailed (1), *i.e.* filters were presoaked in 5%, rather than 10%, albumin solutions and the wash buffer was 20 mM Tris-acetate, pH 6.0, containing 2.5% albumin. Specific binding was measured by subtracting values obtained from control incubations containing either no membranes or unlabeled TSH at a 10,000- or 100,000-fold excess over labeled TSH.

Other Assays and Materials—Adenylate cyclase activity was measured by following the procedure of Krishna *et al.* (15) as modified by Wolff and Cook (16, 17). Protein was measured colorimetrically (28) using albumin crystallized 5 times as the standard in order to insure their solubilization. Membranes were heated to 90° for 30 min in 1 N NaOH prior to being assayed for protein.

[α-^{32}P]ATP for adenylate cyclase assays was obtained from the International Chemical and Nuclear Corporation; cyclic [^{3}H]adenosine 3′:5′-monophosphate was from Schwarz/Mann; and [^{125}I]iodine used for TSH labeling procedures was a 300 mCi/ml preparation from Amersham/Searle Corporation. Crude bovine TSH (used to prepare purified TSH preparations) was obtained from either Miles-Pentex or Organon (Oss, Holland). All other reagents were of the highest purity available from commercial sources.

RESULTS

Properties of ^{125}I-TSH Binding Compared to Properties of [^{3}H]TSH Binding to Thyroid Plasma Membranes—Binding of TSH to its thyroid membrane receptor had the same pH dependency whether ^{125}I-TSH was used at 1.5×10^{-10} M concentrations of whether [^{3}H]TSH was used at 5×10^{-6} M concentrations (Fig. 2). Binding in both cases was sensitive to the choice of buffer; for example, binding was better in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid at pH 7.4 than in Tris-chloride at pH 7.4 (Fig. 2). Binding of ^{125}I-TSH to the membranes was as sensitive to the buffer and salt concentration in the incubation medium as was the binding of [^{3}H]TSH (Figs. 3 and 4). Optimal binding with either labeled TSH preparations was thus between 0.005 and 0.03 M Tris-acetate or Tris-maleate at pH 6.0, and in both cases magnesium and calcium salts were significantly more inhibitory than sodium or lithium salts. Concentrations of magnesium chloride which are optimal for adenylate cyclase activation, *i.e.* 2 to 5 mM (15, 17, 29, 30), were 80 and 95 to 98% inhibitory, respectively, when binding was assayed (Fig. 4). Calcium inhibited binding to even a greater extent; thus, 0.1 mM $CaCl_2$ inhibited binding over 50% and 1 mM abolished it completely (not shown).

^{125}I-TSH binding at 1.5×10^{-10} M and 37° was effectively immediate as was the binding of [^{3}H]TSH at 5×10^{-6} M (Fig. 5). In both cases, however, continued incubation at 37° resulted in a decrease in specific binding (Fig. 5). As previously reported (1), this decrease in binding activity at 37° was not a function of degradation of the TSH by membrane enzymes. Instead it appeared to be a function of membrane stability since preincubation of the membranes at 37° resulted in an analogous loss of binding ability whereas preincubation of the TSH at 37° was without effect on binding (Table II). This effect of temperature was partially prevented by the presence of TSH in the incubation medium and was reversible by returning the membranes to 0° for 1 hour prior to assay (Table II).

Binding of ^{125}I-TSH at 1.5×10^{-10} M and at 0° was

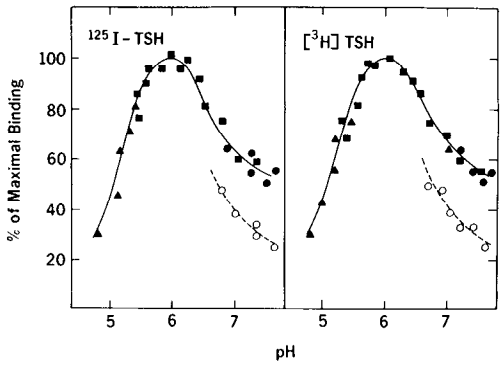

FIG. 2. Binding of ^{125}I-TSH and [^{3}H]TSH as a function of pH. Binding of ^{125}I-TSH was at a hormone concentration of 1.5×10^{-10} M; binding of [^{3}H]TSH was at a hormone concentration of 5×10^{-6} M. The buffer in all cases was 0.025 M in concentration; binding conditions were otherwise standard. The buffers used were acetate (▲), Tris-acetate or Tris-maleate (□), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (●), and Tris-chloride (○).

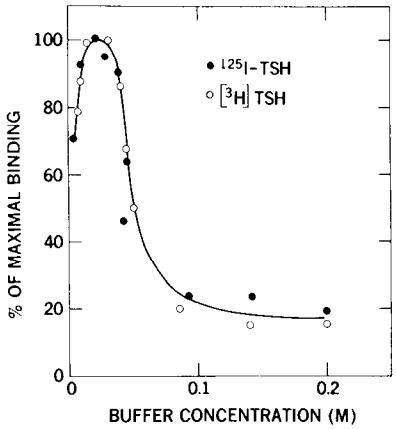

FIG. 3. Binding of ^{125}I-TSH (●) and [^{3}H]TSH as a function of buffer concentration. The ^{125}I-TSH and [^{3}H]TSH concentrations were the same as in Fig. 2 and the buffer was Tris-acetate at pH 6.0. Assay conditions were otherwise standard.

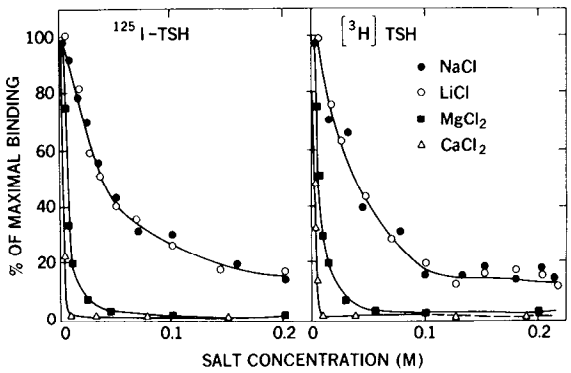

FIG. 4. Binding of ^{125}I-TSH and [^{3}H]TSH to bovine thyroid plasma membranes as a function of salt concentration. Hormone concentrations were those noted in Fig. 2; assay conditions were optimal (see "Materials and Methods") except for the inclusion of the sodium, lithium, calcium, or magnesium chloride at the noted concentrations.

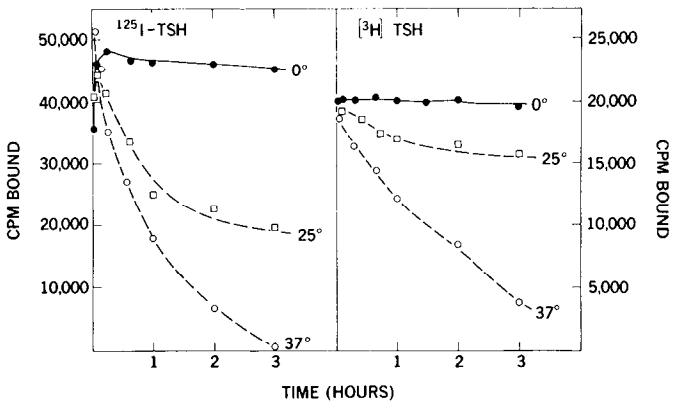

FIG. 5. Binding of ^{125}I-TSH and [^{3}H]TSH to thyroid plasma membranes as a function of temperature. Conditions were standard; labeled hormone concentrations were the same as in Fig. 2.

TABLE II

Effect of preincubation at 37° on membrane and TSH stability

Preincubation conditions	Preincubation components	[^{3}H]TSH bound at 0° after 1 min of incubation[a]
		cpm
None		22,000
0° for 1 hour	Membranes + TSH	22,000
	Membranes − TSH	21,400
	TSH only	20,600
37° for 1 hour	Membranes + TSH	12,300
	Membranes − TSH	7,100
	TSH only	21,400
37° for 1 hour and 0° for 1 hour	Membranes + TSH	20,500
	Membranes − TSH	17,200
	TSH only	21,700

[a] Preincubations were performed using the noted conditions for time and temperature. In a volume of 90 μl, the preincubation mixture contained the noted components, as well as the buffer, and the albumin contained in a standard binding assay incubation. The binding assay was started by adding in a volume of 10 μl either sufficient [^{3}H]TSH to yield a final assay concentration of 5×10^{-6} M or an appropriate amount of membrane. Binding assay conditions were thus optimal and the same as described under "Materials and Methods." Binding incubations were for 1 min before filtration.

time-dependent with an optimum of 30 min (Fig. 5). At 0° and 5×10^{-11} M, ^{125}I-TSH time dependence increased to over 1 hour (not shown) whereas at 5×10^{-6} M, both ^{125}I-TSH and [^{3}H]TSH binding at 0° was immediate (Fig. 5). Although these data are compatible with a simple second order binding reaction ($A + B \rightleftarrows AB$) which exhibits time dependence as the concentration of one reactant (TSH) decreases, they in no way prove that the TSH-receptor interaction has such a simple mechanism. Since there was no loss of binding activity at 0°, this temperature was chosen as the standard condition of incubation.

As was previously reported for [^{3}H]TSH (1), binding with ^{125}I-TSH was a linear function of the concentration of membrane protein. Also as previously reported (1), [^{3}H]TSH binding at 0° appeared to be a linear function of hormone concentration with an apparent binding constant of 0.5×10^8 M^{-1} at pH 7.5 in 0.02 M Tris-chloride. At pH 6.0, in 0.025 M Tris-acetate, [^{3}H]TSH binding at 0° yielded an apparent binding constant of 0.25×10^8 M^{-1}. In contrast, ^{125}I-TSH binding yielded a complex curve (Fig. 6) which suggested that the TSH receptor sites were heterogeneous in their ability to bind TSH. If this heterogeneity were explained by the existence of a group of "high affinity" and "low affinity" binding sites, which did not interact, *i.e.* a discrete group with a binding constant of 0.25×10^{10} M^{-1} and a separate and independent group with a binding constant of 0.25×10^8 M^{-1} as suggested in Fig. 6, *inset*, a Hill plot of these data should be linear and the line should have a slope of unity (32–34). A Hill plot with a value greater than unity would indicate that the receptor sites were not independent but had a positively cooperative relationship whereas a plot with a value less than unity would indicate a negatively cooperative relationship (32–34). As noted in Fig. 7, the Hill plot of these data yields a line with a slope of 0.65, *i.e.* the TSH receptors yield complex Scatchard plots not because of the existence of discrete groups of sites with different binding constants but because of a relationship among the sites such that the affinity for hormone

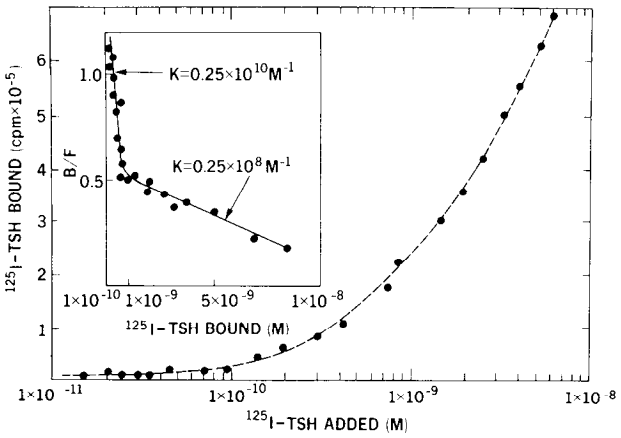

FIG. 6. Binding of ^{125}I-TSH as a function of hormone concentration. *Inset* is a Scatchard plot (31) of the data. Optimal assay conditions were used.

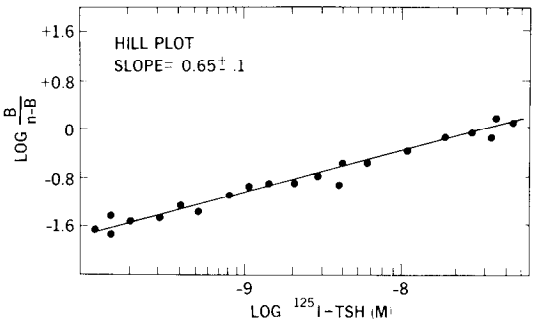

FIG. 7. Hill plot (32–34) of the data in Fig. 6.

decreases as the hormone concentration increases (32–35). These data would thus suggest that ^{125}I-TSH and [^{3}H]TSH binding at 1.5×10^{-10} M and 5×10^{-6} M concentrations, respectively, are in fact examining related receptor sites whose different binding constants are simply a reflection of the hormone concentration used in their examination. It is notable, therefore, that both classes of sites have the same pH optima, the same buffer optima, the same salt inhibition phenomena, and the same temperature effects at 37°.

^{125}I-TSH binding was a reversible process as previously reported for [^{3}H]TSH binding (1), *i.e.* unlabeled TSH at a 100,000-fold excess could displace ^{125}I-TSH bound during a prior incubation (Table III). There was negligible specific ^{125}I-TSH binding to plasma membranes from either bovine skeletal muscle or bovine adrenocortical tissue (Table III).

Inhibition of ^{125}I-TSH Binding by LH, Subunits of TSH, and by γ-Globulin from Patients with Malignant Exophthalmos—Inhibition of binding using [^{3}H]TSH as the labeled hormone is relatively simple to interpret since only 2 to 3% of the hormone added is bound at a 10^{-6} M concentration of hormone. Inhibition kinetics is thus performed at saturating hormone levels where effectively all of the unlabeled hormone will act as a competing agent. In the case of ^{125}I-TSH, however, inhibition is performed under nonsaturating conditions where as much as 40 to 50% of the total hormone added to the incubation can be bound even after the addition of unlabeled hormone; accordingly, nonlinear inhibition curves will be obtained. This is evident in Fig. 8, where ^{125}I-binding is measured in the presence of unlabeled TSH, unlabeled luteinizing hormone (LH), and the unlabeled subunits of TSH. When, however, corrections are made for the ^{125}I-TSH bound by considering the total of the labeled and unlabeled TSH concentration present

TABLE III

Reversibility of ^{125}I-TSH binding to thyroid, muscle, and adrenocortical plasma membranes

TSH added during first incubation[a]	TSH added during second incubation[b]	^{125}I-TSH bound		
		Thyroid membranes	Muscle membranes	Adreno-cortical membranes
^{125}I-TSH, 1.5×10^{-10} M[c]	None	38,600	1,200	980
^{125}I-TSH, 1.5×10^{-10} M	Unlabeled TSH, 5×10^{-6} M[d]	4,800	840	890
^{125}I-TSH, 1.5×10^{-10} M, + unlabeled TSH, 5×10^{-6} M	None	2,300	950	960

[a] Incubation conditions were standard, *i.e.* in 0.025 M Tris-acetate, pH 6.0, for 1 hour at 0°. Reactions were started by addition of membranes.

[b] At the end of the first incubation, 10 μl of the incubation buffer or 10 μl of incubation buffer containing unlabeled TSH was added, and the incubation was continued another hour at 0°.

[c] Final concentration.

[d] Prepared as described (36).

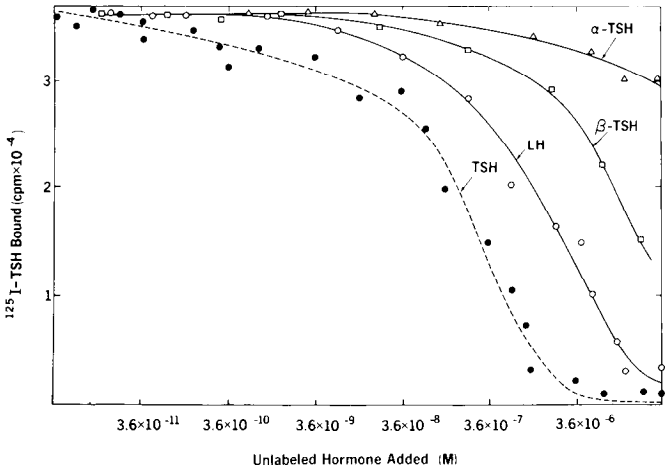

FIG. 8. Inhibition of ^{125}I-TSH binding to thyroid plasma membranes by unlabeled TSH (●), LH (○), β-TSH (□), α-TSH (△), and either albumin, prolactin, adrenocorticotropic hormone, insulin, glucagon, or growth hormone (■). Assay conditions were optimal; ^{125}I-TSH was at 1.5×10^{-10} M. The *dashed line* drawn through the binding values obtained in the presence of unlabeled TSH is a theoretical curve, derived by compensating for the amount of labeled hormone which would be bound at the concentrations of total hormone, labeled and unlabeled, present in the assay mixture. This is an important correction since in the 1×10^{-9} M total concentration range, over 40% of the total hormone added continues to be bound under these conditions.

in the assay and the percentage of this total that should be bound, a theoretic curve can be derived. As noted in Fig. 8, the theoretic curve (*dashed line*) derived for unlabeled TSH inhibition of ^{125}I-TSH binding encompassed the actual data points reasonably well.

By comparing the concentration of LH, β-TSH, or α-TSH necessary to achieve 50% inhibition of ^{125}I-TSH binding, a table of their relative binding affinities could be calculated (Table IV). The similarity of these values obtained at a 1.5×10^{-10} M concentration of ^{125}I-TSH and those obtained at a 5×10^{-6} M concentration of [^{3}H]TSH (2) again suggest that, despite the different binding constants of the TSH receptors being examined under these two conditions, the characteristics of the receptors are the same.

Lissitzky *et al.* (37) have reported the displacement of bound ^{125}I-TSH from TSH receptors by the subunits of TSH and by LH. In that study they used whole porcine cells, an approximately 0.1 M medium with a pH of 7.4, and 15-min incubations at 35°. Despite these differences in conditions and the major difference of whole cells as opposed to plasma membranes, the factors calculated in Table IV can be applied to their data reasonably well. Thus, the conversion of their LH or subunit

TABLE IV

Relative affinity of TSH, LH, and subunits of TSH for receptor on bovine thyroid membranes

Hormone	Relative affinity toward TSH receptor[a]	
	From ^{125}I-TSH data[b]	From [^{3}H]TSH data[c]
TSH	100	100
LH	10	12.5
β-TSH	2.1	2.5
α-TSH	<0.5	0.4

[a] The activity of the subunit or LH preparations when they are at the same concentration as TSH and when TSH affinity is set at 100.

[b] From Fig. 8.

[c] From Wolff *et al.* (2).

concentrations to equivalent TSH concentrations using the factors of Table IV and the calculation of the inhibition which this equivalent TSH concentration actually exhibited in their assay yields inhibition values very close to their experimental determinations.

As noted earlier, studies of [^{3}H]TSH binding to plasma membranes of retro-orbital tissue have shown that γ-globulin from patients with malignant exophthalmos could increase the [^{3}H]TSH binding (9, 10). This effect was not seen with normal γ-globulin or γ-globulin from patients with Graves' disease without exophthalmos (9, 10), and this effect was not seen when [^{3}H]TSH binding was examined in thyroid plasma membranes (1). In the present study ^{125}I-TSH binding to thyroid plasma membranes also was not enhanced by γ-globulin from patients with malignant exophthalmos when the γ-globulin was tested at concentrations which enhanced ^{125}I-TSH binding in retro-orbital tissue membranes (9, 10).

Effect of Trypsin on TSH Receptor of Bovine Thyroid Plasma Membranes—During the course of our studies on the solubilization of the TSH receptor from thyroid membranes (38), it was noted that trypsin did not destroy binding activity as did other proteases; in contrast, trypsin did destroy the binding of TSH to receptors on intact thyroid plasma membranes (Fig. 9*A*). This discrepancy was resolved when binding in the supernatant phase was measured as well as binding to the plasma membranes (Fig. 9*A*). As noted, the loss in receptor binding activity on the plasma membranes correlates with the appearance of receptor binding activity released by trypsin into the supernatant.

When the binding activity released by trypsinization of the bovine plasma membranes was eluted on Sephadex G-100, its

estimated molecular weight was 15,000 to 30,000 based on standards eluted on this same column (Fig. 9*B*). The trypsin solubilized receptor activity eluted from the columns was specific for ^{125}I-TSH binding in that results analogous to those in Table III were obtained when binding activity was measured in the presence of unlabeled TSH. Albumin, adrenocorticotropic hormone prolactin, human growth hormone, and insulin did not displace ^{125}I-TSH or [^{3}H]TSH bound to the trypsin released receptor. Muscle and adrenal cell plasma membranes (36) similarly treated with trypsin yielded no specific TSH binding component in the supernatant solutions. The optimum conditions for binding either ^{125}I-TSH or [^{3}H]TSH to this 15,000 to 30,000 molecular weight receptor fragment were the same as those described for the solubilized TSH receptor in an accompanying report (38).

DISCUSSION

In previous reports using [^{3}H]TSH (1, 2, 9), we have demonstrated a specific TSH receptor on plasma membranes isolated from bovine thyroid and guinea pig retro-orbital tissues. We further demonstrated what appeared to be an important difference between these two receptors, *i.e.* an increase in TSH binding to retro-orbital tissue receptors but not to thyroid receptors when γ-globulin from patients with malignant exophthalmos was included in the incubation medium. In our present studies we had hoped that ^{125}I-TSH binding studies would allow a more detailed characterization of the TSH receptors in thyroid and retro-orbital tissue and that this characterization would uncover biochemical differences which would either explain or amplify our previous results (1, 2, 7–9). However, as noted in this and an accompanying report (10), ^{125}I-TSH and [^{3}H]TSH binding studies demonstrate no significant difference between the TSH receptors in these two tissues; on the contrary, they emphasize their similarity. Both have similar pH optima, buffer optima, salt inhibition phenomena, and temperature effects; both exhibit nonlinear Scatchard plots which can be explained by negative cooperativity among the receptor sites.

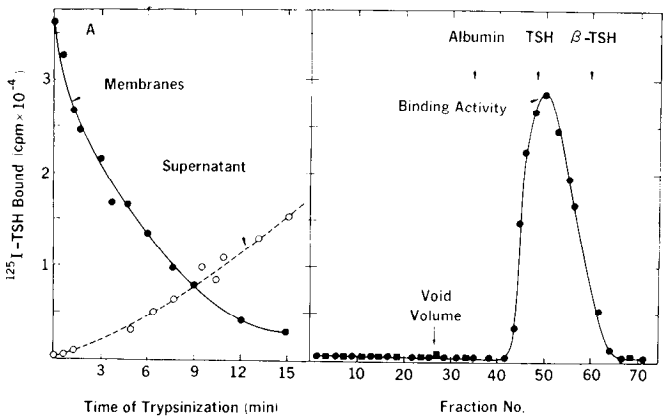

FIG. 9. *A*, loss of binding activity in thyroid plasma membranes (●) after exposure to trypsin and the release of "solubilized" receptor activity (○) into the supernatant solution. Membranes were exposed to L-1-tosylamido-2-phenylethyl chloromethyl ketone trypsin at room temperature, at a 20:1 weight ratio of membranes to trypsin, and in 0.025 M Tris-maleate buffer, pH 7.8. At the noted times, aliquots were removed and mixed with an amount of cold soybean trypsin inhibitor (Calbiochem) 5-fold in excess of the trypsin concentration, and the suspensions were chilled to 2–4°. One aliquot from this suspension was immediately assayed for binding activity using optimal conditions and a 1.5×10^{-10} M ^{125}I-TSH concentration (●); a second aliquot was centrifuged using a Beckman microfuge, and the supernatant was assayed (○) using a modified binding assay developed for our studies of solubilized TSH receptors (38). Controls included duplicate incubations and assays containing trypsin or trypsin plus trypsin inhibitor at identical concentrations but either in the absence of membranes or with membranes added at the time of assay. *B*, chromatography on Sephadex G-100 of the TSH receptor activity (●) released into the supernatant by trypsinization of bovine thyroid plasma membranes. Binding activity was measured on aliquots of the noted fractions using optimal conditions for the solubilized receptor assay (38) and 1.5×10^{-10} M ^{125}I-TSH. Solubilized receptor activity from bovine plasma membranes was prepared as described in *A*; the supernatant after 15 min of a large scale incubation was used. The elution peaks of the three markers, albumin, TSH, and β-TSH, are noted by the *arrows;* their molecular weights are 67,000, 27,000 to 28,000, and 13,000 to 14,000, respectively. The column was 50 × 0.9 cm in size and was equilibrated and eluted with 0.025 M Tris-acetate, pH 6.0. The elution rate was 20 ml per hour. Fractions of 0.4 ml were collected. The void volume is the peak of elution of a dextran blue dye marker of >200,000 molecular weight.

This study is nevertheless interesting in three regards: the effect of trypsin on the TSH receptor; the relationship of binding to adenylate cyclase activation; and the unusual conditions which optimize *in vitro* binding of TSH to the TSH receptor. In regard to the former effect these results show that trypsin can release from thyroid plasma membranes a 15,000 to 30,000 molecular weight component which has specific TSH binding activity. Work by Levey *et al.* (39) has indicated that receptors of such small size may not be a unique phenomena, since these workers have recovered glucagon receptor activity in similarly sized units released from cat myocardium. As is pointed out in an accompanying report (38), the low molecular weight TSH receptor component described in this report appears to be a fragment of a higher molecular weight TSH receptor which can be solubilized from thyroid plasma membranes and has slightly modified TSH binding properties. Of extreme interest is our finding in a second accompanying report (10), that analogous trypsinization experiments with retro-orbital tissue TSH receptors release a much larger receptor fragment.

In regard to the relationship of TSH binding to TSH stimulation of adenylate cyclase activity, these studies contain several important observations. TSH binding is inhibited over 85% by 2 mM magnesium and at least 95 to 98% inhibited by 5 mM magnesium, *i.e.* concentrations which have been used for optimal adenylate cyclase activation of thyroid plasma membranes (15–17, 29, 30). TSH binding is also inhibited at least 2- to 3-fold for Tris-chloride buffers at pH values greater than 7.4. The consequence of these observations is that only 1 to 10% of the TSH added to adenylate cyclase incubations is binding to the membranes and that K_m determinations relating the effect of TSH to adenylate cyclase stimulation have therefore been seriously in error in the past and must be revised. As an example, double reciprocal plots of the data in Fig. 1 yield a nonlinear curve (analogous to the Scatchard plot in Fig. 7), from which two K_m values for TSH can be calculated, one at 1×10^{-6} M and one at 1.25×10^{-8} M. If, however, consideration is given to the fact that no more than 1/10 and as little as 1/100 the TSH is really binding to the membranes, these values can be lowered to values very close to the binding constants derived from ^{125}I-TSH binding studies in this report. This observation has been confirmed by Moore and Wolff, who have used our TSH and [^{3}H]TSH preparations to more closely study the relationship of TSH binding to adenylate cyclase activation and the effects of nucleotides on the two processes (40).

The optimal conditions for TSH binding *in vitro*, *i.e.* a pH of 6.0 to 6.5, a buffer concentration of less than 0.05 M, and a

temperature of 0°, are obviously far from what are presently assumed to be *in vivo* conditions. The question can be raised, therefore, as to the significance of such measurements in regard to the "real" TSH receptor *in vivo*. The clearest evidence that *in vitro* binding studies such as these at present are measuring physiologically important receptors comes from an accompanying report (23) and from the work of Macchia and Meldolesi (41). In our own studies (23) the loss and regrowth of TSH receptors on thyroid cells exposed briefly to trypsin is paralleled by the loss and return of TSH-stimulable adenylate cyclase activity in the absence of effects on basal adenylate cyclase activity. In the work by Macchia and Meldolesi (41), TSH-stimulable functions are lost in a thyroid tumor whose plasma membranes when assayed *in vitro* as described herein cannot bind TSH. Thus in both studies the loss of the *in vitro* assayable receptor is coupled with the loss of TSH-stimulable functions *in vivo*.

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